Université de Montréal

PHYSIOPATHOLOGIES CARDIOMÉTABOLIQUES ASSOCIÉES À L'OBÉSITÉ : MÉCANISMES SOUS-JACENTS ET THÉRAPIE NUTRITIONNELLE

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Thèse présentée en vue de l'obtention du grade de PhD
en Nutrition

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Cette thèse a été évaluée par un jury composé de

Président rapporteur : Dr Pierre Haddad
Membre du jury : Dre Chantal Bemeur
Directeur de recherche : Dr Emile Levy
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Résumé

Le tractus digestif et le foie interagissent continuellement, non seulement à travers les connexions anatomiques, mais également par des liens physiologiques/fonctionnels. Le déséquilibre de l'axe intestin-foie apparait de plus en plus comme un facteur primordial dans les désordres cardiométaboliques, à savoir l'obésité, le syndrome métabolique, le diabète de type 2 et la stéatose hépatique non alcoolique (NAFLD), pour lesquels la prévalence demeure alarmante, les mécanismes moléculaires encore méconnus, et les traitements peu efficaces.

L'hypothèse centrale du présent projet de recherche est que la combinaison d'anomalies génétiques et nutritionnelles affecte la sensibilité de l'insuline intestinale, ce qui conduit à une surproduction des chylomicrons, à une dyslipidémie, une insulinorésistance systémique et des répercussions sur le foie. Dans cet agencement, le foie développe une NAFLD progressive, impliquant plusieurs sentiers métaboliques intrinsèques et des mécanismes comprenant le stress oxydatif, l'inflammation et l'insulinorésistance. En revanche, des nutriments, comme les acides gras polyinsaturés (AGPI) n-3, peuvent présenter des effets bénéfiques en ciblant plusieurs circuits pathogéniques.

L'objectif central de cette thèse consiste à : *(i)* Démontrer que des gènes codant pour les protéines intestinales clés associées au transport des lipides, comme c'est le cas du *Sar1b GTPase*, peuvent interagir avec l'environnement nutritionnel pour produire l'obésité et des dérangements cardiométaboliques, incluant la NAFLD ; *(ii*) Explorer les mécanismes hépatiques sous-jacents à la NAFLD; et *(iii)* Identifier les effets et les cibles thérapeutiques des AGPI n-3 sur la NAFLD. Ces objectifs seront soutenus par une prospection de la littérature scientifique disponible dans les champs du syndrome métabolique et de la NAFLD afin d'en disséquer les forces et les faiblesses au bénéfice de la communauté scientifique.

À ces fins, nous avons utilisé des modèles animaux et cellulaires manipulés génétiquement, des animaux exposés de façon chronique à des diètes riches en lipides, des

spécimens de tissus hépatiques obtenus durant la chirurgie bariatrique d'obèses morbides, et une cohorte d'adolescents obèses souffrant de NAFLD et qui seront traités avec les AGPI n-3.

L'ensemble de nos expériences ont soutenu nos hypothèses et ont mis en évidence les concepts et mécanismes suivants : *(i)* L'abondance d'un gène crucial (notamment *Sar1b GTPase*) au niveau de l'intestin, en synergie avec une alimentation obésogène, perturbe l'homéostasie locale et mène à des dérangements cardiométaboliques, défiant même l'axe intestin-foie ; *(ii)* Les causes développementales de la NAFLD comprennent les dérangements du métabolisme des acides gras, du statut redox et inflammatoire, de la sensibilité à l'insuline, des sentiers métaboliques (lipogenèse, β-oxydation, gluconéogenèse) et de l'expression des facteurs de transcription; et *(iii)* Les AGPI n-3 représentent un robuste arsenal thérapeutique des dérangements cardiométaboliques, notamment la NAFLD, en agissant sur plusieurs cibles pathogéniques.

Globalement, nos résultats montrent le rôle indéniable de l'intestin comme organe insulino-sensible interagissant de près avec les aliments et capable de déclencher des troubles métaboliques. Plusieurs mécanismes gouvernant les désordres métaboliques ont été dévoilés par nos travaux. En outre, nos études cliniques ont pointé la force thérapeutique des AGPI n-3 qui interviennent dans de nombreux processus de régulation métaboliques et notamment dans le stress oxydatif et l'inflammation.

Mots-clés : Obésité; Syndrome métabolique; Nutrition; NAFLD; Stéatose; Lipogenèse; Gluconéogenèse; Résistance à l'insuline; Inflammation; Stress oxydatif; Dysfonction mitochondriale; Endotoxémie; Traitement; Aliments fonctionnels; Acides gras Omega-3; Protéomique; Cardiométabolisme.

Abstract

The digestive tract and liver interact continuously, not only through anatomical connections, but also through physiological / functional links. The imbalance of the intestine-liver axis is increasingly emerging as a key factor in cardiometabolic disorders (CMD), namely obesity, metabolic syndrome, type 2 diabetes, and non alcoholic fatty liver disease (NAFLD), for which prevalence remains alarmingly high, molecular mechanisms are poorly understood, and treatments are largely inefficient.

The central hypothesis of this research project is that the combination of genetic and nutritional abnormalities affect intestinal insulin sensitivity, leading to overproduction of chylomicrons, dyslipidemia, systemic insulin resistance and dysregulated intestine-liver axis. In this situation, the liver develops progressive NAFLD, implicating several intrinsic metabolic pathways and mechanisms, including oxidative stress, inflammation and insulin resistance. In contrast, functional foods, such as omega-3 polyunsaturated fatty acids (n-3 PUFA), may have beneficial effects by targeting several pathogenic pathways.

The central objective of this thesis is to: *(i)* Demonstrate that genes coding for key intestinal proteins associated with lipid transport, as is the case with Sar1b GTPase, can interact with the nutritional environment to produce obesity and CMD, including hepatic steatosis; *(ii)* explore the mechanisms underlying NAFLD; and *(iii)* identify the effects and therapeutic targets of n-3 PUFA. These objectives will be supported by a critical review on metabolic syndrome and NAFLD in order to dissect their strengths and weaknesses for the benefit of the scientific community.

For these purposes, we used genetically engineered animal and cell models, chronic exposure of animals to high-fat diets, liver tissue specimens obtained during bariatric surgery of morbidly obese patients, and treatment of obese NAFLD adolescents with n-3 PUFA.

All of our experiments supported our hypotheses and highlighted the following concepts and mechanisms: (i) The abundance of a crucial gene (notably *Sar1b GTPase*) in the intestine, in synergy with an obesogenic diet, disrupts local homeostasis and leads to CMD, challenging even the intestine-liver axis; (ii) Developmental causes of NAFLD include disturbances of fatty acid metabolism, redox and inflammatory status, insulin sensitivity, metabolic pathways (lipogenesis, β-oxidation, gluconeogenesis), and expression of transcription factors; and (iii) n-3 PUFA represent a robust therapeutic arsenal of CMD, including NAFLD, by acting on several pathogenic targets.

Overall, our results show the undeniable role of the intestine, as an insulin-sensitive organ, interacting closely with obesogenic food, and capable of triggering CMD, including perturbations of the intestine-liver axis. Several mechanisms governing metabolic disorders have been unveiled by our work. In addition, our clinical studies have pointed to the therapeutic potential of n-3 PUFA involved in many regulatory processes, including oxidative stress and inflammation.

Keywords : Obesity; Metabolic syndrome; Nutrition; NAFLD; Steatosis; Lipogenesis; Gluconeogenesis; Insulin resistance; Inflammation; Oxidative stress; Mitochondria dysfunction; Endotoxemia; Treatment; Functional foods; Omega-3 Fatty acids; Proteomic profile; Cardiometabolic disorders.

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Liste des abréviations

AA	Acide arachidonique
ACC	Acetyl-CoA carboxylase
AG	Acide gras
AGCC	Acide gras à chaîne courte
AGPI	Acides gras polyinsaturés
AGT	Acide gras *trans*
AL	Acide linolénique
ALA	Alpha-linolénique
ALT	Alanine aminotransférase
AMPK	«Adenosine 5'-monophosphate-activated protein kinase »
Apo	Apolipoprotéine
AST	Asparate aminotransferase
ChREBP	«Carbohydrate response element binding protein »
CM	Chylomicron
CoQ	Coenzyme Q
CRP	« C Reactive Protein »
DHA	Acide docosahexaénoïque
DT2	Diabète de type 2
EPA	Acide eicosapentaénoïque
FAS	«Fatty acid synthase»
FoxO1	«Forkhead box O1»
GGT	Gamma-glutamyltransférase
GPR	«G protein-coupled receptors»
GPx	«Glutathione peroxidase»
HDL	high-density lipoprotein cholesterol
HNE	«4-hydroxy-2,3-nonenal»
HTA	Hypertension artérielle
IMC	**I**ndice de masse corporelle

JS	Jonction serrée
LPS	Lipopolysaccharide
MCV	Maladies cardiovasculaires
MDA	Malondialdehyde
NAFLD	Stéatose hépatique non alcoolique
NASH	Stéatohépatite
NF-kB	«Nuclear factor-kappa B»
NLRP3	«NOD-like receptor family, pyrin domain containing 3»
NOX	«NADPH oxydase»
Omega-3	n-3
oxLDL	LDL oxydés
PL	Phospholipides
PGC-1α	«peroxisome proliferator-activated receptor-γ coactivator-1α»
PNPLA3	«Patatin like phospholipase domain containing 3»
PPAR	«Peroxisome proliferator-activated receptor»
PPRE	«PPARG response element»
PUFA	Polyunsaturated FA
RE	Réticulum endoplasmique
RI	Résistance à l'insuline
ROS	Espèces d'Oxygène Réactives
SCD1	«Stearoyl-CoA desaturase 1»
SOx	Stress oxydatif
SREBP-1c	«Sterol regulatory element-binding protein-1c»
SyM	Syndrome métabolique
TG	Triglycérides
TNF-α	«Tumor Necrosis Factor-α»
UCP2	«Uncoupling protein 2»
VLDL	«Very low density lipoproteins»

“Connaître, ce n'est point démontrer, ni expliquer. C'est accéder à la vision”

Antoine de Saint-Exupéry/Pilote de guerre

«Nul ne peut atteindre l'aube sans passer par le chemin de la nuit»

-Gibran Khalil Gibran-

À Ceux qui m’ont quittée trop tôt pour mieux veiller sur moi!

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Introduction

La surcharge pondérale et l'obésité sont étroitement associées à de nombreux désordres métaboliques et à des risques élevés de développer des maladies chroniques. Les répercussions les plus répandues de l'excès de poids incluent le syndrome métabolique (SyM), le diabète non insulino-dépendant ou diabète de type 2 (DT2) et les maladies cardiovasculaires (MCV) (1-7). Actuellement, les défis de taille pour les scientifiques et les intervenants en santé consistent non seulement à réduire substantiellement la prévalence de l'obésité notamment en s'attaquant aux habitudes de vie, mais également en améliorant la prise en charge des sujets obèses menacés par la gravité des morbidités énoncées (1, 6, 8-18). L'approche nutritionnelle demeure le moyen par excellence privilégié pour atteindre un indice de masse corporelle acceptable (17, 19-22). Depuis la dernière décennie, l'engouement de toutes les couches de la société se maintient pour les aliments fonctionnels, un puissant moyen naturel de combattre les troubles métaboliques. Sans porter atteinte à l'alimentation saine, la tendance de plusieurs professionnels de la santé est de recommander certains aliments fonctionnels ou produits nutraceutiques qui vraisemblablement s'élèvent au-dessus des propriétés de base nutritive, en agissant en tant que nutriments thérapeutiques. Ainsi, des composantes bioactives sont présentement conseillées car elles exerceraient des effets bénéfiques sur la santé et le bien-être. Cependant, il est primordial de préciser les principaux sentiers et mécanismes impliqués dans le développement de l'obésité et ses complications afin de découvrir si les aliments fonctionnels peuvent efficacement les cibler et les corriger.

Le but central de cette thèse est *(i) d'apporter plus de lumière sur certains défauts cellulaires et moléculaires intervenant dans le tractus gastro-intestinal et le système hépatique dans des conditions cardiométaboliques anormales telles que l'obésité, le SyM et la stéatose hépatique non alcoolique (NAFLD); et (ii) d'examiner les mécanismes et l'action préventive/thérapeutique des acides gras polyinsaturés oméga-3 (AGPI n-3), un agent puissant antioxydant et anti-inflammatoire.*

Pour ce faire, un modèle animal et des cohortes humaines ont été utilisés pour évaluer les mécanismes édifiant le lien entre l'obésité et ses complications cardiométaboliques associées à l'environnement nutritionnel : 1) Un modèle de souris transgéniques soumises à une diète riche en lipides pour évaluer la prédisposition génétique face à une nutrition défavorable; 2) Des patients obèses avec NAFLD subissant une chirurgie bariatrique, mettant ainsi à notre disposition des spécimens biologiques (foie) pour dévoiler les mécanismes sous-jacents à la stéatose; et finalement 3) Un groupe d'enfants/adolescents avec obésité et NAFLD faisant partie d'un essai clinique pour examiner l'impact des AGPI n-3 sur plusieurs paramètres cardiométaboliques.

En outre, la dernière tâche de cette thèse est de prospecter la littérature scientifique disponible dans les champs du SyM et de la NAFLD afin d'en disséquer les forces et les faiblesses et d'en faire des synthèses extrêmement critiques publiables dans des journaux prestigieux au bénéfice de la communauté scientifique.

1. SYNDROME MÉTABOLIQUE

1.1 Définition

Le SyM représente, par définition, la coexistence d'un ensemble de conditions métaboliquement anormales qui augmentent considérablement le risque de développer le DT2, les MCV et la mort précoce. Selon le NCEP ATP III (National cholesterol education program-Adult Treatment Panel III), l'Organisation Mondiale de la Santé, et Fédération Internationale du Diabète, le SyM comprend au moins trois composantes parmi les critères suivants dans un contexte de stress oxydatif (SOx) et d'inflammation : obésité abdominale, intolérance au glucose/résistance à l'insuline (RI), dyslipidémie et hypertension artérielle (HTA) (**Tableau 1**), mais d'autres désordres peuvent s'y ajouter (prématurité, malformation cardiaque, cancer, etc.). L'intérêt grandissant pour le SyM proviendrait de sa prévalence très élevée (ex. USA : 22-24% et Canada : 18% chez les 17-79 ans) (23, 24), du risque de deux et cinq fois plus grand de causer le DT2 ou les MCV, respectivement (25-27), accompagnés de coûts socioéconomiques excessifs (28).

Tableau 1: Critères d'évaluation du syndrome métabolique

Critères	NCEP ATP III (Adultes)	OMS (Adultes)	FID (Adultes)	FID (E/A)
Obésité abdominale	Tour de taille: >102 cm (H) >88 cm (F)	Taille/hanche: >0,90 (H) >0,85 (F)	Tour de taille: ≥90-94 cm (H) ≥80 cm (F)	6-15 ans ≥ 90ème percentile 16+ ans Critères adultes
Tension artérielle	≥130/85 mm Hg	≥140/90 mm Hg	≥130/85 mm Hg	6-15 ans: si HF/DT2 16+ ans Critères adultes
Glycémie **À jeun**	≥5,6 mM	DT2 IG/RI	≥5,6 mM Diagnostic de DT2	6-15 ans: si HF/DT2 16+ ans Critères adultes
TG	≥1,7 mM	≥1,7 mM	≥1,7 mM	6-15 ans: si HF/DT2 16+ ans Critères adultes
HDL-C	<1,0 mM (H) <1,3 mM (F)	<0,9 mM (H) <1,0 mM (F)	<1,0 mM (H) <1,3 mM (F)	6-15 ans: si HF/DT2 16+ ans <1,03 mM

A : adolescents; CT : Cholestérol total; DT2 : diabète de type 2; E : enfants; F : femme; FID : Fédération Internationale du Diabète; H : homme; HF : histoire familiale; IG : intolérance au glucose; TG : triglycérides; OMS : Organisation Mondiale de la Santé

Même si la combinaison des caractéristiques cliniques du SyM indique une étiologie commune, l'évidence demeure encore vague. Certains mécanismes sont suggérés et, parmi eux, on souligne l'idée que l'obésité centrale et la RI sont au premier plan de la physiopathologie. À eux deux, ces facteurs peuvent faire affluer les autres composantes du SyM en faisant intervenir les dérangements hormonaux et la dérégulation de la signalisation cellulaire (29). Par ailleurs, il n'est pas improbable de penser que ces composantes, une fois amorcées, intensifieraient à leur tour l'accumulation de la graisse adipeuse et la perte de la sensibilité à l'insuline. Par exemple, la dyslipidémie ou mobilisation intense des acides gras (AG), en réponse à la RI, induirait l'intolérance au glucose et diminuerait davantage la sensibilité à l'insuline (30). D'autres écoles de pensée situent la RI à la base de la plupart des caractéristiques du SyM (31). En fait, la résistance aux actions métaboliques de l'insuline peut aisément expliquer l'intolérance au glucose, l'hypertriglycéridémie, les faibles concentrations des lipoprotéines à haute densité (HDL), le DT2 et même l'athérogénèse (31), ce qui conduirait donc à la maladie coronarienne et à la maladie cérébro-vasculaire associées toutes deux au SyM. Il est à espérer qu'au fur et à mesure que la compréhension de la pathogenèse du SyM évolue, les approches thérapeutiques soient plus percutantes. C'est d'ailleurs un des desseins de ce travail de doctorat.

1.2 Obésité

1.2.1 Épidémiologie de l'obésité

L'obésité est une préoccupation de santé publique mondiale et résulte d'une accumulation excessive de graisse corporelle qui peut nuire à la santé (4, 32). L'augmentation globale de la prévalence de l'obésité a des conséquences graves, contribuant à un nombre important de maladies dont le SyM, le DT2, les MCV, la NAFLD et même certains cancers (4, 6). Au-delà

des comorbidités, l'obésité a un impact social important et des coûts directs et indirects dramatiques dans les services de soins de santé (6). Plusieurs études définissent l'obésité comme un déséquilibre entre l'apport énergétique et la dépense énergétique (33). Aujourd'hui, cette définition apparait simpliste car le bilan énergétique représente un conglomérat de traits, chacun d'entre eux étant influencé par de nombreuses variables telles que le comportement, le régime alimentaire, l'environnement spatial, le statut économique, les structures sociales, et les facteurs métaboliques et génétique (34). Le résultat de cette interaction complexe entre l'ensemble de ces variables contribue vraisemblablement aux différences individuelles dans le développement de l'obésité.

Le constat actuel indique que la prévalence de l'obésité augmente continuellement malgré les campagnes de sensibilisation et les efforts pour convaincre les personnes avec surpoids de réduire l'apport énergétique et d'augmenter la dépense énergétique (35). Si l'augmentation de la prévalence de l'obésité a commencé à émerger dans les années 80, de plus en plus de pays s'incorporent maintenant dans la ''pandémie mondiale'' de l'obésité (35, 36). Entre 1980 et 2008, le changement global par décennie pour l'IMC moyen normalisé selon l'âge a été accru de ~0.4 kg/m^2 chez les hommes et ~ 0,5 kg/m^2 chez les femmes (35). En 2013, la prévalence globale du surpoids (IMC > 25 kg/m^2) et l'obésité (IMC > 30 kg/m^2) chez les hommes et les femmes était de 36,9 et 38,0 %, respectivement, rivalisant avec 28,8 et 29,8 %, respectivement, en 1980 (7). Si on ne considère que la prévalence de surpoids et d'obésité chez les adultes (> 18 ans) dans le monde entier, on note un bond de 857 millions de personnes en 1989 à 2,1 milliards en 2013 (36). Malheureusement, les enfants et adolescents ne sont pas épargnés puisqu'à la fois la prévalence dans cette couche de la société, le surpoids et l'obésité ont grimpé de 1980 à 2013 dans les pays développés de 23,8% chez les garçons et de 22,6 % chez les filles. Ironiquement, les pays en développement connaissent également une hausse de la prévalence d'obésité d'environ 5 % (1/4) pour l'ensemble des garçons et des filles car ils adoptent progressivement le mode de vie occidental. Ces valeurs représentent une augmentation moyenne d'environ 41% en 33 ans (36). Encore plus exagérée (80%) est présentement la prévalence chez les indiens Pima de l'Arizona depuis qu'ils ont adopté le mode de vie occidental alors que l'obésité était inexistante durant leur mode de vie traditionnel (8).

1.2.2 Étiologie de l'obésité

1.2.2.1 Facteurs environnementaux

Comme mentionné auparavant, l'obésité est définitivement une maladie complexe qui résulte d'une interaction entre une multitude de facteurs génétiques et environnementaux (4, 37). Si les progrès combinés de la génétique quantitative, de la génomique et de la bio-informatique ont permis de mieux comprendre les bases génétiques et moléculaires de l'obésité, la multitude de travaux concentrés sur l'environnement a permis d'en définir plusieurs facteurs (38-40).

Pour de nombreux pays, l'environnement est caractérisé par (i) un accès facile aux aliments peu nutritifs et très caloriques (gras, excès de sel et de sucre); (ii) une sédentarité et un milieu quotidien peu approprié et des aménagements peu favorables à la pratique de l'exercice (ex. vélo et marche); et (iii) le stress (38-40). À cela s'ajoute l'effet du genre, de l'âge, le tableau hormonal, les habitudes alimentaires et l'histoire familiale (13, 41, 42). Tous ces facteurs sont modifiables et nous disposons d'un bagage de connaissances formidable pour agir de façon préventive sur tous ces éléments de risque connus et abondamment documentés qui influencent notre « niche écologique » (**Figure 1**).

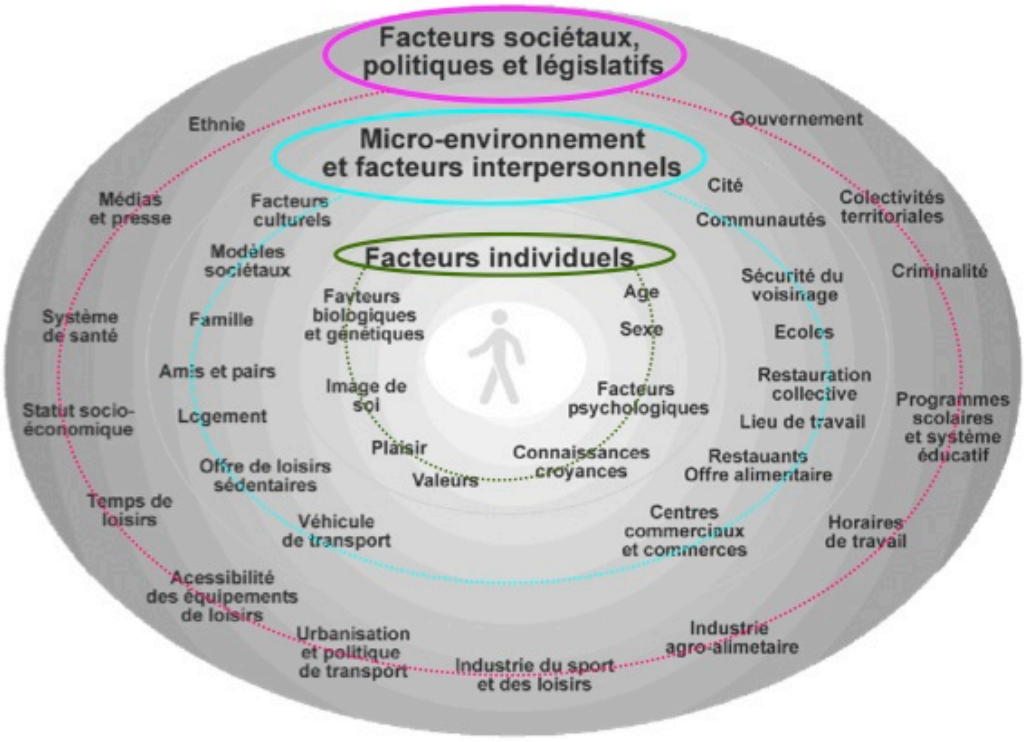

Figure 1. Facteurs influençant l'obésité via la niche écologique des individus.

La niche écologique d'un individu s'étend de son environnement personnel, comme le sexe, la tradition, la génétique, en passant par son environnement immédiat; le quartier, les amis, l'école, etc. jusqu'à l'environnement spatial et social élargi, incluant les lois gouvernementales, les infrastructures bâties ou économiques dans lequel vit l'individu, adaptée de (43, 44).

1.2.2.2 Facteurs génétiques

Les éléments qui militent en faveur d'une dimension génétique de l'obésité sont : (i) la concentration de cas dans une même famille étant donné que le risque d'obésité est ~2-8 fois plus élevé chez un individu présentant des antécédents familiaux comparativement à un individu sans histoire familiale d'obésité; (ii) le taux élevé de concordance chez les jumeaux monozygotes en ce qui a trait à la masse corporelle; et (iii) la découverte de gènes associés à l'obésité.

S'il existe des différences dans la susceptibilité de l'obésité chez les individus exposés au même environnement obésogène, impliquant les facteurs de risque génétiques, seulement un nombre limité de gènes défectueux a été décrit jusqu'à maintenant (45). Ces cas génétiques s'inscrivent dans le répertoire d'obésité monogénique qui fait référence à un certain nombre de formes rares d'obésité sévère résultant des mutations d'un gène individuel avec un effet amplifié (**Tableau 2**). Malgré la rareté de ces formes monogéniques de l'obésité, l'examen de leur base génétique sous-jacente a contribué significativement à notre compréhension de la pathogenèse de l'obésité puisqu'il a mis en lumière plusieurs voies et mécanismes impliqués dans le développement de cette condition (46-49). En outre, l'étude des formes monogéniques de l'obésité a également modifié notre perception de l'obésité, considérée actuellement comme une maladie mettant en évidence la contribution des facteurs neurologiques et endocriniens à son développement.

Tableau 2: Génétique de l'obésité monogénique

Gène	**Région**	**Phénotype**	**Mutation/ individus**	**Références**
LEP	7q32.1	Obésité sévère, Hyperphagie, hyperinsulinémie	2/6	(50, 51)
LEPR	1p31.3	Obésité sévère, croissance anormale	1/3	(50)
POMC	2p23.3	Obésité sévère, hyperphagie	3/8	(52)
MC4R	18q21.32	Obésité sévère, hyperinsulinémie	51/143	(50, 53)
MC3R	20q13.2-3		1/2	(54)
PCSK1	5q15	Obésité sévère chez les enfants, homéostasie anormale du glucose, hyperinsulinémie	1/1	(55)
SIM1	6q16.3	Obésité sévère	2/2	(56)

Mis à part ces cas rares, les formes les plus courantes d'obésité sont sans doute dues à la forme polygénique de l'obésité. C'est la combinaison de ces polymorphismes avec le milieu obésogène, qui entraîne le développement de l'obésité (**Tableau 3**). Environ 127 variantes

génétiques ont été proposées d'être associées avec l'obésité ou des phénotypes d'obésité (9), mais la réplication n'a pu être vérifiée que pour quelques-uns des polymorphismes (**Tableau 3**). Probablement, le manque de validation, évoqué par plusieurs études, pourrait provenir des différences dans la taille de l'échantillon, la caractérisation des patients, la puissance statistique des travaux, et les critères d'inclusion et d'exclusion (57). Pour ne donner qu'un exemple, mentionnons le gène *FTO* dont les variantes génétiques sont largement associées à l'obésité. Les études fonctionnelles montrent sa localisation dans le noyau cellulaire et son rôle dans la déméthylation de l'ADN, ce qui suggère son potentiel à réguler l'expression de plusieurs gènes (58). Les analyses du profil d'expression de *FTO* ont, de leur côté, révélé son expression dans de nombreux tissus, y compris l'hypothalamus, en association avec le volume du cerveau (59-61).

Tableau 3: Variantes génétiques de gènes spécifiques associées à l'obésité

Gènes	**Région**	**Polymorphismes**	**Références**
ADCY3-DNAJC27	2p23.3	rs6545814	(62, 63)
ADCY9	16p13.3	rs2531995	(63)
ALPK1	4q25	rs4833407	(64)
ARG1	6q23.2	rs2807278	(65)
ASAH1	8p22	rs17126232	(65)
BC041448	4q28.2	rs4864201	(64)
BDNF	11p14.1	rs2030323, rs988712, rs10767664, rs6265	(63, 65-68)
C5orf37	5q13.3	rs2307111, rs2112347	(63)
CADM2	3p12.1	rs13078807	(63, 68)
CAMK1G	1q32.2	rs12130212	(69)
CMYA5	5q14.1	rs259067	(70)
DNAJC27	2p23.3	rs4665736	(71)
ETV5	3q27.2	rs9816226, rs1516725	(63)
FANCL	2p16.1	rs887912, rs6731302	(63, 68)
FAT1, MTNR1A	4q35.2	rs925642	(65)

FBN2	5q23.3	rs374748	(72)
FLJ20309	2q33.3	rs7603514	(72)
FLJ39743	15q26.3	rs970843	(69)
FLJ35779, HMGCR	5q13.3	rs2112347	(68)
FTO	16q12.2	rs8043757, rs7185735, rs1421085, rs1558902, rs17817449, rs9941349, rs9939609, rs8050136, rs12149832, rs7202116, rs1121980, rs9930506, rs9940128	(63, 72-75)
GIPR	19q13.32	rs1800437, rs10423928	(63)
GNAT2	1p13.3	rs17024258	(63)
GNPDA2	4p12	rs10938397, rs13130484	(63, 68)
GPRC5B	16p12.3	rs12444979, rs11639988, rs12446554	(63, 68)
HNF4G	8q21.11	rs4735692	(63)
HOXB5	17q21.32	rs9299	(64)
HS6ST3	13q32.1	rs7989336	(63)
INHBB	2q14.2	rs7581710	(75)
IRS1	2q36.3	rs2943650	(76)
ITPR3	6p21.31	rs999943	(72)
KC6	18q12.3	rs17697518	(64)
KCNMA1	10q22.3	rs2116830	(65)
KCTD15, CHST8	19q13.11	rs29941	(66, 68)
LHFPL3	7q22.3	rs10953454	(75)
LINGO2	9p21.1	rs10968576, rs1412239	(63)
LOC144233	12q13.12	rs7138803, rs10875976	(63)
LRP1B	2q22.2	rs2890652	(68)
LRRN6C	9p21.1	rs10968576	(68)
MAF	16q23.2	rs1424233	(73)
MAP2K5	15q23	rs8028313, rs2241423	(63, 68)

MC4R	18q21.32	rs538656, rs11152213, rs10871777, rs17782313, rs571312, rs12970134	(63, 66, 68, 73)
MDFIC	7q31.2	rs7784447	(70)
MLN	6p21.31	rs2274459	(72)
MRPS33P4	20q13.2	rs13041126	(63)
MUC15	11p14.2	rs12295638	(72)
NCAM2	21q21.1	rs11088859	(75)
NEGR1	1p31.1	rs7531118, rs2815752, rs2568958	(63, 66, 68)
NPC1	18q11.2	rs1805081	(73)
NRXN3	14q31.1	rs7141420, rs2370983	(63)
NRXN3	14q24.3	rs11624704	(75)
OLFM4	13q14.3	rs9568867, rs9568856	(63, 64)
PCDH9	13q21.32	rs17081231	(75)
PITPNB	22q12.1	rs5762430	(75)
PRF1	10q22.1	rs10999409	(72)
PROX1	1q32.3	rs1704198	(70)
PTER	10p13	rs10508503	(73)
RABEP2,SH2B1	16p11.2	rs7184597	(63)
RAFTLIN	3p24.3	rs12635698	(72)
RARB	3p24.2	rs1435703	(72)
RGS6	14q24.2	rs699363	(69)
RPL27A	11p15.4	rs11042023	(63)
RPTOR	17q25.3	rs7503807	(63)
RTN4	2p16.1	rs6726292	(72)
SEC16B	1q25.2	rs633715, rs543874, rs10913469	(63, 66, 68)
SH2B1	16p11.2	rs7498665	(63, 73)
TFAP2B	6p12.3	rs2206277, rs987237, rs734597	(63, 68, 69)
TMEM18	2p25.3	rs6711012, rs10189761, rs3810291, rs2867125, rs6548238, rs7561317	(63, 68, 69)
TNNI3K	1p31.1	rs1514177, rs1514174, rs1514175	(63, 68)

TRAM1L1	4q26	rs10433903	(72)
UBE2E3	2q31.3	rs16867321	(75)
WWOX	16q23.1	rs9923451	(75)
ZNF248	10p11.21	rs7474896	(72)
ZZZ3	1p31.1	rs17381664	(63)

Les connaissances actuelles permettent de conclure que les facteurs génétiques peuvent être impliqués dans l'étiologie de l'obésité, et qu'à l'exception de très rares cas d'obésité sévère, les gènes en cause sont sans doute des gènes qui interagissent avec les facteurs de l'environnement. Des investigations supplémentaires sont absolument requises pour l'identification de polymorphismes fonctionnels (mutations), ce qui permettra de mieux cerner l'étiologie de l'obésité et de ses complications métaboliques, de repérer les individus ou les groupes à risque en fonction de leur profil génétique, d'évaluer les résultats des traitements actuels de l'obésité, de mettre en place la prise en charge personnalisée, et de développer des stratégies de prévention et de traitements individualisés.

1.2.3 Comorbidités associées à l'obésité

Les personnes obèses peuvent manifester divers symptômes et anomalies médicales dépendamment de la sévérité de l'obésité et des facteurs individuels. Nous ne mentionnerons que certains parmi les plus fréquents :

- Complications cardiométaboliques et cardiovasculaires: les marqueurs susceptibles de révéler ces anomalies sont; l'hyperglycémie à jeun, la RI, le DT2, un bilan lipidique anormal/dyslipidémie (comprenant l'élévation de facteurs comme les triglycérides (TG), l'HTA, le cholestérol total et le LDL-cholestérol en plus de la baisse du HDL-cholestérol (5, 77-79).
- Problèmes hépatiques: des cholestases et une NAFLD qui se complique en évoluant vers une stéatohepatite non-alcoolique (NASH) (12, 80);

- Troubles respiratoires: essoufflement important, syndrome d'apnée du sommeil associées à une somnolence, maladie pulmonaire obstructive chronique (81-84);
- Désordres squelettiques, articulaires et rhumatologiques : maux de dos, scoliose, arthrose de la colonne vertébrale, hernies discales, arthrose de la hanche et des genoux, etc.;
- Reflux gastro-œsophagien (85-87);
- Certains types de cancers, ex. le cancer de la prostate et de l'intestin pour les hommes, le cancer du sein et de l'utérus pour les femmes (88-90);
- Dérèglements endocriniens: TSH, cortisol, syndrome des ovaires polykystiques (règles irrégulières, acné, pilosité excessive), anomalies de la puberté : retard pubertaire chez le garçon et puberté avancée chez la fille, gynécomastie (développement des seins chez le garçon) et infertilité (91-93).

En plus des anomalies cliniques, l'obésité est aussi liée à des problèmes psychosociaux comme la faible estime de soi, la dépression, les troubles anxieux, la déscolarisation, l'isolement social et les troubles du comportement alimentaire. Des dizaines d'articles scientifiques et synthèses alertent quant au fardeau économique de l'embonpoint et de l'obésité en mettant en évidence les coûts directs liés à la prestation de services de santé et les coûts indirects relatifs aux pertes de productivité résultant de l'absentéisme du travail, de l'invalidité ou de la mortalité prématurée. Ces coûts indirects sont surtout calculés sur la base de la contribution que ces individus auraient pu apporter à l'économie, en l'absence de leurs problèmes de santé. Pour le Canada, on estime que les coûts de soins de santé supplémentaires associés à l'embonpoint et l'obésité représentaient en 2006 près de 4,1 % des dépenses totales de santé du pays (1). L'estimation du fardeau économique équivalait à 1,5 milliard de dollars pour l'année 2011, et supposément ces résultats sont des estimations conservatrices puisque les montants annuels n'incluent pas les autres coûts directs tels que ceux dus à la consommation de médicaments ou les services de professionnels de la santé autres que les médecins, ni d'ailleurs les coûts indirects tels que l'absentéisme et l'invalidité (94).

1.3 Obésité dans le Syndrome Métabolique

Si l'indice de masse corporelle (IMC) sert de mesure pour définir l'obésité, c'est principalement l'obésité abdominale ou centrale qui est qualifiée de « moteur » du SyM (95). L'IMC, équivalant au rapport du poids (en kg) sur la taille au carré (en mètre^2), se situe entre 18,5 et 24,9 pour le poids normal, entre 25 et 29,9 pour le surpoids, et $\geqslant$ 30 pour l'obésité. Cette dernière prend de nos jours la forme de pandémie et est devenue dans plusieurs pays un problème majeur de santé publique en vue de sa contribution à de nombreuses maladies chroniques (96). Si on prend comme exemple le risque d'infarctus du myocarde non fatal, il semble que l'obésité abdominale contribue à elle seule à 20% du risque (97). Par conséquent, plusieurs intervenants insistent sur la mesure de la circonférence (tour) de la taille et le ratio taille/hanche (**Tableau 1**) pour définir plus précisément les risques liés à l'obésité (98-101). Cette recommandation n'est pas superflue car elle tient compte du lien de l'obésité avec les désordres du métabolisme des lipides et des hydrates de carbone, ainsi que des perturbations de l'HTA, de l'inflammation et du SOx.

1.3.1 Obésité et résistance à l'insuline

L'accumulation des graisses à l'intérieur de la cavité abdominale (tissu adipeux intra-abdominal) est associée à l'intolérance au glucose et à une hyperinsulinémie découlant vraisemblablement d'un état de RI due à l'incapacité de l'insuline à stimuler l'entrée du glucose dans les tissus, à réduire la production hépatique de glucose et à réguler son métabolisme (102). La réaction amoindrie de l'organisme aux effets de l'insuline pour garder le contrôle sur les taux de glucose sanguin est surtout mise en évidence par le test d'intolérance au glucose qui révèle un état pré-diabétique et qui accroit les risques de MCV et d'HTA (103). Comme mentionné précédemment, les mécanismes impliqués dans la RI liée à l'obésité incluent les défauts pré-récepteurs, récepteurs et post-récepteurs. Ces derniers limitent par conséquent l'incorporation de l'insuline au muscle, secondaire à la surcharge des AG (pré-récepteur), à une modulation négative du récepteur de l'insuline suite à l'hyperinsulinémie (récepteur) et

l'inhibition des cascades de signalisation intracellulaire par plusieurs facteurs (AG, adipokines) liés à l'adiposité (post-récepteur) (104). Les AG en grande concentrations dans le tissu adipeux abdominal peuvent atteindre le foie via la veine porte induisant la RI hépatique et par conséquent la stimulation de la production de glucose (105). De même, les cytokines inflammatoires, libérées par la graisse viscérale dans la veine porte, provoquent l'insulinorésistance hépatique et même systémique (106). Dans le cas d'obésité avec la capacité réduite du tissu adipeux sous-cutané à se développer, il y aurait un débordement des AG (« spillover ») vers le compartiment graisseux viscéral et dans les tissus non adipeux (foie, muscle, pancréas, rein, os), résultant en une RI, lipotoxicité et apoptose cellulaire, compromettant ainsi la fonction des organes impliqués (107-109). D'autres facteurs interviennent dans le développement de l'insulino-résistance, tels que les aberrations de clairance de l'insuline circulante (104), le statut de la vitamine D dans l'altération de l'action de l'insuline (110), et l'expression du gène CEACAM1, un régulateur clé de la clairance hépatique de l'insuline (111), pour n'en citer que quelques-uns.

1.3.2 Obésité et hypertension

Un constat largement rapporté par la littérature est que 30% des patients avec une surcharge pondérale ou obèses présentent une HTA et que, d'un autre côté, 30% des hypertendus deviendront obèses (112, 113). Même si les deux entités apparaissent inter-reliées sur le plan épidémiologique, les liens causaux sous-tendant cette association sont insuffisamment établis. Certains mécanismes potentiels par lesquels l'obésité exerce une influence sur l'HTA ont été évoqués: (i) surcroît du tonus vasculaire dicté par le SOx accru (114); (ii) augmentation des concentrations de la diméthylarginine asymétrique (un inhibiteur endogène de l'oxyde nitrique) (115); et (iii) renforcement de l'expression de l'angiotensinogène par le tissu adipeux menant à une activation du système rénine-angiotensine (116). Néanmoins, le lien entre obésité et l'HTA est largement influencé par le type de tissu adipeux et sa distribution puisque les individus avec une obésité androïde présentent plus souvent des HTA plus sévères (117), soulignant que les facteurs induisant l'augmentation de l'HTA dans ces conditions sont ceux qui augmentent la fréquence cardiaque

et donc le débit ou les résistances vasculaires périphériques. Comme le nombre de cas d'obésité est en ascension depuis 1975 selon l'OMS, la prévalence de l'HTA a augmenté de façon spectaculaire (118). Par conséquent, le focus est sur le développement de stratégies censées prévenir ces maladies non transmissibles, notamment le SyM et l'obésité, et de traiter leurs complications telles que l'HTA, ce qui amoindrira les décès prématurés.

1.3.3 Obésité et inflammation

Dans le cas de l'association causale de l'obésité à l'inflammation, l'exemple souvent cité est surtout celui de la cytokine inflammatoire TNF-α (Tumor Necrosis Factor-α) étant donné (i) sa présence constante dans le tissu adipeux; (ii) son hyper-expression dans l'obésité; (iii) son effet médiateur de l'insulinorésistance dans de nombreux modèles animaux (119); et (iv) la restauration de la sensibilité à l'insuline via sa neutralisation (119). Cela démontre le concept du rôle de l'obésité dans la pathogenèse de la RI via l'intervention de mécanismes inflammatoires. Le contenu d'autres facteurs inflammatoires en grandes concentrations dans la circulation renforce ce concept (120-123). Le contenu de nombreux médiateurs inflammatoires a été documenté dans le tissu adipeux. Par conséquent, l'expansion de l'obésité crée un environnement pro-inflammatoire capable d'influencer l'émergence de la RI et d'autres composantes du SyM. Dans ce contexte, il est à noter que les macrophages résidant dans le tissu adipeux peuvent également être une source de facteurs pro-inflammatoires aptes à moduler l'activité sécrétoire des adipocytes (124). Un élément important qui peut contribuer à l'inflammation métabolique est le facteur inhibiteur de la migration des macrophages des cytokines (MIF). Plusieurs observations suggèrent la libération de MIF par le tissu adipeux et son implication dans les processus métaboliques et inflammatoires qui sous-tendent le développement de pathologies liées à l'obésité (125-128) (**Figure 2**).

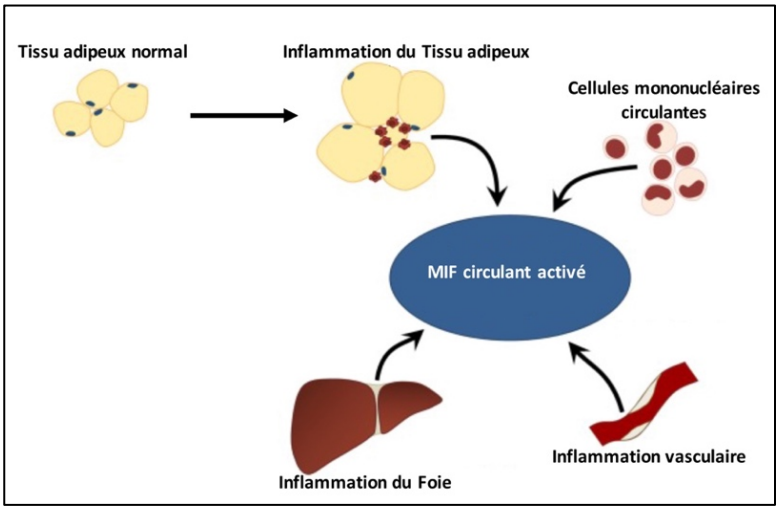

Figure 2. L'inflammation métabolique accentuée par l'activation du MIF dans l'obésité.

Le facteur inhibiteur de la migration des macrophages des cytokines (MIF) est libéré par le tissu adipeux et participe au processus inflammatoire et au développement des pathologies liées à l'obésité, adaptée de (125).

Par ailleurs, on ne pourrait passer sous silence les adipokines telles que la leptine, l'adiponectine et la résistine qui exercent une influence métabolique évidente dans ce contexte (129). Si la leptine est connue comme signal de satiété bien capable d'inhiber la consommation alimentaire et d'exercer une modulation de la fonction sexuelle et immunitaire, elle détient également un rôle pro-inflammatoire et pro-agrégatif plaquettaire (129, 130). Dès lors, des concentrations élevées de leptine peuvent contribuer à l'état pro-inflammatoire de l'obésité et à l'athérogenèse à long terme. Par contre, l'adiponectine, sécrétée en abondance par les adipocytes chez les sujets sains, est anti-inflammatoire et potentiellement anti-athérogène. Contrairement à la leptine, sa concentration diminue avec le gain pondéral et encore plus avec l'obésité (130, 131). On considère actuellement que la production de médiateurs inflammatoires par un tissu adipeux dysfonctionnel entraîne le développement de formes plus complexes de désordres tels que le DT2 et la NAFLD (125).

1.3.4 Obésité et stress oxydatif

La prévalence croissante de l'obésité est liée au dérèglement de l'apport calorique face à la dépense énergétique. Un bilan énergétique positif favorise le SOx qui représente probablement un lien notable entre l'obésité et les complications connexes, y compris la RI. Par exemple, les AG libres qui résultent d'un apport élevé en graisses et en hydrates de carbone chez les rongeurs et les humains obèses peuvent favoriser la production d'espèces réactives oxygénées (ROS) en raison de la saturation de la chaîne de transport des électrons dans les mitochondries (132, 133). À son tour, le SOx peut amplifier l'accumulation de graisse en stimulant la prolifération pré-adipocytaire, la différenciation adipocytaire et la taille des adipocytes matures (133-135). Il a également été observé que le SOx contrôle la prise alimentaire et le poids corporel en exerçant divers effets sur les neurones hypothalamiques avec un impact concomitant sur la satiété et la faim (136). Lorsque l'obésité s'installe, le tissu adipeux devient un site idéal capable de produire des ROS via la modulation de NADPH oxydase (NOX) (133, 137).

De faibles niveaux de molécules antioxydantes (138, 139) ainsi que l'augmentation des produits protéiques à oxydation élevée, des lipoprotéines de basse densité oxydées (oxLDL), des substances réactives à l'acide thiobarbiturique (140) et d'autres indicateurs cellulaires/systémiques ont été rapportés chez des sujets obèses. Les activités des enzymes antioxydantes, y compris le glutathion peroxydase (GPx) et la catalase, se sont révélées être nettement diminuées dans l'obésité (141, 142). Les défenses antioxydantes, qui normalement protègent contre les processus infectieux et contribuent aux fonctions normales telles que la prolifération, la différenciation et la signalisation cellulaire (143-146), sont moins capables de piéger les ROS lorsqu'elles sont diminuées. Bien qu'une relation inverse ait été observée entre la capacité antioxydante totale et la graisse viscérale, indépendamment des autres variables (147), la relation inverse entre l'IMC et le pourcentage de graisse corporelle totale n'a pas pu être clairement démontrée (148). Une explication possible est que différents tissus tentent à travers des mécanismes compensatoires d'augmenter les défenses antioxydantes afin de contrer le SOx.

Les NOX dans les cellules vasculaires constituent un système de ROS majeur qui peut déclencher des composantes en aval telles que l'oxyde nitrique synthase et l'induction du SOx dans les mitochondries (149). Fait intéressant, des ROS vasculaires élevées, chez des souris surexprimant la p22phox et sous un régime riche en graisses, déclenchent l'obésité et même un phénotype du SyM en augmentant l'inflammation, en favorisant l'adipogenèse et en développant une intolérance à l'exercice (150). Le SOx induit une accumulation de graisse et participe à la pathogenèse du SyM associé à l'obésité par la synthèse dérégulée d'adipocytokines (**Figure 3**).

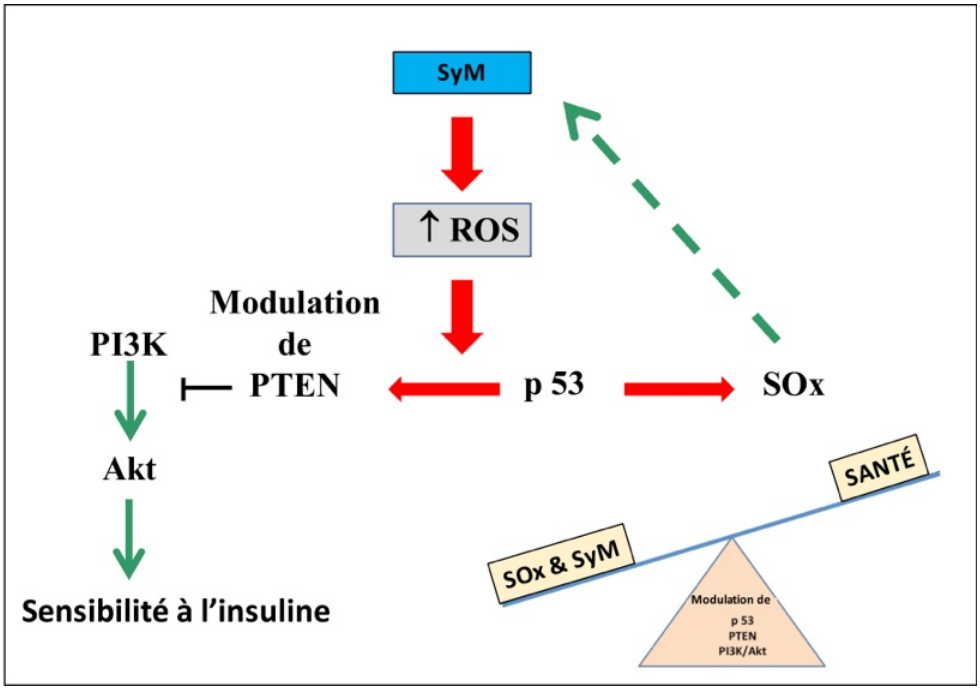

Figure 3. Rôle du SOx dans le SyM.

Les concentrations élevées de ROS pourraient inactiver PTEN « Phosphatase and tensin homolog », un membre de la famille des protéines tyrosine phosphatase, soit de manière directe en oxydant son résidu de cystéine, soit indirectement en stimulant sa phosphorylation, interférant ainsi avec la présence de p53 à la membrane plasmique et entraînant l'activation de la cascade de signalisation PI3K/Akt (151), (© Spahis).

Par ailleurs, l'accumulation de graisse dans le tissu adipeux induit une production de ROS qui affecte l'intégrité génomique et modifie l'expression de p53, conduisant à une inflammation du tissu adipeux, à une RI locale et à des troubles cardiométaboliques.

Alors que plusieurs groupes conviennent que l'obésité peut causer des dérangements métaboliques et qu'elle précède et prédispose au développement du SyM via la résistance à la leptine (150), l'induction de la résistance à la leptine par les ROS vasculaires peut altérer la biogenèse et la sécrétion de l'insuline par les cellules β, constituant ainsi un cycle vicieux exacerbant le gain de poids (152). Un appui supplémentaire de ce concept peut être reflété par la diffusion de ROS généré par NOX aux mitochondries pour activer le SOx, provoquant ainsi un dysfonctionnement mitochondrial, limitant la capacité d'exercice musculaire et favorisant le gain de poids (153). En conséquence, les souris dépourvues de l'enzyme antioxydante mitochondriale « manganese-dependent superoxide dismutase (MnSOD) » présentent de graves perturbations (154). Collectivement, ces résultats suggèrent l'existence de multiples mécanismes d'élaboration du SOx par les ROS dans le SyM.

1.4 Dyslipidémie dans le Syndrome Métabolique

Comme mentionné auparavant, le foisonnement des AG libres dans la circulation contribue fortement à la pathogenèse de la RI. Les fortes concentrations bloquent la transduction du signal d'insuline par des actions spécifiques (155, 156). Par ailleurs, l'accumulation des AG libres entraîne l'induction du SOx, de l'inflammation et de la réactivité vasculaire, en plus de provoquer une RI (157). La RI active la lipase hormono-sensible adipocytaire qui stimule la lipolyse des TG dans le tissu adipeux et augmente la concentration de AG libres, ce qui finit par produire un cercle vicieux de lipolyse, d'augmentation d'AG libres, de RI, de SOx et d'inflammation.

Une des caractéristiques du SyM est la faible concentration des HDL en association avec le taux élevé des TG circulants. On pense que c'est le résultat d'une diminution de la biosynthèse

de l'apolipoprotéine (Apo) A-I étant donné la présence de la RI (158); l'insuline étant l'activateur de la transcription du gène Apo A-I (159). Une autre raison de la baisse des niveaux des HDL dans la circulation sanguine proviendrait de l'hydrolyse des TG (comme composantes des HDL) par la lipase hépatique, réduisant ainsi la taille des particules de HDL qui sont alors filtrées par les reins (160).

En parallèle avec la prévalence du SyM, la dyslipidémie s'accroit de façon déconcertante, et plus de 85% des adultes de race blanche présentent une élévation légère à modérée des TG et un faible taux de HDL-cholestérol circulants (161). Il est probable que la perte de la sensibilité à l'insuline attise la production des lipoprotéines hépatiques de très basse densité (VLDL), tout en limitant l'activité de la lipoprotéine lipase, ce qui favorise l'hypertriglycéridémie (162, 163). En outre, des patients avec le SyM sont caractérisés par des hausses de LDL petits et denses connues pour accélérer l'athérosclérose (164). La **Figure 4** illustre bien le portrait des anomalies lipoprotéiniques dans les conditions de SyM.

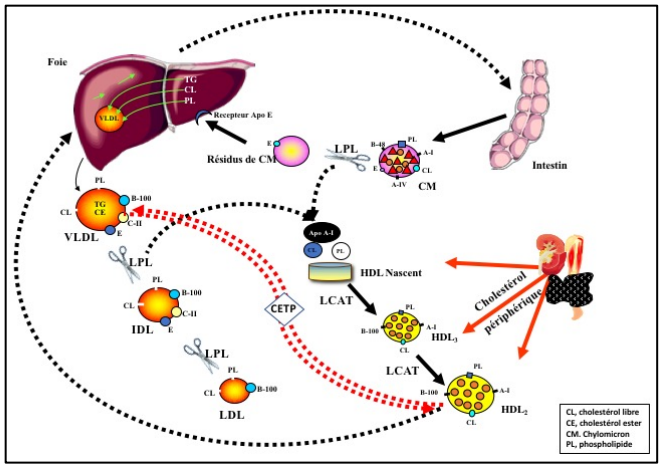

Figure 4. Anomalies lipidiques dans le SyM.

Augmentation de la production hépatique des VLDL riches en triglycérides, des LDL petites et denses et un enrichissement en cholestérol des LDL et HDL (© Spahis).

Un profil lipidique normal (A) se présente avec une sécrétion minime de lipoprotéines VLDL & LDL et une plus grande richesse en HDL. Dans un état de dyslipidémie (B), il y a une production accrue de lipoprotéines (VLDL & LDL) petites et denses, riches en TG et une diminution de quantité de HDL Adapté de (165).

Comme l'hypertriglycéridémie engendre un risque non négligeable d'induire des accidents vasculaires, il devient important d'être vigilant à son égard surtout chez les sujets porteurs de SyM. D'ailleurs, des études de randomisation mendélienne montrent le rôle des TG dans l'association entre la variation génétique des gènes APOA5 et LPL et l'infarctus du myocarde (166, 167). Un des mécanismes causaux de cette manifestation pourrait être l'infiltration des résidus de chylomicrons (CM) dans l'espace sous-endothélial de la paroi artérielle, conduisant à leur phagocytose rapide par les macrophages qui se transforment en cellules spumeuses et initient le processus athérosclérotique (168). D'autre part, l'hypertriglycéridémie contribue au dysfonctionnement endothélial, à la coagulopathie, à la thrombose, à la stéatose hépatique, à l'inflammation et au SOx (169, 170).

L'autre désordre lipidique contribuant à l'augmentation du risque cardiométabolique dans le SyM est la dyslipidémie postprandiale, surtout dans les conditions d'excès du tissu adipeux viscéral et de l'insulinorésistance (171-177) (**Figure 5**).

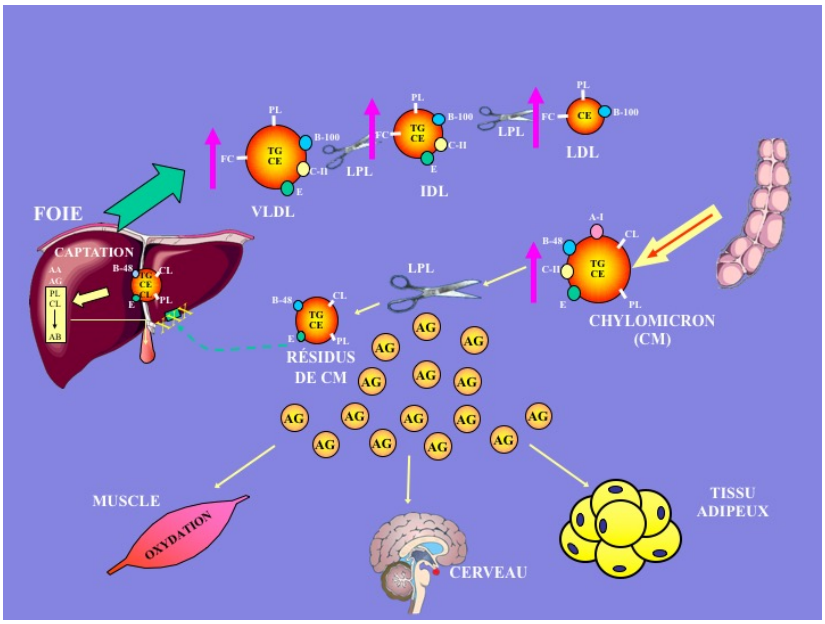

Figure 5. Dyslipidémie postprandiale et émergence du SyM.

La surproduction hépatique de particules VLDL riches en TG, induite par l'excès des AG libérés du tissu adipeux, l'intestin grêle est responsable du développement de la dyslipidémie postprandiale (© Spahis).

La réponse exacerbée de l'intestin en période postprandiale a été démontrée dans le modèle murin (172, 178, 179), les gerboises *Psammomys obesus* (171, 175, 180, 181) et chez les obèses morbides (177), tous affichant les caractéristiques du SyM. Nous avons proposé que l'insulinorésistance des entérocytes soit à l'origine des aberrations de l'absorption des graisses alimentaires. Des efforts ont été déployés dans le présent projet de recherche pour approfondir les mécanismes de la surproduction des CM dans les conditions de SyM et complications cardiométaboliques tout en évaluant l'impact des aliments fonctionnels.

1.5 Stress Oxydatif dans le Syndrome Métabolique

Comme pour l'obésité, les patients avec SyM se caractérisent par une augmentation inappropriée des AG libres (182, 183) capables d'activer la production de ROS (184). Bien que le mécanisme soit encore assez mal compris, il a été démontré que l'augmentation des AG libres intensifie la production de précurseurs de radicaux libres tels que les anions superoxydes ($O_2^{\bullet -}$), les radicaux hydroxyles (OH•) et les peroxydes d'hydrogène (H_2O_2) dans les cellules musculaires lisses et endothéliales vasculaires (184). D'autre part, l'exposition des tissus adipeux au SOx dans des conditions de SyM entraîne une diminution de l'adiponectine anti-inflammatoire et une augmentation des cytokines proinflammatoires (185), ce qui compromet la signalisation de l'insuline via l'induction de la phosphorylation en sérine des récepteurs de l'insuline et la détérioration de la translocation du transporteur de glucose 4 et de la transcription de son gène (186).

Des études supplémentaires ont rapporté le rôle régulateur du facteur de transcription p53, un suppresseur de tumeur et un agent protecteur du génome, en relation avec l'expression de GPX1, l'isoforme le plus abondant de la famille GPX capable de détoxifier H_2O_2 (187). Dans des conditions pathophysiologiques telles que le SyM, la suppression de p53 régule à la baisse l'expression basale de GPX1 et de SOD2, ce qui déclenche la production cellulaire de ROS, conduisant à des dommages oxydatifs, alors que la restauration des niveaux physiologiques de p53 rétablit les enzymes antioxydantes et décroit la formation des ROS (188 , 189 , 190) **(Figure 6)**.

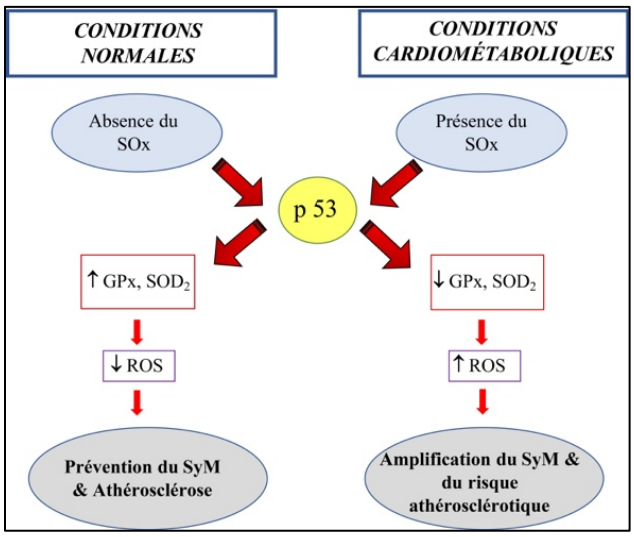

Figure 6. Implication du p53 en présence ou absence du SOx.

L'expression du p53 a de larges incidences sur la santé. La protéine p53 peut diminuer ou augmenter le SOx, influençant ainsi le contrôle ou la progression de l'obésité, du SyM et des MCV. p53 peut aussi agir à la fois comme un sensibilisateur et un activateur du SOx. Dans ce contexte, ROS peut induire p53, qui favorise l'activité antioxydante dans des conditions normales et l'activité pro-oxydante dans les conditions cardiométaboliques (© Spahis).

1.6 Inflammation dans le Syndrome Métabolique

De nombreuses preuves sont présentes pour souligner l'association étroite entre l'inflammation et le SyM (191). Les marqueurs inflammatoires ont la prépotence d'instruire sur la sensibilité des patients avec le SyM à développer les processus athérosclérotiques et à connaître les évènements coronariens et cardiovasculaires (192). Ces marqueurs inflammatoires comprennent, par exemple, la C Reactive Protein (CRP), le fibrinogène, les interleukines, le TNF-α, le PAI-1, l'amyloïde sérique A et les adipokines (leptine, resistine, adiponectine). Ces derniers peuvent, en présence du SOx, déployer l'effet multiplicatif des composantes du SyM à engendrer les lésions vasculaires (193, 194). À l'image du CRP, les protéines inflammatoires montrent un pouvoir de prédiction et une excellente corrélation, non seulement avec la RI mais aussi avec les caractéristiques du SyM (195-197) (**Figure 7**).

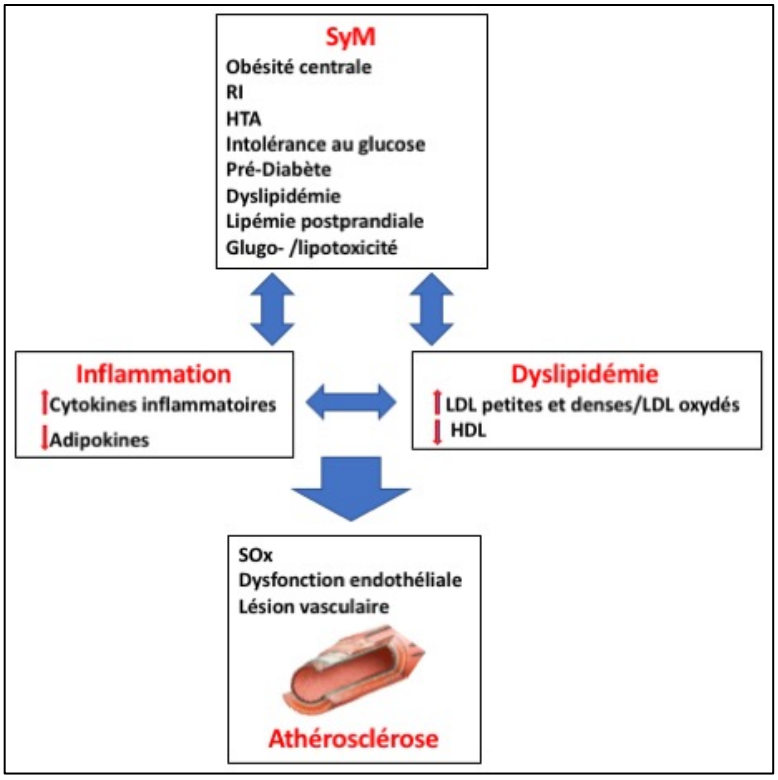

Figure 7. Dyslipidémie, Inflammation émergente et processus athérosclérotique dans le SyM.

L'inflammation constitue le lien entre les composantes du SyM et les événements cardiovasculaires, adaptée de (198).

Il est important de documenter sur la portée de l'inflammation émanant du tissu adipeux dans le SyM. L'obésité induit l'accumulation marquée de cellules immunitaires (p. ex. macrophages) dans le tissu adipeux des humains et des rongeurs (199-201). Bien qu'il ait été démontré que les adipocytes eux-mêmes produisent des cytokines pro-inflammatoires et les chimiokines (202), les macrophages au niveau du tissu adipeux participent activement à l'état pro-inflammatoire, et leur recrutement est corrélé avec le degré d'obésité et l'inflammation systémique, la RI et le SyM (203). De plus, un nombre plus faible de lymphocytes T régulateurs anti-inflammatoires a été rapporté dans le tissu adipeux viscéral d'individus obèses avec SyM comparativement à des individus obèses sans SyM (métaboliquement sains) (203). D'autres tissus, comme le foie et le muscle, peuvent être porteurs de facteurs pro-inflammatoires et

contribuer, durant le SyM, à la progression de NAFLD/NASH (204, 205). Finalement, l'inflammasome NLRP3 « NOD-like receptor family, pyrin domain containing 3 » constitue une autre voie contribuant à la RI et au SyM (203, 206) (**Figure 8**).

L'inhibition du NLRP3 a montré des effets pléiotropiques combinant une meilleure signalisation de l'insuline dans le tissu adipeux, le foie et le muscle squelettique, et une augmentation de la sécrétion d'insuline par le pancréas (206, 207). Actuellement, on considère le SyM comme une maladie inflammatoire de bas grade (193).

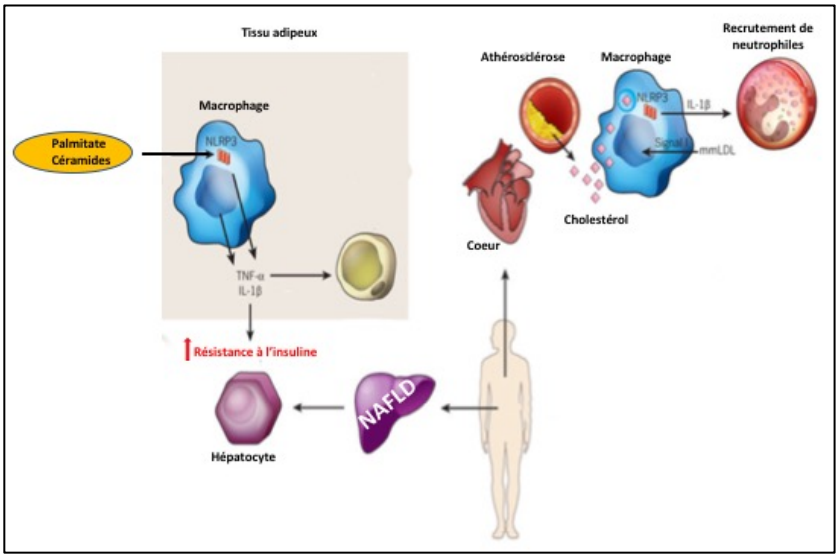

Figure 8. Rôle de l'inflammasome NLRP3 dans le SyM.

Durant l'obésité, l'inflammasome NLRP3 est activé dans de multiples tissus. Dans le tissu adipeux, le palmitate et les céramides activent NLRP3 dans les macrophages, ce qui conduit à une augmentation de la résistance à l'insuline et l'émergence de la NAFLD. De plus, des cristaux de cholestérol dans les artères activent NLRP3 dans les macrophages, favorisant l'infiltration des cellules inflammatoires et l'augmentation de la progression de l'athérosclérose, adaptée de (208).

2. BARRIÈRE INTESTINALE ET ENDOTOXÉMIE

Selon la définition la plus communément acceptée, la muqueuse intestinale fait figure de barrière intestinale. Si la fonction première de la muqueuse intestinale est d'assumer la fonction d'absorption des aliments, elle assume simultanément un contrôle rigoureux de la translocation du contenu luminal, empêchant ainsi le passage de produits indésirables ou nocifs à la circulation. Dans le cas d'obésité et de troubles cardiométaboliques, des perturbations de la barrière intestinale ont été rapportées, ce qui entraine son dysfonctionnement et permet l'accès des microbes, des produits microbiens et des antigènes étrangers à la paroi intestinale, à la circulation sanguine et à l'ensemble de notre organisme (209).

2.1 Épithélium Intestinal

Les travaux des dernières années ont révélé la compartimentation impressionnante et la complexité structurelle et fonctionnelle de l'épithélium intestinal. Ce dernier est composé de différents types cellulaires tels que les entérocytes absorbant les nutriments, les cellules caliciformes « goblet cells » produisant du mucus, les cellules de Paneth synthétisant les peptides antimicrobiens et les cellules entéro-endocriniennes générant les peptides gastro-intestinaux régulateurs. Ces diverses cellules sont toutes enfouies dans la monocouche épithéliale et se renouvellent tous les 4-5 jours (210). Par ailleurs, cette monocouche et le tissu sous-jacent abritent une pléthore de cellules immunitaires telles que les cellules B, les cellules lymphoïdes innées, les macrophages, les cellules dendritiques, les cellules Tγδ et les cellules Tαβ, qui s'intercalent entre les cellules épithéliales et juste au-dessous de la membrane basale (211, 212). Cependant, l'élément central de la barrière intestinale demeure la cellule épithéliale absorptive (entérocyte) qui migre à partir des cellules souches à la base de la crypte jusqu'à la pointe des villosités. (**Figure 9**).

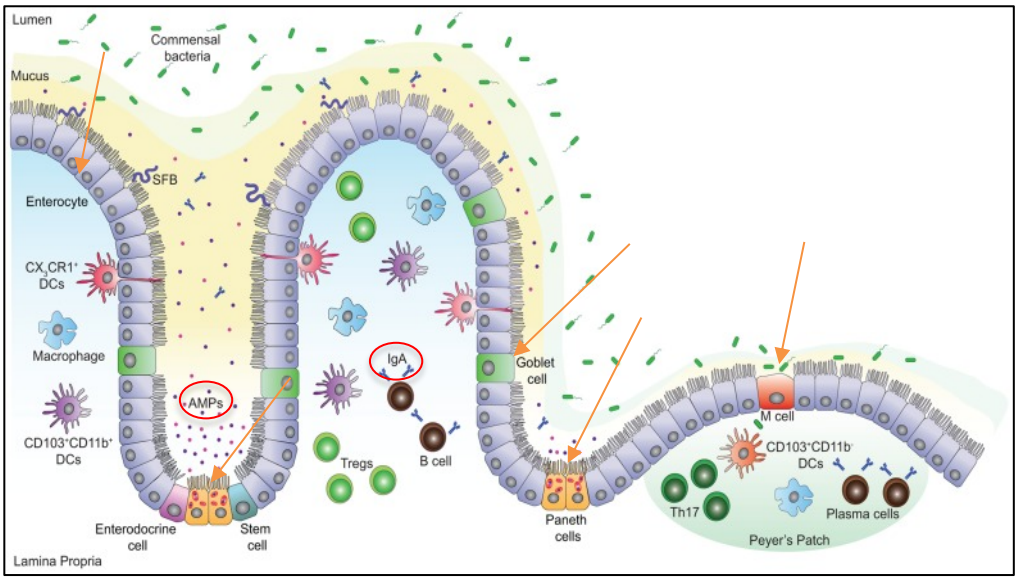

Figure 9. Muqueuse intestinale en état d'équilibre homéostatique.

La barrière épithéliale intestinale est une muqueuse de surface hautement organisée qui empêche l'entrée des microbes dans la lamina propria. L'épithélium est constitué d'une seule couche de cellules épithéliales intestinales (CEI) recouvertes d'une nappe de mucus stratifiée. Contrairement à la couche de mucus externe qui est colonisée par des espèces commensales, la couche de mucus interne est principalement dépourvue de bactéries. Elle contient l'immunoglobuline A (IgA) et des peptides antimicrobiens (AMP) qui empêchent les espèces commensales d'interagir avec la surface des CEI. Il existe cinq lignées de CEI : des entérocytes, des cellules caliciformes productrices de mucus, des cellules entéroendocrines fournissant les peptides hormonaux, des cellules de Paneth formant l'AMP à la base des cryptes, et enfin des cellules M qui échantillonnent les antigènes de la lumière intestinale pour les présenter aux cellules immunitaires voisines. Un grand nombre de lymphocytes T, de macrophages, de cellules B sécrétant des IgA et de plasmocytes sont présentes dans la lamina propria et dans les plaques de Peyer. De plus, les cellules dendritiques (CD) favorisent le développement des cellules T auxiliaires (Th17) et T régulatrices (Treg), adaptée de (213).

Pour maintenir l'homéostasie et l'intégrité de l'axe crypte-villosités, l'exfoliation des cellules épithéliales après le processus apoptotique est contrebalancée par la division cellulaire

dans la région de la crypte (214, 215). La migration épithéliale est intimement liée à l'excrétion cellulaire impliquant des mécanismes complexes régulés par des intégrines (216, 217), des héparanes sulfate protéoglycanes (218), des facteurs de croissance (219), des cytokines (220) et des récepteurs de chimiokines (221) ainsi qu'une matrice extracellulaire formée entre autres des laminines et du collagène IV (222).

2.1.1 Jonctions serrées

Les cellules épithéliales sont mises en réseau par des contacts adhésifs protéiniques appelés jonctions. Ce sont les zones étroitement associées de deux cellules dont les membranes se rejoignent pour former une barrière pratiquement imperméable aux fluides. Ce scellement paracellulaire, constitué de 5-7 sites de fusion membranaire appelés « points d'embrassement » dicte le flux d'ions à travers le tissu et maintient la polarité cellulaire (223, 224). En fait, les jonctions serrées (JS) régulent d'une part le flux des ions et des solutés « gate function », et maintiennent d'autre part la polarité cellulaire « barrier function » (225). Remarquablement, les JS servent de barrière aux bactéries et aux produits bactériens en arrimage avec les protéines de la membrane plasmique apicale et avec les mucines glycocalyx, au niveau de la lumière des cellules épithéliales. Comme exemple, citons les protéines de la famille des claudines, des composants essentiels aux JS. Les claudines (~24 types chez l'homme) forment des brins de JS en se polymérisant dans la membrane plasmique et en se dimérisant avec des claudines sur des cellules apposées, à travers l'espace extracellulaire, pour générer le scellement paracellulaire (226). On pense que la perméabilité des JS dérive au moins en partie des quantités relatives de claudines formant ou scellant les pores dans l'architecture décrite (225). Outre les claudines, il existe trois classes supplémentaires de protéines transmembranaires dans les JS: l'occludine, la tricelluline et les molécules d'adhésion jonctionnelles (227). Une "plaque" dense de molécules d'échafaudage est également ancrée aux protéines transmembranaires et comprend les protéines Zonula occludens « ZO-1 » et les protéines de la famille MAGUK (225). Ces protéines d'échafaudage relient les protéines transmembranaires aux kinases et aux molécules de signalisation qui se localisent à la jonction. Ces dernières contrôlent à leur tour non seulement

les contacts cellule-cellule, mais aussi la machinerie de polymérisation de l'actine et l'appareil de contractilité de l'actine et de la myosine apicales (225). Des observations associent un dérèglement de la barrière mucosale à l'architecture des JS et à l'expression des claudines, en particulier dans l'étiologie des maladies cardiométaboliques (228, 229) (**Figure 10**).

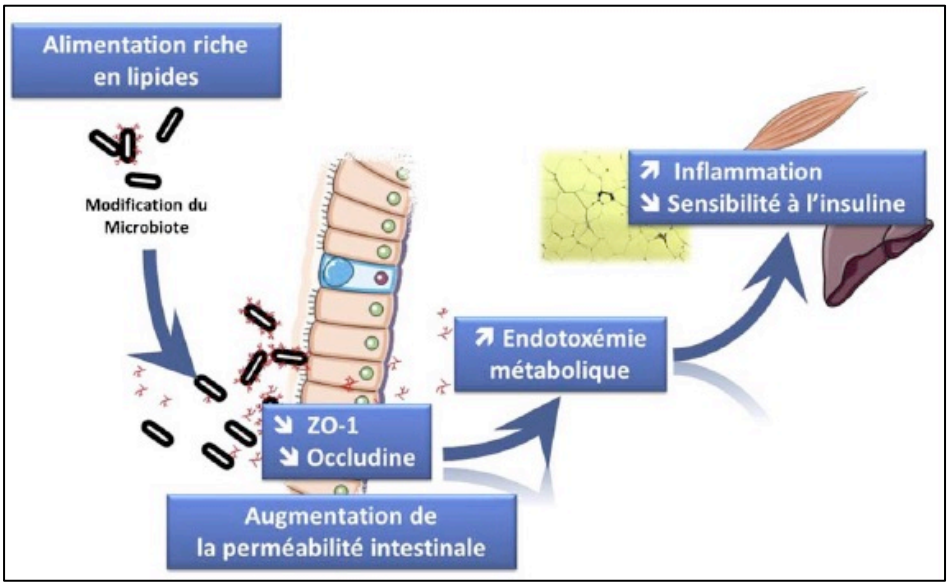

Figure 10. Altération de la barrière intestinale dans l'obésité.

Une alimentation riche en gras et en sucre peut altérer la barrière intestinale, en diminuant les protéines clés des JS (ZO-1, occludine), en augmentant l'endotoxémie, tout en activant l'inflammation et l'instauration d'une RI systémique, adaptée de (230).

2.1.2 Endotoxémie

Récemment, l'endotoxémie métabolique est apparue comme un facteur possible dans l'étiologie du SyM. Les endotoxines circulantes produites par la microflore intestinale et absorbées dans la circulation peuvent stimuler les récepteurs de type Toll, entraînant l'activation

du facteur nucléaire κB (NF- κB), la sécrétion de cytokines pro-inflammatoires et la RI (231, 232). L'endotoxémie est probablement causée par 2 facteurs principaux. Premièrement, les altérations de la microflore intestinale sont susceptibles d'augmenter la production d'endotoxines dans l'intestin. Effectivement, des études animales et humaines ont démontré qu'en présence d'un régime alimentaire riche en graisses, l'obésité et le DT2 peuvent altérer la composition du microbiote intestinal et la production d'endotoxines intestinales (230, 233-240). Deuxièmement, la perméabilité intestinale élevée facilite l'absorption des endotoxines paracellulaires dans la circulation via les JS épithéliales (232, 241). Une confirmation est obtenue chez les souris exhibant une altération de l'expression des protéines épithéliales des JS, concomitamment à l'élévation de la perméabilité dans des conditions de SyM, d'obésité et de diabète (230, 236, 238) (**Figure 11**). Actuellement, on considère que la santé intestinale est devenue une nouvelle frontière en médecine préventive et thérapeutique visant divers troubles gastro-intestinaux et métaboliques.

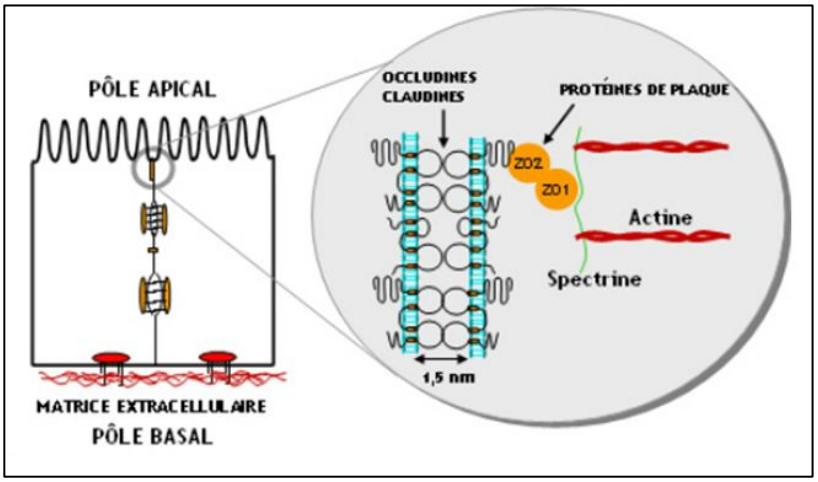

Figure 11. Principales familles de molécules d'adhésion dans l'intestin.

Les JS sont composées de plusieurs types d'occludines, claudines et de protéines d'adhésion jonctionnelle (JAM). Toutes les protéines jonctionnelles transmembranaires interagissent avec des protéines d'échafaudage intracellulaires (telles que ZO-1, -2 et -3) qui interagissent avec d'autres protéines, y compris l'actine dans le cytosquelette, adaptée de (242).

2.1.3 Microbiote intestinal

L'intestin humain fourmille de nombreux microbes tels que les bactéries, les virus et les levures composant le microbiote, précédemment nommée flore intestinale. On estime que 100 000 milliards de bactéries colonisent de façon permanente le tractus digestif d'un seul individu et appartiennent à ~1000 espèces, ce qui illustre bien la grande diversité taxonomique (243). Le métagénome ou nombre total de gènes du microbiote semble dépasser de 150 fois le génome humain (3,3 millions vs. 25000); le côlon abritant la plus importante concentration de bactéries, avec une densité atteignant 10^{11} bactéries pour un gramme de contenu (244, 245). L'exploration de cette écologie microbienne a connu une expansion considérable grâce au développement de techniques dites métagénomiques faisant appel au séquençage de l'ADN (**Figure 12**).

Des équipes françaises ont analysé 396 échantillons de selles provenant d'espagnols et de danois et ont été capables d'identifier 741 espèces de bactéries dont 85% étaient jusque-là inconnues (240). L'analyse métagénomique a aussi conduit à la reconstitution du génome complet de 238 bactéries intestinales et la mise en évidence de maintes associations de dépendance entre ces différents groupes bactériens (246). La multitude de connaissances émergeant de nombreuses études nous approchent de la compréhension du fonctionnement global de la population microbienne intestinale et de ses interactions avec l'organisme qui les héberge. Par contre, seules certaines espèces peuvent être cultivées à partir des selles alors que la majorité demeure non cultivable (247).

(A) Arbre phylogénétique de 5,088 séquences d'ARNr 16S associées au cæcum de souris rapportées dans cette étude et 11 831 séquences d'ARNr 16S associées au côlon humain. Les divisions dominantes chez les souris et les humains sont colorées en rouge, les divisions plus rares en bleu et les divisions non détectées en noir. B) Arbre phylogénétique de la bactérie montrant les divisions décrites. (C) Arbre de parcimonie maximale montrant des taxons représentatifs des cyanobactéries en vert, y compris les séquences chloroplastiques des eucaryotes. Les séquences détectées dans les tractus gastro-intestinaux des animaux sont en brun, et celles détectées dans d'autres environnements sont en violet.

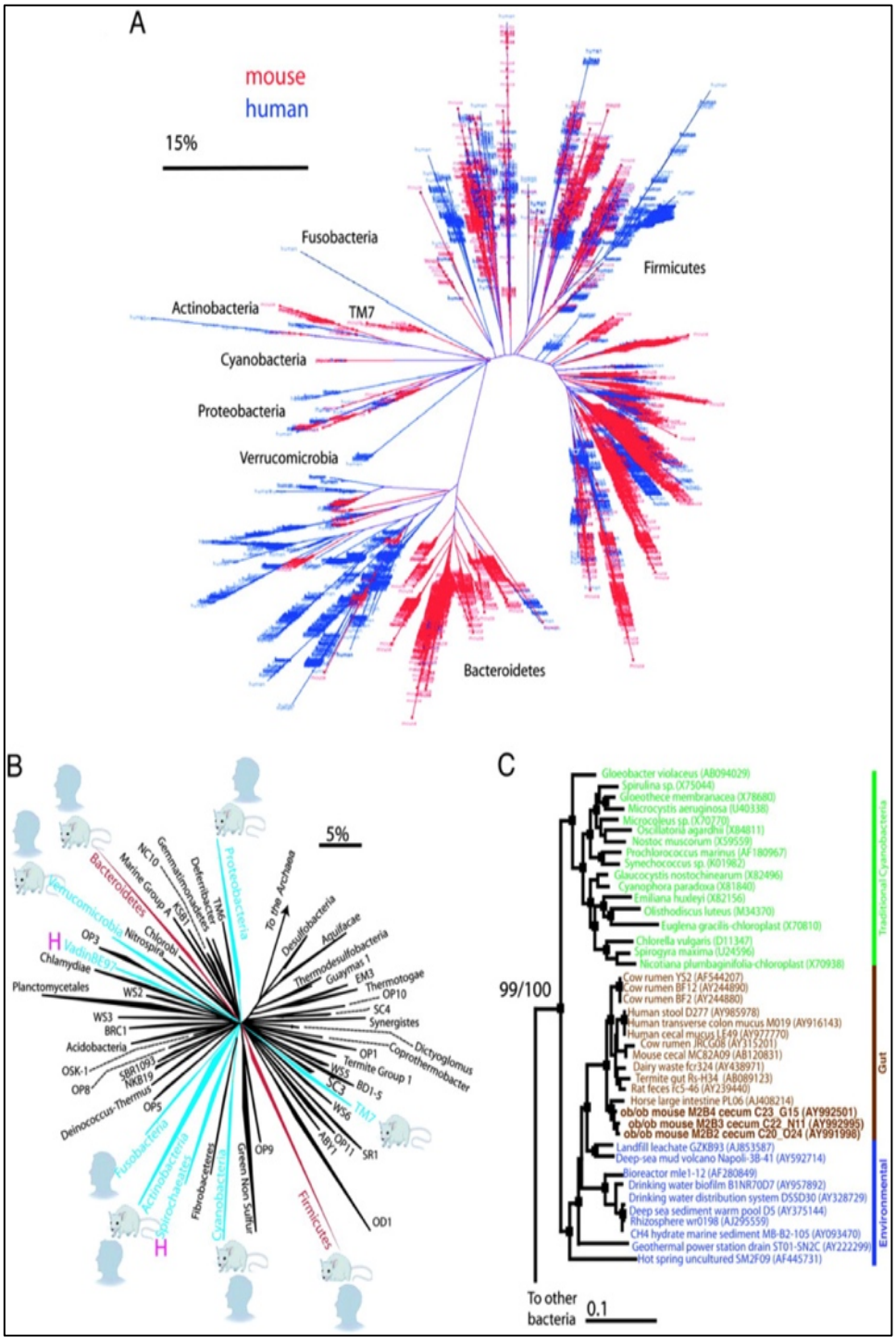

Figure 12. Diversité bactérienne chez l'humain et la souris, adaptée de (240).

La communauté de microorganismes dont le poids est de 1 à 5 kg vit en symbiose avec son hôte et s'alimente de substrats végétaux et animaux issus de notre alimentation, ainsi que de substrats endogènes (mucines) propres à l'hôte. De façon intéressante, on se rend de plus en plus compte que la composition de la flore s'adapte aux changements de nourriture. Par exemple, contrairement à ce que l'on pourrait penser, le poids du microbiote dépend bien plus du type d'alimentation que du poids total de l'individu (248, 249). À cet effet, la privation alimentaire et l'apport de protéines ou de fibres solubles à long terme modifient la biodiversité des bactéries, stimulent la dépense énergétique et prévient l'accumulation des lipides (249-252). Par contre, on réalise que des comportements alimentaires déviants ou des écarts radicaux des saines habitudes alimentaires menacent la biodiversité des microorganismes commensaux qui assurent une symbiose avec l'hôte et le maintien de sa santé et de son bien-être (248, 250). De même, la sédentarité, la surconsommation, la prise régulière de médicaments et l'exposition aux polluants causent une dysbiose intestinale et affecte considérablement la santé (248-251, 253).

La diversité du microbiome a servi d'agent comparatif dans l'identification des signatures associées à diverses maladies. En fait, les corrélations entre l'existence/disparition de souches bactériennes dans des désordres spécifiques permettent de formuler des postulats reliant la dysbiose et l'étiologie d'un certain nombre de pathologies. C'est ainsi que le débalancement du microbiote a aidé à détecter des mécanismes relatifs aux troubles chroniques non transmissibles majeures (254-258). Par exemple, dans l'obésité, le SyM et le DT2, la dysbiose microbienne du tractus gastro-intestinal est en mesure de modifier le profil de fermentation intestinale et l'intégrité de la paroi intestinale tout en provoquant une endotoxémie métabolique, une inflammation de bas grade, une auto-immunité en association avec d'autres anomalies métaboliques (230, 233, 236, 239, 240, 243).

On est finalement arrivé à la conclusion que les symbiotes résidents contrôlent le métabolisme de l'hôte de multiples façons, intégrant l'homéostasie physiologique, la signalisation immuno-inflammatoire et la compliance énergétique (230, 233, 234, 236, 239, 243). Il est important de noter que la fermentation microbienne recueille de l'énergie pour la croissance microbienne et génère des monosaccharides et des acides gras à chaîne courte (AGCC). Ceux-ci sont capables de se lier aux récepteurs GPR41 (ffar2) et GPR43 (ffar3)

couplés aux protéines G et de les activer afin d'inhiber les cytokines inflammatoires (259, 260). Il a été démontré que les AGCC, composés essentiellement de butyrate, propionate et acétate, peuvent avoir des fonctions complémentaires. Le butyrate est principalement utilisé comme source d'énergie par les entérocytes alors que l'acétate et le propionate sont transférés au foie pour la lipogenèse et la gluconéogenèse. La supplémentation en butyrate chez les souris prédiabétiques obèses a significativement amélioré la barrière épithéliale intestinale et la sécrétion d'insuline des cellules bêta, tout en diminuant l'adiposité corporelle ainsi que le gain de poids, la RI, l'hyperinsulinémie et l'hyperglycémie (261). De leur côté, les aliments prébiotiques fonctionnels qui altèrent le profil de fermentation du microbiome, améliorent l'équilibre énergétique et la sensibilité à la leptine en modulant les sécrétions des cellules entéro-endocrines dans les modèles d'obésité et de souris diabétiques (262) (**Figure 13**).

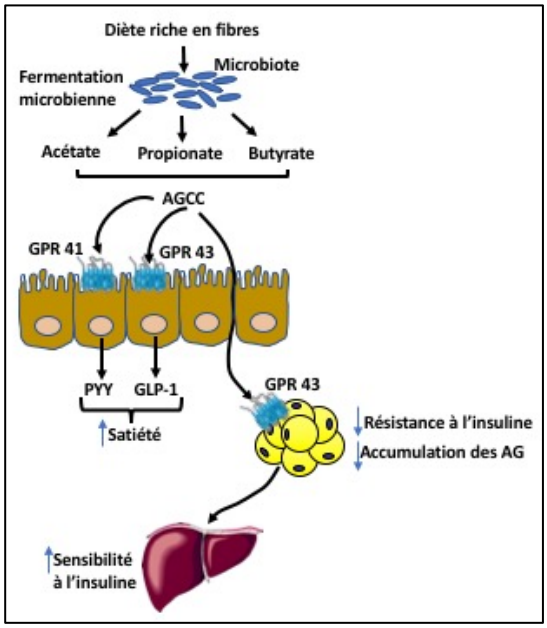

Figure 13. Régulation du microbiote intestinal et du métabolisme de l'hôte.

L'activation de GPR41 et GPR43 par les AGCC (i) stimule la production des neuropeptides (PYY et GLP-1) pour augmenter (i) la satiété, (ii) réduit la signalisation de l'insuline dans le tissu adipeux; assurant ainsi la prévention subséquente de l'accumulation de graisse ectopique, et (iii) augmente la sensibilité à l'insuline au niveau du foie, adaptée de (263).

D’autres évidences montrent que la colonisation de rongeurs axéniques (exempts de germes) avec des microbes intestinaux, obtenus à partir de rongeurs conventionnels (en bonne santé métabolique), a favorisé l'expression de gènes impliqués dans l'absorption des nutriments, l'intégrité de la barrière muqueuse, l'angiogenèse et les enzymes du métabolisme xénobiotique (264).

2.2 Axe Intestin-Foie

L’anatomie du foie permet de nombreuses interactions physiologiques avec l’intestin, méritant ainsi l’appellation ‘’axe intestin-foie’’ par la communauté scientifique. Alors que le quart de l'apport sanguin provient de la circulation systémique et atteint le foie par l'artère hépatique, les trois autres quarts reflètent le sang riche en nutriments dérivé de l'intestin qui pénètre dans le foie par la veine porte (7). Les nutriments, molécules de signalisation et métabolites bactériens dérivés de l'intestin sont transférés au foie par la circulation portale et contribuent à la santé hépatique en maintenant un métabolisme local optimal. En fait, le flux sanguin ralenti dans les sinusoïdes du foie favorise des interactions entre les substances issues de l'intestin et les cellules hépatiques, soit les hépatocytes et cellules immunitaires. On arrive actuellement à la conclusion que le dysfonctionnement de l'axe intestin-foie constitue un facteur critique dans le développement et la progression de plusieurs désordres hépatiques tels que la NAFLD (265-268).

2.2.1 Composantes de l’axe intestin-foie

Les cellules immunitaires hépatiques incluent les cellules de Kupffer, les cellules tueuses naturelles, les cellules dendritiques, les lymphocytes, et même les cellules non parenchymateuses (endothéliales et étoilées) (269). De solides observations ont particulièrement démontré que les composantes microbiennes dérivées de l'intestin constituent des signaux de

danger pour les cellules hôtes dans le foie puisqu'ils activent la cascade inflammatoire des cellules immunitaires et détériorent la fonction des cellules parenchymateuses (270).

Par ailleurs, les microbes influencent le système immunitaire de la muqueuse qui assiste l'hôte dans la distinction des signaux de sécurité et de danger (271). Étant donné que le foie est irrigué entre autres par le sang de provenance intestinale, les bactéries intestinales peuvent également exercer un effet sur le milieu immunitaire dans le foie. En effet, l'exposition des cellules hépatiques aux produits de la digestion, aux antigènes et aux métabolites bactériens (lipopolysaccharides, ADN microbiens, peptidoglycans, PAMP et même des bactéries intactes) contenus dans le sang veineux portal (272) est susceptible d'altérer la tolérance et de moduler l'environnement immunitaire dans le foie (273, 274). Par exemple, des modifications dans la composition du microbiome en association avec les altérations de la perméabilité intestinale favorisent la translocation des bactéries dans la circulation portale (7). Ainsi, les changements compositionnels du microbiote intestinal en association avec les altérations de la perméabilité intestinale peuvent renverser l'environnement tolérogène hépatique via l'impact sur les cellules immunitaires hépatiques (269).

Nous avons décrit comment la dysbiose est impliquée dans la pathogenèse de désordres hépatiques et de leurs complications. Cependant, la maladie du foie peut de son côté modifier le profil des bactéries intestinales. La proposition d'un cercle vicieux est possible à l'instar de la cirrhose qui altère le microbiote, ce dernier contribuant, de son côté, à de nombreuses complications de la cirrhose.

2.3 Microbiote et Maladies du Foie

Indiscutablement, le microbiote intestinal recèle de fonctions critiques pour la physiologie du foie et l'homéostasie métabolique (270). Ses produits toxiques bactériens dérivés de l'intestin peuvent agir comme déclencheurs d'inflammation hépatique et aggraver la stéatose hépatique (275, 276). Inversement la stérilisation intestinale aide à prévenir la stéatose hépatique

après ligature des voies biliaires (276). Le microbiote intestinal pourrait aussi participer à la progression de la NAFLD vers le NASH en favorisant la fibrose (277, 278). Par ailleurs, la défaillance de l'homéostasie intestinale contribue aux maladies hépatiques auto-immunes (279). Même si le foie est capable de réparer ses propres lésions via la prolifération hépatocytaire, la formation de tissu fibreux pourrait s'y développer et remplacer le parenchyme hépatique normal (280). Ce sont surtout les cellules stellaires hépatiques qui sont les plus responsables de ces événements, car ils acquièrent un phénotype myofibroblastique et produisent du collagène, le principal composant du tissu fibreux (281).

L'axe intestin-foie est également impliqué dans la cirrhose. La translocation bactérienne est l'une de ses manifestations (282, 283). Les mécanismes qui sous-tendent l'émergence de la translocation bactérienne dans la cirrhose comprennent : (1) la prolifération des bactéries intestinale associée au degré de dysfonction hépatique; (2) le dysfonctionnement immunitaire; (3) l'altération des facteurs luminaux (ex. acides biliaires, immunoglobuline A, mucines, défensines, lysozyme, phospholipase A2); les acides biliaires produisant notamment un effet trophique sur la muqueuse intestinale avec en plus l'inhibition de la prolifération des bactéries anaérobies (284), alors que l'administration d'acides biliaires conjugués à des rats cirrhotiques avec ascite entraîne une réduction de la prolifération bactérienne intestinale, de la translocation bactérienne et de l'endotoxémie tout en augmentant la survie des animaux (285); (4) la modification de la perméabilité intestinale impliquant l'augmentation exagérée de l'acide nitrique qui altère l'intégrité de la muqueuse intestinale par l'expansion des TJ, la destruction du cytosquelette d'actine et la réduction de l'adénosine triphosphate (286). On constate donc qu'une multitude de facteurs intestinaux, conduisant à des effets locaux et systémiques délétères en aval, jouent un rôle majeur dans les issues cliniques défavorables chez les personnes atteintes de problèmes hépatiques.

2.3.1 Mécanismes d'action du microbiote sur la NAFLD

L'excès de certains nutriments et leur transport intestinal cause l'accumulation de lipides au niveau du foie comme c'est le cas dans la NAFLD. En effet, l'apport alimentaire de fructose, de graisses saturées et de cholestérol entraînent une altération de la β-oxydation et une augmentation de la lipogenèse (287). D'autres évidences ont ciblé les AGCC dans les maladies hépatiques et particulièrement la NAFLD, car ils peuvent représenter une source des calories supplémentaires. Les monosaccharides activent le foie via la protéine de liaison de l'élément de réponse glucidique (ChREBP), ce qui augmente la lipogenèse hépatique et l'accumulation de graisse (268).

Le microbiote intestinal est responsable de la conversion de l'acide cholique et de l'acide chénodésoxycholique en acides biliaires secondaires. En régulant les pools d'acides biliaires, le microbiote contrôle le métabolisme des lipides et du glucose dans le foie. Les acides biliaires fonctionnent également comme des molécules de signalisation et se lient aux récepteurs cellulaires. Il est bien établi que la synthèse des acides biliaires contrôle l'activation du récepteur nucléaire farnésoïde X et du récepteur 5 couplé à la protéine G (288). Ces deux facteurs sont fortement impliqués dans la modulation du métabolisme du glucose (289, 290).

Il est maintenant acquis que les endotoxines dérivant de l'intestin activent les macrophages hépatiques via la signalisation des « Toll like receptors », stimulant ainsi la production de cytokines pro-inflammatoires (ex. TNF-α et IL-8) aptes à déclencher la migration hépatique des neutrophiles et des monocytes (291) (**Figure 14**). Cet événement mène finalement à une lésion hépatique et à une inflammation systémique. Il induit également un dysfonctionnement immunitaire inné qui prédispose à l'infection et au développement de complications décompensatrices telles que l'hémorragie et la septicémie (292). Des analyses quantitatives de métagénomique ont récemment été utilisées pour démontrer que 75245 gènes microbiens diffèrent en terme d'abondance entre les patients atteints de problèmes hépatiques et les individus en bonne santé (293).

De ce fait, la dysbiose entérique est un joueur clé dans l'étiologie des maladies hépatiques. Des changements défavorables dans le microbiote intestinal ont été causalement liés à la pathogenèse de la NAFLD/NASH et à la progression vers une maladie hépatique avancée (273, 294, 295).

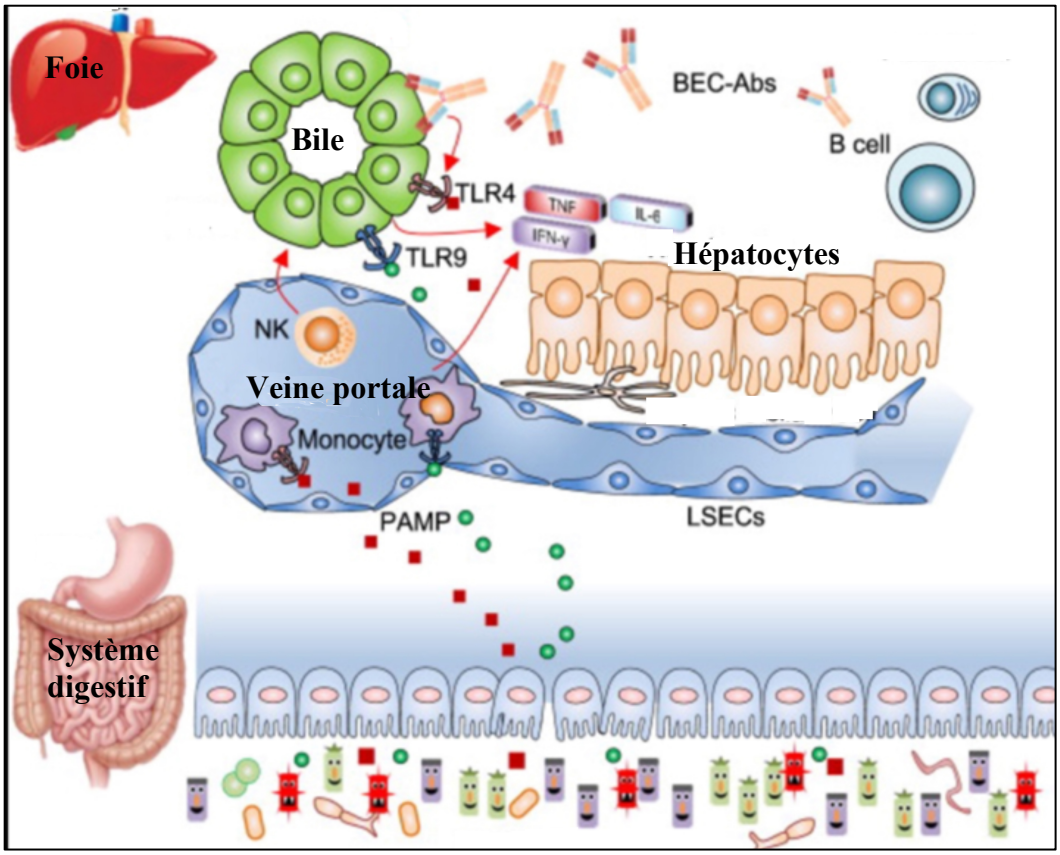

Figure 14. Le microbiote intestinal participe à l'initiation et au maintien de maladies hépatiques auto-immunes en modulant le système immunitaire inné.

Les endotoxines dérivant de l'intestin activent les macrophages hépatiques via la signalisation des TLRs, stimulant ainsi la production de cytokines pro-inflammatoires, menant à une lésion hépatique, adaptée de (296).

3. STÉATOSE HÉPATIQUE NON ALCOOLIQUE (NAFLD)

3.1 Définition et Prévalence de la NAFLD

La NAFLD est définie comme une accumulation excessive de graisse hépatique (hépato-stéatose), principalement sous forme de lipides neutres constitués de TGs, d'esters de cholestérol et de diacylglycérols. La NAFLD est aussi considérée par la communauté scientifique comme la manifestation hépatique du SyM, s'associant à l'obésité, la RI et la dyslipidémie (297) ainsi qu'au SOx (298) et à l'inflammation (299). Elle constitue aussi un problème majeur de santé publique (300, 301) et sa prévalence continue de progresser en parallèle avec l'épidémie d'obésité dans les sociétés occidentales (302-304), atteignant 20 à 30 % dans la population générale, 80 à 90 % chez les adultes obèses, 30-50 % chez les sujets atteints de T2D, et jusqu'à 90 % chez les sujets atteints d'hyperlipidémie (301 , 305). Dans plusieurs cohortes pédiatriques, l'estimation de la prévalence de la NAFLD est de 3 à 10 %, passant à 40-70 % chez les enfants et adolescents en surpoids ou obèses (300 , 306), avec un ratio mâle-femelle 2 :1 (307). La NAFLD manifeste une variété de symptômes allant d'une stéatose simple à l'aspect histologique simultanée de l'inflammation et d'hépatocytes ballonnés, qui définit la NASH (308, 309); ces symptômes ayant une prévalence de 10 à 56 % dans la population obèse (310). Jusqu'à 20 % des patients qui développent le NASH peuvent progresser vers la fibrose, la cirrhose et le carcinome hépatocellulaire (311-313) (**Figure 15**).

La NASH est considérée comme la troisième cause commune de la transplantation hépatique. Aux États-Unis, on estime que la NASH se projette comme la principale indication de la transplantation du foie d'ici 2020 (314). Une approche multidimensionnelle apparaît nécessaire pour cibler la NAFLD associée à plusieurs risques liés à la santé.

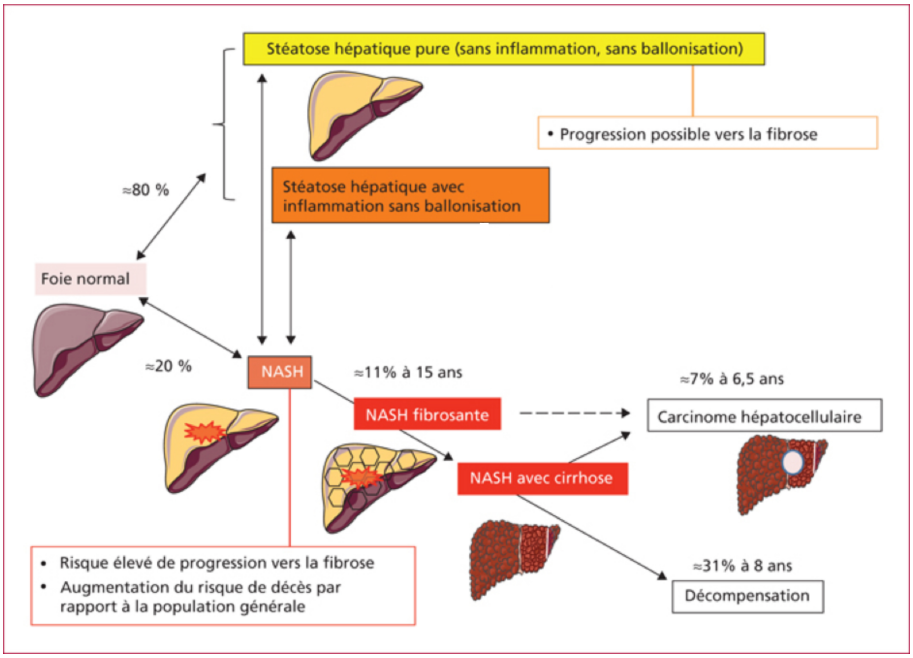

Figure 15. Spectrum et évolution de la stéatose hépatique non alcoolique.

La NAFLD manifeste une variété de symptômes allant d'une stéatose simple à l'aspect histologique simultanée de l'inflammation et d'hépatocytes ballonnés, qui définit la NASH, adaptée de (315).

3.2 Causes de la NAFLD

Même en absence de consommation d'alcool et d'infection virale, le foie peut accumuler les lipides de manière substantielle. La NAFLD en est un remarquable exemple, qui semble définitivement être associée à de mauvaises habitudes alimentaires, à la sédentarité et notamment aux désordres cardiométaboliques (297). Cependant, de nombreux sujets porteurs d'obésité, de SyM et de DT2 ne présentent pas de NAFLD/NASH. Inversement, ce ne sont pas l'ensemble des patients avec une NAFLD/NASH qui présentent une RI, une obésité et SyM. Actuellement, plusieurs équipes biomédicales à travers le monde sont activement engagées à élucider la pathogenèse, surtout que la prévalence de la NAFLD a doublé ces 20 dernières

années, alors que celle d'autres maladies hépatiques chroniques est restée stable ou a même diminué (316). Malgré les limitations dans la compréhension de la pathogenèse, on continue à se servir de facteurs de risque (DT2, IR, obésité, HTA, hyper-cholestérolémie et hypertriglycéridémie) pour dépister la présence de la NAFLD. En particulier, l'interaction réciproque entre le dépôt lipidique dans le foie et la RI est un élément clé dans le développement de la NAFLD. D'ailleurs, 50 à 60% des patients avec DT2 et affichant une RI présentent une NAFLD et, réciproquement, 20 à 30% des sujets avec une NAFLD sont diabétiques (317). Le modèle à deux étapes (ou « two-hit hypothesis » en anglais) « tient toujours la route » pour expliquer la pathogenèse de la NAFLD (318). La première étape est caractérisée par l'émergence de la RI qui conduit à la mobilisation des AG par le tissu adipeux et à la lipogenèse hépatique *de novo*. Dans la seconde étape, l'afflux élevé de ROS et l'apport de cytokines inflammatoires génèrent le SOx et un environnement inflammatoire, respectivement, concourant ainsi à produire un environnement cytotoxique favorisant la NASH et la fibrose (319). Un troisième ''hit'' a également été proposé et est basé sur la mort progressive des hépatocytes et au déclin de la réplication des hépatocytes matures, ce qui conduit à la cirrhose et au carcinome hépatocellulaire (318, 320) (**Figure 16**). Par conséquent, l'accumulation de lipides au niveau des hépatocytes, probablement due à un déséquilibre entre l'afflux important d'AG (par l'alimentation, la lipolyse dérivant des tissus adipeux et de la lipogenèse hépatique), et l'amenuisement de l'élimination et de l'oxydation des AG sont responsables de la NAFLD.

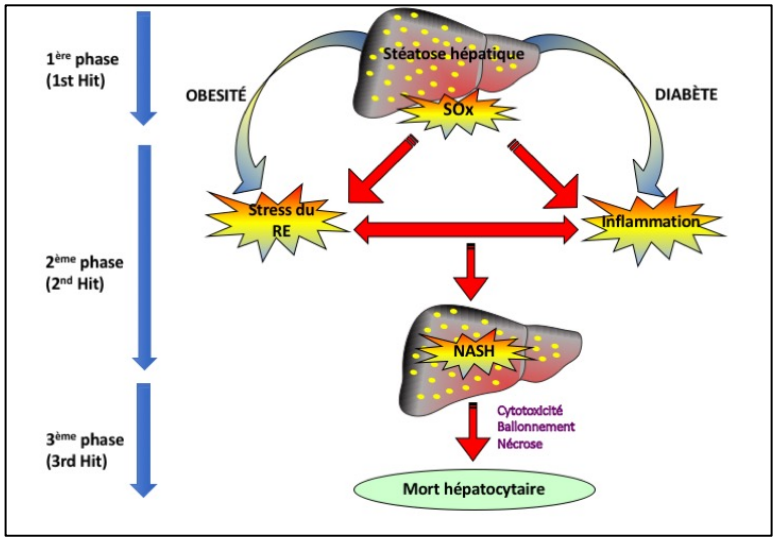

Figure 16. Pathogenèse de la stéatose hépatique non alcoolique selon les atteintes ou « Hits » proposés (© Spahis)**.**

Il est raisonnable de penser que l'accumulation des TG intrahépatiques interfére avec les voies de signalisation intracellulaires de l'insuline et provoque donc une résistance à l'action de l'insuline au niveau hépatique. Un autre mécanisme physiopathologique observé dans la NAFLD est la dérégulation de la biogenèse et du fonctionnement des mitochondries, causant ainsi une baisse de leur capacité oxydative et participant au développement de la RI (321, 322). Globalement, l'accumulation de graisse dans le foie (1st hit), accompagnée d'une peroxydation lipidique, accroît la vulnérabilité au SOx (2nd hit), qui déclenche l'inflammation, le stress du réticulum endoplasmique (RE), le dysfonctionnement mitochondrial et l'incapacité des hépatocytes à synthétiser des antioxydants endogènes (323).

3.3 Symptômes et Diagnostique de la NAFLD

Comme nous l'avons mentionné, la NAFLD en tant qu'hépato-stéatose cliniquement bénigne peut varier en gravité. Elle évolue en NASH avec une inflammation et même des lésions hépatiques. La NASH est la forme progressive de la maladie qui peut conduire à des changements de la morphologie hépatique (ballonnement des hépatocytes) avec complications (mort cellulaire et fibrose) (**Figure 15**). Dans la grande majorité des cas, la NAFLD est asymptomatique, mais durant son évolution, on peut constater un foie augmenté en volume, une sensation d'inconfort au niveau de l'abdomen, une sensibilité du foie lors de la palpation dans la région de l'hypocondre, des nausées et des vomissements.

En 2012, la Société européenne de gastroentérologie pédiatrique, hépatologie et nutrition (ESPGHAN) a publié ses lignes directrices pour le diagnostic de la NAFLD (324). La biopsie du foie est considérée comme un étalon-or pour le diagnostic de la stéatose, mais elle n'est pratiquée qu'en cas de nécessité, étant donné son caractère invasif et coûteux. En outre, le criblage demeure difficile pour déterminer la gravité de l'altération du foie et pour arriver à distinguer entre la pathogénèse de NAFLD et de NASH (324). De ce fait, pour atteindre une précision dans le diagnostic, on fait appel à un test de dépistage non invasif essentiellement basé sur des marqueurs communs pour le diagnostic de la NAFLD. Les enzymes hépatiques [aspartate aminotransférase (AST) et alanine aminotransférase (ALT)], couramment utilisées dans les études de NAFLD/NASH pédiatriques (325, 326) ainsi que l'échographie abdominale, la résonnance magnétique (IRM) et l'ultrasonographie (327) contribuent à donner plus de lumière au diagnostic sans être envahissant. Généralement, les ALT et AST sont élevées de deux à trois fois la limite supérieure de la normale avec une activité enzymatique d'ALT supérieure à celle de l'AST. L'activité de la gamma-glutamyl-transférase (GGT) sérique est souvent en hausse. L'échographie est en mesure d'indiquer la présence d'une augmentation de la taille du foie qui s'accompagne souvent d'un aspect hyperéchogène, mais l'utilité d'une échographie abdominale dans les cas non compliquées demeure controversée. Les autres tests d'imagerie tels que l'IRM ayant une valeur quantitative sont aptes à confirmer l'accumulation de graisses dans

le foie. Dans certains cas, on a recours à l'histologie suite à la biopsie hépatique afin de déterminer avec certitude le pourcentage d'atteinte hépatique.

3.4 Voies Métaboliques dans la NAFLD

Comme discuté précédemment, le SyM, particulièrement chez les adultes, est défini par la présence d'obésité abdominale (tour de taille) et deux ou plusieurs des facteurs suivants: hypertriglycéridémie, faible taux de HDL, glycémie élevée à jeun/RI et HTA. En outre, alors que le SyM dans la population pédiatrique n'est pas clairement compris, deux facteurs apparaissent comme cruciaux dans sa pathogénèse : obésité centrale avec accumulation de gras dans le tissu adipeux et la RI avec dyslipidémie, inflammation et augmentation des AG libres dans la circulation (328). De plus, de nombreuses études ont montré que le SyM et les risques élevés de MCV (329) sont associés à la stéatose et la fibrose hépatiques chez les enfants et les jeunes adolescents (330, 331).

3.4.1 Dyslipidémie et NAFLD

Les lipides occupent une place centrale dans la pathophysiologie de la NAFLD, et les TG sont les premiers à être emmagasinés conséquemment à la lipogenèse hépatique et à la mobilisation des AG du tissu adipeux (332). De plus, des désordres du bilan lipidique sont présents chez la majorité des patients souffrant de NAFLD, se manifestant par une hypertriglycéridémie et des taux circulants élevés d'AG libres (333, 334).

3.4.2 Insulinorésistance et NAFLD

La RI est l'un des facteurs les plus fortement associés à la NAFLD (335). En effet, il a été rapporté que 21 à 55% des patients adultes obèses, diagnostiqués avec une NAFLD, ont un DT2

ou une intolérance au glucose (336). De plus, la majorité des études, portant sur la NAFLD associée à l'obésité et au SyM, démontrent l'instauration d'une RI chez tous les sujets avec NAFLD, indépendamment de l'existence d'une hyperglycémie (333, 334, 337). La composition corporelle chez l'enfant comme chez l'adulte et, par conséquent, la masse du tissu graisseux viscéral, influencent le degré de RI (338). Cette dernière favorise des anomalies métaboliques capables d'induire une surcharge lipidique au foie. Ainsi, elle induit une lipolyse périphérique, augmentant la libération d'AG libres dans la circulation et une production hépatique de glucose en stimulant la néoglucogenèse. À l'inverse, le traitement de l'adulte par des agents hypoglycémiants, comme la metformine, diminue l'insulinorésistance hépatique et améliore les taux sanguins des transaminases hépatiques (339). Pourtant, peu d'études se sont attardées sur le traitement de la RI chez l'enfant ou l'adolescent obèse avec NAFLD. D'autre part, le tissu adipeux est de plus en plus considéré comme un organe endocrinien et non seulement comme un tissu de stockage des TG. Une étroite association a été démontrée entre la taille des adipocytes, les adipokines et la NAFLD (340-342).

3.4.3 Inflammation et NAFLD

L'obésité est associée à une inflammation de bas grade chez l'enfant comme chez l'adulte avec des niveaux augmentés de la CRP (14). De plus, une CRP plasmatique élevée est un facteur prédicteur de la NAFLD chez l'adulte (343), mais peu de données sont disponibles pour l'enfant ou l'adolescent. D'autres marqueurs de l'inflammation ont été étudiés pour la NAFLD, comme l'IL-6 qui est produite par les adipocytes et est capable d'induire la synthèse hépatique de la CRP (344). Ceci n'a pas pour autant attirer l'attention sur le besoin d'effectuer des études supplémentaires chez l'enfant. De son côté, le TNFα est capable d'induire les lésions histologiques de la stéatose hépatique (345). De nombreux travaux portant sur des biopsies hépatiques de sujets obèses avec NAFLD montrent une corrélation étroite entre l'expression génique du TNFα et la sévérité histologique de l'atteinte hépatique (346).

3.4.4 Stress oxydatif

Une augmentation du niveau de SOx est une des hypothèses physiopathologiques les plus fréquemment évoquées de la NAFLD et, particulièrement, de son évolution vers la NASH (347). En effet, les ROS caractérisant le SOx sont capables d'induire des perturbations hépatiques en interagissant avec les lipides et les protéines, en altérant leurs structures et fonctions. D'autre part, les ROS sont capables d'induire des anomalies histologiques identiques à celles observées dans le foie lors d'une stéatose. Par exemple, les ROS peuvent activer des facteurs de transcription impliqués dans la mort cellulaire hépatocytaire, tandis que les aldéhydes produits par la peroxydation des lipides, comme le 4-hydroxynonenal (HNE) ou le malondialdehyde (MDA), ont le potentiel de se lier de manière covalente à des protéines hépatiques et d'induire ainsi une réponse immunitaire dirigée contre les hépatocytes (345). Un niveau accru de SOx a été démontré par plusieurs études autant chez l'animal que chez des adultes obèses au niveau sérique et au niveau hépatocytaire (348-350). Chez l'enfant, relativement peu d'études ont exploré les voies du SOx.

3.4.5 Adipokines

Outre l'obésité centrale, les taux circulants des adipokines sont aussi impliqués dans la NAFLD comme dans le SyM chez les enfants. Fait intéressant, les taux sériques de la leptine augmentent en parallèle avec le grade de la stéatose et les complications de la pathogenèse de la NAFLD, particulièrement en présence de la RI (351, 352). Ce champ d'investigation demeure encore peu élaboré, et les résultats controversés portent à confusion. Par exemple, le groupe de Nobili a montré que les taux circulants d'adiponectine (favorable métaboliquement) et de la résistine (non favorable métaboliquement) sont corrélés négativement avec la stéatose (351). Indubitablement, des études plus approfondies sont nécessaires afin de clarifier le rôle des adipokines dans le développement et la progression de la NAFLD.

3.4.6 Mitochondrie

Selon certains groupes, le SOx n'est pas seulement une des principales causes de l'accumulation de graisse dans le foie (353), mais il est également impliqué dans le développement de la fibrose (354 , 355). Il a été observé que dans la NAFLD, l'atteinte de la chaîne respiratoire mitochondriale, les altérations ultrastructurales mitochondriales (356 , 357) et le déficit de synthèse de l'ATP au niveau du foie (358 , 359) sont engendrées par la présence accrue du SOx et l'absence de la défense antioxydante (360) (**Figure 17**). Le déséquilibre redox, causé par une surproduction de ROS au niveau de la mitochondrie, occasionne i) une altération de l'ADN mitochondrial ; (ii) une augmentation des taux de MDA et de 4-HNE, provenant de la peroxydation lipidique; (iii) une inhibition du cytochrome c, entrainant un dommage à la membrane mitochondriale (361 , 362); iv) une réduction de l'expression du facteur de transcription « peroxisome proliferator-activated receptor-γ coactivator-1α » (PGC-1α), un important régulateur de la biogenèse mitochondriale (363), affectant de ce fait, les autres facteurs de transcription mitochondriaux (TFAM TFB1M, TFB2M) (363 , 364 , 365) ; (v) la régulation négative de PGC-1α dans la NAFLD qui résulte en l'inactivation de la défense antioxydante mitochondriale, y compris, le nuclear factor erythroid 2 (NRF2), la SOD, la catalase et le GLPx (366); et finalement (vi) le déclenchement d'une inflammation et l'instauration d'une RI hépatique.

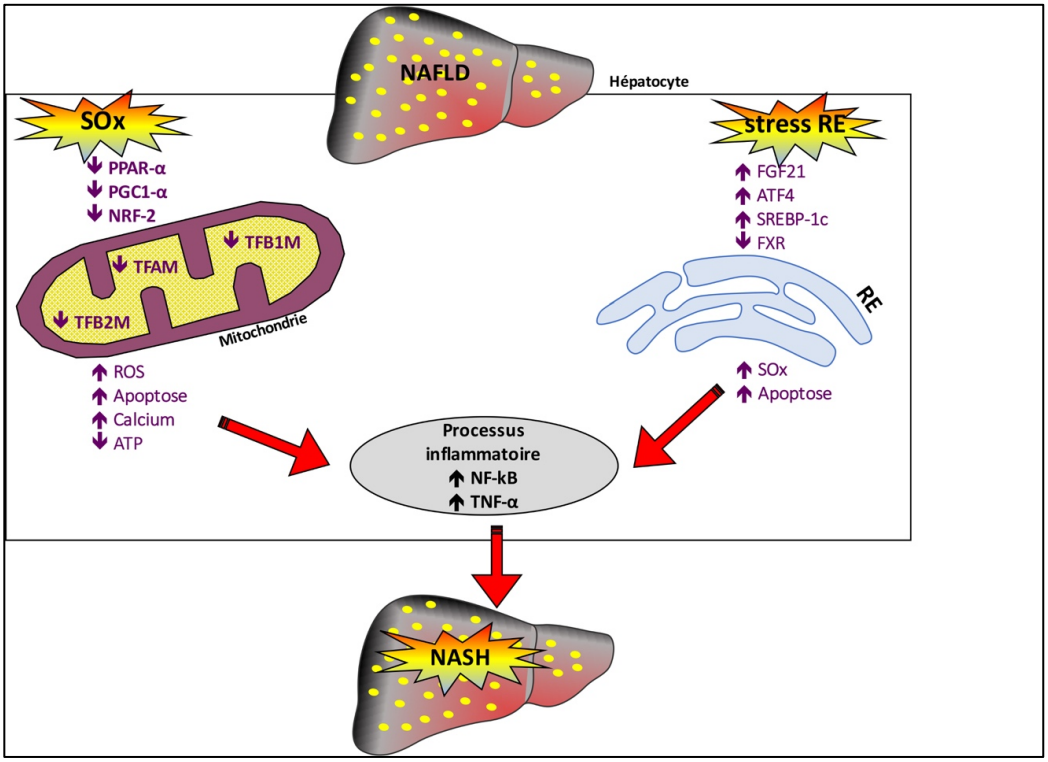

Figure 17. Processus intracellulaires liés à la NAFLD.

Le processus inclut le stress oxydatif mitochondrial (SOx) et les dysfonctionnements ainsi que le stress du RE, déclenchant l'inflammation via l'activation du facteur nucléaire (NF-κB). Des facteurs de transcription supplémentaires sont impliqués dans les mécanismes moléculaires menant au développement de la NASH (© Spahis)**.**

3.4.7 Facteurs génétiques

Le spectre de la NAFLD est multifactoriel et très complexe. Son interaction avec les facteurs génétiques, épigénétiques et environnementaux entraînent le développement et la progression de la pathologie, particulièrement, dans la population pédiatrique (367, 368). Comme pour l'obésité, il existe certainement une composante génétique dans la pathogenèse de la NAFLD, où différents polymorphismes génétiques confèrent un risque de SyM et de RI

étroitement associés à la stéatose. En fait, des études épidémiologiques et les investigations sur des jumeaux ont bien confirmé la susceptibilité génétique associée à la NAFLD (369).

Le plus important gène documenté est le « patatin like phospholipase domain containing 3 » (PNPLA3) (370) où un de ses variants génétiques, rs738409, qui contribue à l'émergence de la NAFLD chez les enfants obèses (371). Il a été aussi rapporté que le gène PNLP3 pourrait être modulé par des facteurs alimentaires, particulièrement la consommation d'AGPI n-3 (372). D'autres mutations ont été identifiées dans les gènes impliqués dans le métabolisme des lipides et les désordres reliés au SyM, à la RI, à l'inflammation et au SOx (373) (**Tableau 4**).

Tableau 4: Gènes impliqués dans la NAFLD en association avec les évènements cardiométaboliques

Marqueurs	**Gène**	**Implication/Mécanisme**	**Référence**
Stéatose	*KLF6*	–27G>A (rs3750861 est associé à une NAFLD modérée	(374))
	AGTR1	Implication de rs3772622 dans la NASH et la fibrose Interaction avec le gène PNPLA3 rs2276736, rs3772630 et rs3772627 sont protecteurs pour la stéatose	(375, 376)
SOx	*ABCC2 (MRP2)*	Peroxydation lipidique	(377)
	GCLC; GCLM	129C> T, rs17883901 est associé à une stéatose simple	(378)
	HFE	rs1800562, Cys282Tyr est associé à la NASH et une fibrose hépatique avancée	(379-381)
	SOD2	rs4880, codant pour Ala16Val est associé à une fibrose hépatique avancée	(382)
Inflammation	*TNF*	-238G> A, rs361525 est associé dans la transition de la NAFLD vers la NASH	(383, 384)

		1031T> C, rs1799964 et -863C> A, rs1800630 sont plus fréquents dans la NASH	
	IL6	–174G>C, rs1800795 est associé à la NASH	(385)
	ADIPOQ	rs266729 et rs2241766 (+45 T/G) sont associés à la sévérité de la NAFLD	(386)
	LEPR	rs8179183, Lys656Asn est associé à une RI dans la NAFLD rs1137100 est associé à un risque accru de NAFLD/NASH	(387 , 388)
Endotoxémie	*CD14*	rs2569190 augmente l'expression de CD14 dans la NAFLD	(389, 390)
Lipides	*MTTP*	-493G> T; rs1800591 prédispose à la NAFLD/NASH	(378, 391, 392)
	NR1I2	rs7643645 et rs2461823 sont associés à la NAFLD et prédisent à la sévérité de la maladie	(393, 394)
	PEMT	rs7946, codant pour Val175Met est associé à la NAFLD	(395, 396)
	PNPLA3	rs738409, codant pour Ile148Met est associé à la NAFLD/NASH	(370, 397)
	PPARA	rs1800234, codant pour Val227Ala est associé à la NAFLD	(398)
	SLC27A5	rs56225452 améliore la triglycéridémie et l'hyperglycémie associées à la NAFLD	(399)
	APOC3	455T>C; rs2854116 et -482C> T; rs2854117 sont associés à une augmentation de la RI et de la stéatose	(400-402)
	APOE	Le génotype ApoE3/3 est associé à la NAFLD ApoE3/4 avait un effet protecteur	(403-405)
	SREBP1/2	rs11868035 est associé au trouble du métabolisme des lipoprotéines et à l'émergence de la NAFLD rs2228314 G> C peut augmenter le risque de NAFLD et sert de biomarqueur potentiel pour le diagnostic de la maladie	(406, 407)
	TM6SF2	rs58542926 est impliqué dans la progression de la maladie et la gravité de la NAFLD	(408)

Glucose et RI	*ENPP1; IRS1*	rs1044498, codant pour Lys121Gln et rs1801278, codant pour Gln972Arg réduisent l'activité d'Akt et provoquent la RI associée à la NAFLD	(409, 410)
	GCKR	rs780094 est associé à l'accumulation des TG dans le foie	(411, 412)
	LPIN1	Les variantes de ce gène sont impliqués dans le SyM associé à la NAFLD	(413, 414)
	PPARG	Une perte de fonction de rs1805192, codant pour Pro12Ala) affecte la sensibilité à l'insuline dans la NAFLD	(415)
	SLC2A1	3,072 SNPs sur 92 gènes candidats ont identifié le variant *SLC2A1* comme associé à la NAFLD indépendamment de la RI ou le DT2	(416)
	TCF7L2	Gène clé dans la signalisation des Wnt et est impliqué dans la RI dans la NAFLD	(417)

SLC2A1 : «solute carrier family 2» qui facilite le transport du glucose

TCF7L2 : «transcription factor 7-like 2».

3.4.8 Lipogenèse

Comme nous l'avons mentionné, l'insulinorésistance est une composante centrale dans la manifestation de NAFLD. En sa présence, le tissu adipeux libère une quantité abondante d'AG, surtout saturés, qui arrivent au foie par la circulation portale et constituent des précurseurs pour la production des TGs (418). En plus de cet apport en AG libres, l'insulinorésistance stimule la synthèse *de novo* des AG tout en inhibant leur catabolisme par la voie de la β-oxydation dans les mitochondries des hépatocytes (419). Ces deux processus, soit l'afflux accru des AG par la mobilisation du tissu adipeux et la lipogenèse hépatique, contribuent significativement à l'accumulation excessive des TG dans le foie (349) (**Figure 18**). Si on se fie aux données disponibles dans la littérature, les AG non estérifiés circulants représenteraient environ 60% de la teneur en TG hépatiques et le restant proviendrait de la lipogenèse *de novo* (26%) ou de l'alimentation (14%) chez les patients atteints de NAFLD (420).

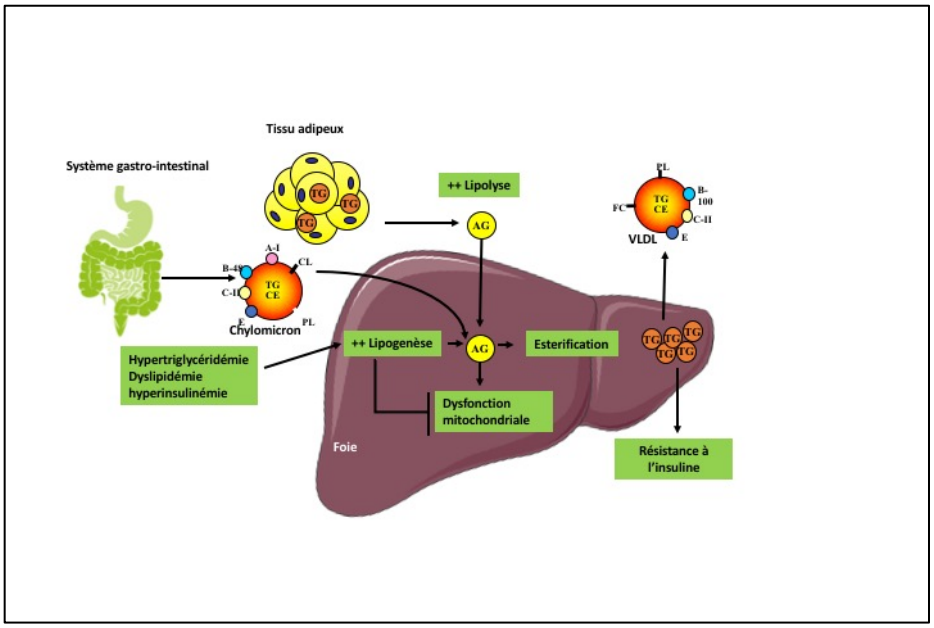

Figure 18. Mécanismes favorisant la NAFLD.

Un apport alimentaire riche en gras et en sucre diminue la β-oxydation des AG (engendrant une dysfonction mitochondriale), et favorise leur accumulation dans le foie, ce qui induit une synthèse *de novo* des TG et une surproduction des VLDL (© Spahis).

Le sentier métabolique de la lipogenèse comprend en premier lieu la conversion du glucose en acétyl-CoA par la glycolyse et l'oxydation du pyruvate. L'acétyl-CoA est ensuite converti en malonyl-CoA par l'acétyl-CoA carboxylase (ACC). Ensuite, la synthase des acides gras (FAS) catalyse la formation d'acide palmitique à partir de la malonyl-CoA et de l'acétyl-CoA. Les autres enzymes critiques de la voie de synthèse des AG sont impliquées dans l'élongation des AG à longue chaîne (ELOVL6/Δ6) et leur désaturation (stéaroyl-CoA désaturase 1 ou SCD1/Δ9) (421, 422). Indubitablement, le glucose et l'hyperinsulinémie favorisent la lipogenèse par activation de la protéine de liaison aux éléments de réponse aux glucides (ChREBP) et de la protéine de liaison aux éléments de régulation des stérols-1c (SREBP-1c), respectivement (423). Par ailleurs, l'hyperinsulinémie favorise davantage la

lipogenèse hépatique en inhibant le facteur de transcription FoxO1 qui régule normalement l'expression de SREBP-1c et favorise la dégradation de ChREBP (424, 425).

Au cours des dernières années, la consommation exagérée de fructose coïncide avec la prévalence de l'obésité, de la NAFLD et du DT2 (426-428). Il semble que son apport exagéré augmente les niveaux de substrats lipogéniques via l'induction de facteurs de transcription tels que le ChREBP qui induit la transcription de FAS, SCD1, ELOVL6 et ACC (429, 430) ainsi que SREBP1c qui exagère l'expression de FAS, ACC, SCD1 et ELOVL6 (431, 432) (**Figure 19**).

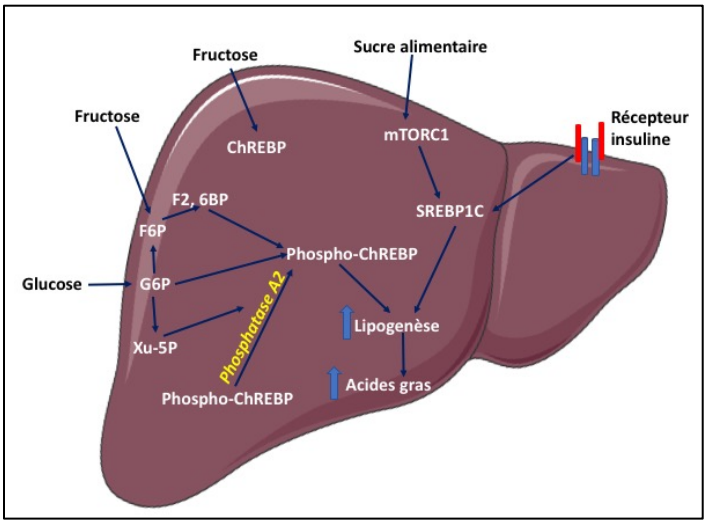

Figure 19. Expression des gènes impliqués dans la lipogenèse hépatique suite à un apport excessif en glucides.

(1) Activation de la protéine de liaison aux éléments sensibles aux glucides (ChREBP) via le xylulose 5-phosphate (Xu-5P), le glucose 6-phosphate (G6P) et le fructose-2,6-bisphosphate (F2,6BP) qui favorisent son expression. (2) La transcription de SREBP-1c et l'expression dépendante des enzymes lipogéniques est médiée par mTORC1qui est induit par la signalisation du récepteur de l'insuline, mais aussi directement par l'apport exogène des glucides. La translocation de SREBP1c dépend de la signalisation de l'insuline, adaptée de (433).

3.4.9 Gluconéogenèse

Comme l'expression des gènes gluconéogéniques est régulée à la hausse en réponse aux AG circulants (434), on peut constater l'élévation de l'expression des enzymes PEPCK et de la G6Pase dans la NAFLD par l'intermédiaire du « peroxisome proliferator-activated receptor-alpha » (PPARα) (435). En effet, l'expression du PPARα hépatique et l'activité transcriptionnelle sont induites par l'alimentation riche en graisses (436, 437) (**Figure 20**).

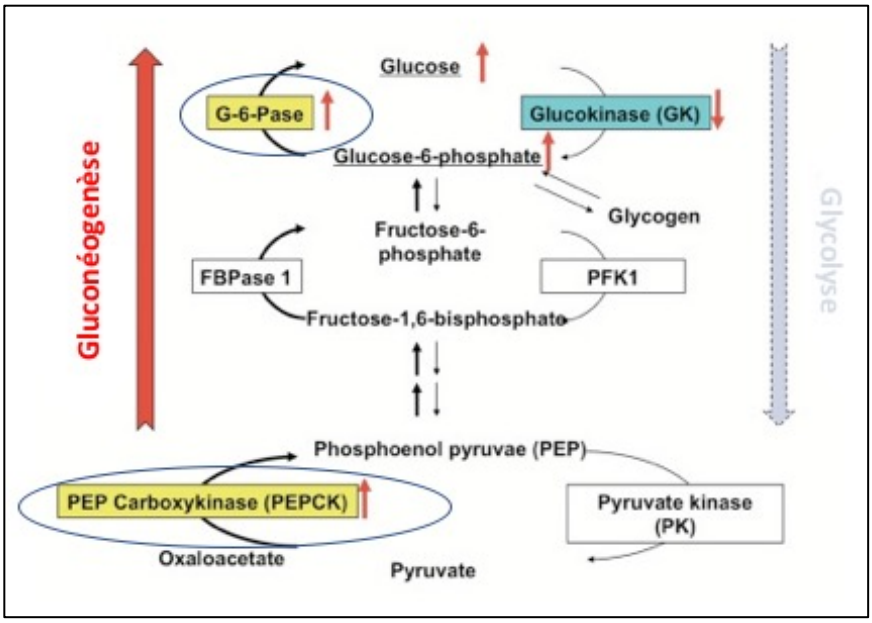

Figure 20. Processus de production du glucose dans le foie par la voie de la gluconéogenèse ou la glycolyse.

L'expression a gluconéogénèse est régulée à la hausse en réponse à l'élévation de l'expression des enzymes PEPCK et de la G6Pase dans la NAFLD, adaptée de (438).

3.4.10 Beta-oxydation

La β-oxydation des AG dans les mitochondries est une voie importante permettant de raccourcir les AG en acétyl-CoA qui peut ensuite être transformé en corps cétoniques (β-hydroxybutyrate ou acétoacétate). Ceux-ci sont ensuite incorporés dans le cycle TCA pour une oxydation complète (439). Pour initier ce processus, les acyl-CoA doivent être transportés au travers des membranes mitochondriales grâce à l'activité de CPT1 qui les convertit en acyl-carnitines dans la membrane externe mitochondriale. Le CPT2 convertit les acyl-carnitines en acyl-CoA pour la β-oxydation des acides gras à l'intérieur de la matrice mitochondriale (**Figure 21**).

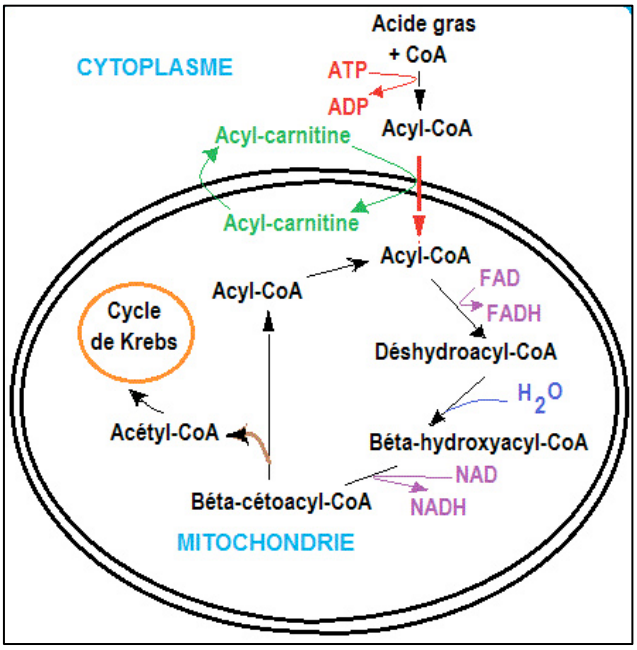

Figure 21. Processus de β-oxydation des AG dans la mitochondrie.

La β-oxydation des AG dans les mitochondries permet de raccourcir les AG en acétyl-CoA, pour être ensuite incorporés dans le cycle TCA pour une oxydation complète, adaptée de (439).

La première étape implique la déshydrogénation β de l'ester d'acyl-CoA par des acyl-CoA déshydrogénases spécifiques de la longueur de chaîne : longue chaîne (LCAD), chaîne moyenne (MCAD) et très longue chaînes (VLCAD). En effet, les souris déficientes en MCAD et VLCAD développent une stéatose hépatique, soutenant le rôle de ces protéines et de la β -oxydation dans la teneur en TG hépatiques (440). Dans les étapes suivantes, les enzymes 2-énoyl-CoA hydratase, 3-hydroxyacyl-CoA déshydrogénase et 3-oxoacyl-CoA thiolase oeuvrent pour achever la conversion de l'ester d'acyl-CoA en acétyl-CoA.

Le PPARα et son co-activateur PGC-1α sont essentiels pour stimuler la β-oxydation en maintenant l'expression des gènes cibles tels que *CPT1, LCAD, MCAD* et *acyl-CoA oxydase*. L'activation de l'AMPK et des sirtuines pourrait également améliorer l'expression de ces gènes en modifiant directement et/ou en activant PGC-1α (441, 442).

Comme cela a été évoqué précédemment, la stéatose dans la NAFLD résulte de l'accumulation de lipides hépatiques suite à une lipogenèse augmentée dans le foie. Par ailleurs, la β-oxydation des AG contribue de façon non négligeable à la pathogenèse de NAFLD comme l'ont rapporté plusieurs équipes (443, 444). Dans le modèle de souris soumises à un régime riche en graisses, la baisse de la β-oxydation des AG a conduit à l'accumulation de lipides hépatiques. En particulier, le PPARα, exprimé surtout dans les tissus riches en mitochondries, agit comme le régulateur principal de la β-oxydation via la modulation de deux enzymes clés, soit CPT1α et acyl-coenzyme A dehydrogenase (445-447). De même, des sujets obèses avec NAFLD montrent un déclin de l'expression de PPARα, combiné avec des changements de microRNA, pouvant donc orchestrer la détérioration de la β-oxydation (448). Parmi les mécanismes sous-jacents se trouve l'insulinorésistance munie du potentiel d'inhiber le récepteur activé par le PPAR-α et entraîner ainsi une réduction de la β-oxydation (449).

4. TRAITEMENTS DE LA NAFLD PAR DES NUTRIMENTS FONCTIONNELS

Depuis les vingt dernières années, la communauté scientifique s'est penchée sur les bienfaits que procurent les aliments sur la santé. En 1998, Santé Canada a proposé une appellation spécifique pour ces aliments qui sont dorénavant dits ''fonctionnels'', étant donné qu'ils procurent des bienfaits physiologiques tout en atténuant le risque de maladies chroniques dans de nombreuses voies métaboliques.

Des progrès récents ont été concentrés sur le rôle de la diète favorable et des nutriments fonctionnels sur la pathogenèse de la NAFLD. Cependant, aucun traitement nutritionnel et/ou pharmacologique n'est officiellement recommandé pour la NAFLD. Dépendamment de la complexité et sévérité de la pathologie, des agents pharmacologiques puissants sont prescrits. Pour ne noter que quelques-uns, il y a la pioglitazone, insulino-sensibilisatrice, qui présente un impact bénéfique sur les paramètres biochimiques et histologiques du foie, mais malheureusement son administration est souvent accompagnée d'événements indésirables (450-453). D'autres médicaments tels que le liraglutide, l'elafibranor, l'acide obéticholique et les statines ont été évalués, mais le nombre d'essais contrôlés randomisés est très limité et des clarifications sont nécessaires quant à leurs avantages et leur innocuité à long terme (454-457). D'autres équipes ont eu recours à une combinaison pharmacologique et nutritionnelle pour améliorer la pathophysiologie. Par exemple, une supplémentation en α-tocophérol et un traitement avec la metformine diminuent les niveaux d'enzymes hépatiques et les complications cardiométaboliques associées à la NAFLD/NASH (458-460). À date, les essais pédiatriques sont eux aussi peu convaincants car ils sont rares et caractérisés par une taille d'échantillon et une durée très limitée. Cependant, de saines habitudes de vie, accompagnées par une alimentation saine, de l'exercice physique et un traitement nutritionnel (non pharmacologique), semblent pouvoir améliorer la stéatose hépatique et ses complications (461-467).

4.1 Vitamine E

Le SOx a été identifié comme un mécanisme central contribuant au développement des lésions hépatiques de la NAFLD (468, 469). La vitamine E est reconnue pour ses propriétés antioxydantes et anti-inflammatoires (469). Chung et collaborateurs ont évalué l'effet médiateur de α- et γ-tocophérol sur des souris avec NAFLD, induite par l'administration de LPS (470). Ils ont observé une augmentation significative de la peroxydation lipidique, du facteur inflammatoire TNF-α, et des taux d'ALT sérique et hépatique, ainsi qu'une exacerbation des AG libres et des TG circulants chez les animaux avec LPS. Par contre, l'apport en α- et γ-tocophérol a contribué à réduire significativement l'inflammation et les transaminases circulantes ainsi que la peroxydation lipidique chez les souris traitées au LPS (470). La vitamine E agit donc au niveau de la seconde phase (2nd HIT) de la stéatose en diminuant les dommages au foie, la peroxydation lipidique et l'inflammation (470) (**Figure 22**). Le traitement pourrait être donc envisagé lorsque la stéatose est installée. D'autres auteurs conviennent de la capacité de la vitamine E à restreindre les taux sériques d'ALT à des valeurs normales en concordance avec les améliorations histologiques (471).

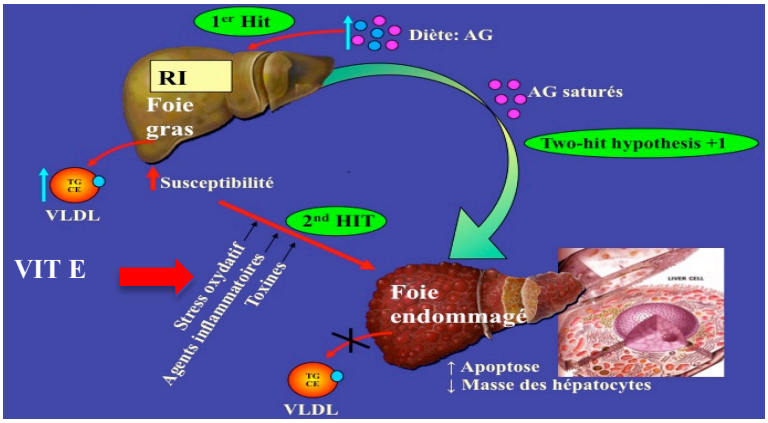

Figure 22. Cibles de la vitamine E dans le sentier métabolique menant à la stéatose.

La vitamine E, en tant qu'antioxydant puissant, peut freiner l'aggravation de la NAFLD vers le NASH, en agissant sur le stress oxydatif et l'inflammation (© Spahis).

Chez l'humain, de nombreuses études cliniques ont corroboré les observations animales. À une dose de 300 UI/j pendant 12 mois, la vitamine E améliore les niveaux de transaminases et les lésions hépatiques (472). De meilleurs résultats ont été obtenus sur le plan de la stéatose avec des doses légèrement plus élevées (400-800 UI/j) et un délai de traitement plus court (473). Ce constat a été confirmé dans un autre essai clinique, soit l'étude PIVENS, où la dose de 800 UI/j de vitamine E a été administrée sur une période de 3 mois, à des patients NAFLD adultes et non-diabétiques. Des améliorations significatives des niveaux de transaminases circulantes et d'inflammation lobulaire ont été remarquées, réduisant ainsi le score d'activité de la NAFLD (453). Ces auteurs ont aussi conclu que la vitamine E pourrait constituer un traitement efficace pour les patients non diabétiques souffrant de NAFLD. En revanche, aucun effet sur les transaminases n'a été rapporté par l'étude multicentrique TONIC de 2 ans, randomisée et en aveugle, auprès d'adolescents atteints de NAFLD, bien qu'une amélioration de l'histologie hépatique ait quand même été notée (458, 459). Chez des enfants avec NAFLD, traités par la vitamine E (400-1200 UI/jour) durant 2-4 mois, les taux de transaminases sériques ont chuté indépendamment des variations de l'indice de la masse corporelle ou de l'échogénicité du foie observés par DEXA ou par échographie (458). Avec une combinaison de plus grandes doses de vitamine E (1000 UI/j) et de la vitamine C (1000 mg/j) sur une période de 6 mois, il y avait également une amélioration de la fibrose sans grands changements des facteurs inflammatoires au niveau du foie de 45 patients diagnostiqués avec NAFLD (474).

L'ensemble de ces observations nous montre que la dose 800 UI/j de vitamine E est suffisante pour exercer un effet positif sur l'histologie du foie. Tout en préconisant la prudence, il est fort nécessaire d'encourager la communauté scientifique à établir la dose optimale et la durée de supplémentation en vitamine E, à la fois chez les adultes et les jeunes obèses atteints de NAFLD, étant donné que (i) la vitamine E n'agit pas contre l'oxydation médiée par les enzymes et les radicaux oxydants comme l'hypochlorite (475); et (ii) qu'une méta-analyse a révélé une mortalité accrue avec l'administration de vitamine E (476).

4.2 Vitamine D

Pour devenir biologiquement active, la vitamine D nécessite deux hydroxylations; une première en position 25 au niveau hépatique et la seconde au carbone 1 dans le rein (477). La 25-hydroxyvitamine D (25(OH)D) est produite exclusivement dans l'hépatocyte par l'entremise des cytochromes hépatiques CYP271 et CYP2R1 (**Figure 23**) ayant une grande affinité pour la vitamine D3. Les effets biologiques de la vitamine D3 sont médiés par le récepteur de la vitamine D (478).

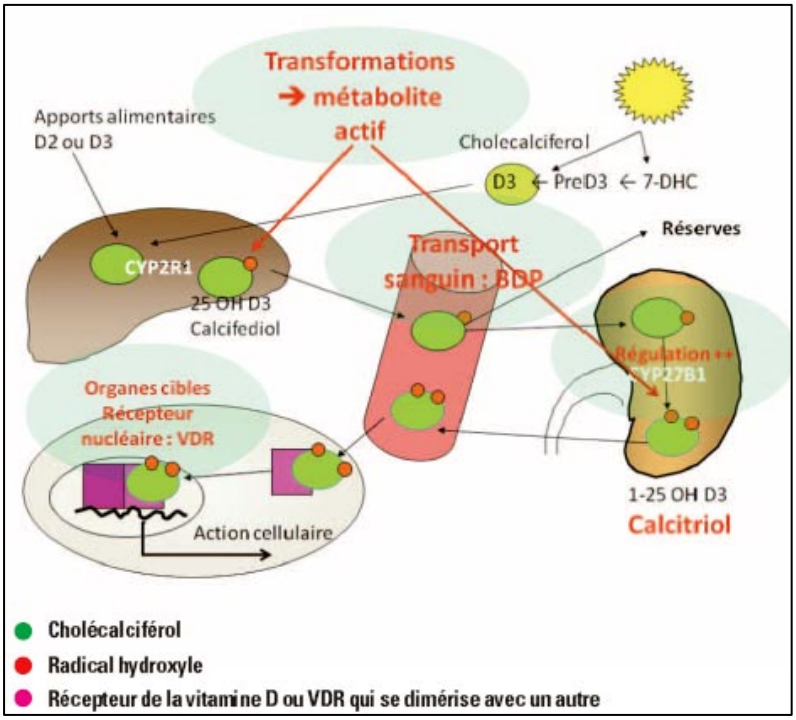

Figure 23. Aperçu du métabolisme de la vitamine D.

La 25-hydroxyvitamine D (25(OH)D) est produite dans l'hépatocyte par l'entremise du cytochrome hépatique CYP2R1 qui a une grande affinité pour la vitamine D3, adaptée de (477).

Les données expérimentales animales suggèrent que la carence en vitamine D est impliquée dans la progression de la NAFLD (479, 480) et, qu'à l'inverse, sa supplémentation exerce un bénéfice thérapeutique (479). Dans ce contexte, le modèle animal de NAFLD induit par une diète riche en lipides et en fructose, mais carencée en vitamine D, exhibe une augmentation significative de la gravité de la NAFLD; celle-ci étant associée par ailleurs à l'expression élevée des gènes médiateurs de l'inflammation. La confirmation de ces données a été obtenue dans un autre modèle animal (481), ce qui appuie l'hypothèse que la carence en vitamine D influence la régulation des gènes impliqués dans la genèse de la NAFLD, tels que ceux reliés au SOx et à l'inflammation hépatique (ex. la voie des récepteurs « Toll-like ») (479). Il est important de noter la corrélation qui a été établie entre la carence en vitamine D et le déclin de la sensibilité à l'insuline (482). D'ailleurs chez l'humain, la faible expression du récepteur de la vitamine D hépatique est étroitement associée à une histologie hépatique plus sévère (483). De plus, les faibles concentrations sériques de 25(OH)D3 ont été observées chez les sujets affectés par la NAFLD (484). Sur la base de ces considérations, le traitement avec la vitamine D pourrait donc prévenir ou même ralentir la progression de la NAFLD.

Cependant, il y a une absence d'essais randomisés, excepté celui utilisant une dose de 50,000 IU auprès de patients adultes avec NAFLD tous les 14 jours durant 4 mois (485). Les auteurs ont alors enregistré une diminution de l'inflammation et l'atténuation de la peroxydation lipidique sans par ailleurs noter l'amélioration des niveaux d'enzymes hépatiques ou de la sensibilité à l'insuline. Ces mêmes auteurs ont donc suggéré que l'apport en vitamine D pourrait servir comme complément à un traitement médicamenteux de la NAFLD (485). Bien que le mécanisme exact soit encore indéterminé, la vitamine D pourrait servir dans la prévention et le traitement de la NAFLD. Néanmoins, il demeure prématuré de la recommander pour le traitement spécifique de la NAFLD.

4.3 Coenzyme Q

L'accumulation des lipides et la RI hépatique influencent l'apparition du SOx et, par conséquent, la surproduction de ROS qui font défaillir les enzymes antioxydantes (486). Des analyses plasmatiques de patients atteints de NAFLD ont révélé une peroxydation lipidique accrue accompagnée d'une réduction de la capacité antioxydante (486). Par conséquent, des suppléments d'antioxydants alimentaires ont été suggérés pour protéger le patient des effets néfastes de la maladie et de ses complications cardio-métaboliques. Parmi les antioxydants lipidiques solubles, le CoQ est un puissant agent de réduction de la peroxydation lipidique à la fois dans les cellules et les tissus (487). De plus, comme la NAFLD est connue pour être associée à une dysfonction mitochondriale (488), elle pourrait probablement bénéficier du CoQ, connu pour son rôle important dans le maintien de la fonction mitochondriale via sa participation à la chaîne respiratoire (487). Toutefois, il est à préciser que les concentrations de CoQ sont susceptibles d'être perturbées par les changements dans les propriétés physicochimiques induits dans les membranes suite à l'accumulation des lipides et, notamment, dans la pathophysiologie de la NAFLD (489). Malgré ces contraintes, une étude animale a mis en évidence un allègement de la progression de la NAFLD suite à l'administration du CoQ, en atténuant les carences en choline et en augmentant l'activité de la NADH-CoQ oxydoréductase dans les mitochondries hépatiques (488). Peu d'études humaines ont évalué l'effet d'une supplémentation en CoQ sur la NAFLD. À date, les observations sont assez mitigées. Cependant, une étude a montré qu'une prise orale de CoQ_{10} (100 mg/j durant 28 jours) contribue significativement à la diminution du tour de taille et des niveaux sériques des transaminases hépatiques (490). Dans l'ensemble, des informations sont en train d'émerger montrant que des perturbations du métabolisme du CoQ sont impliquées dans le développement de la NAFLD, ce qui souligne la nécessité de poursuivre les études humaines.

4.4 Polyphénols

Les polyphénols constituent une famille de molécules organiques, principalement retrouvées dans le règne végétal. Leur rôle d'antioxydants a déjà été étudié pour la prévention du cancer et des MCV, mais ils suscitent de plus en plus d'intérêt pour traiter d'autres désordres cardiométaboliques tels que la NAFLD.

Les flavonoïdes sont des composés naturels souvent étudiés dans des modèles de NAFLD car ils semblent exercer des effets bénéfiques sur la santé en partie grâce à leur pouvoir antioxydant (491). Les avancées scientifiques leur ont attribué des bénéfices additionnels. En effet, ils peuvent agir comme agents anti-inflammatoires et comme régulateurs des voies métaboliques (492).

Des extraits de polyphénols de bleuets sauvages chinois ont démontré une inhibition efficace de l'accumulation des TG dans les cellules HepG2 traitées avec de l'acide oléique pour mimer une diète riche en lipides (493). Les polyphénols agiraient à travers la médiation d'enzymes et de protéines impliquées dans la synthèse des lipides au foie. En fait, les polyphénols peuvent atténuer l'accumulation des TG dans le foie via l'inhibition de l'expression de SREPB-1 (494). Ce facteur de transcription active de multiples gènes impliqués dans le transport et le métabolisme des AG, du cholestérol et des TG, dont la HMG-CoA réductase, le glycérol-3-phosphate acyltransférase, la FAS et l'ACC. Par ailleurs, les polyphénols ont le potentiel de réduire le SOx en stimulant l'expression de PPARα et les enzymes antioxydantes, soient la catalase, la GPx et la SOD (494). Néanmoins, malgré leur efficience *in vitro* et *in vivo*, peu d'études humaines ont validé leur biodisponibilité et efficacité thérapeutique.

4.4.1 Berbérine

La berbérine (BBR), un alcaloïde issu de certaines plantes, s'est montré un puissant composé bioactif intervenant dans de nombreuses voies métaboliques et pathophysiologiques

associées à la NAFLD. Des études récentes soulignent le potentiel de la BBR dans le traitement de la NAFLD chez l'humain et l'animal (495). Pour mieux cerner son implication, il est fort utile d'abord de comprendre les mécanismes modulés par la BBR, ce qui aidera également à saisir son application clinique dans la NAFLD.

La BBR a le potentiel d'abaisser le taux de cholestérol et d'inhiber la synthèse des TGs dans les hépatocytes (496) (**Figure 24**). Par ailleurs, la BBR est capable de stimuler la sécrétion d'insuline dans des cellules HIT-T15 et les îlots pancréatiques, ce qui lui confère un potentiel antidiabétique (497).

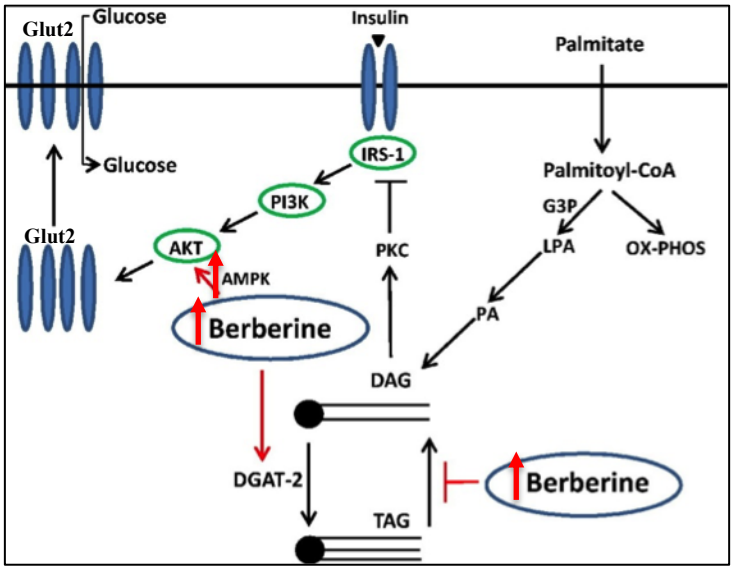

Figure 24. Mécanismes d'action de la Berberine dans le cardiométabolisme.

La BBR stimule l'absorption du glucose par 1) l'activation de la AMPK et des protéines impliquées dans la cascade de la signalisation de l'insuline et par 2) l'atténuation de l'absorption du glucose induite par le palmitate en l'activité de l'enzyme DGAT-2, adaptée de (497).

Le traitement des cellules HepG2 avec la BBR contribue à inhiber la production des cytokines inflammatoires IL-6 et TNF-α (498) qui nuisent au processus sécrétoire et à l'action de l'insuline. Au niveau signalétique, la BBR induit l'expression génique du récepteur de l'insuline et l'activité de la protéine kinase C dans les hépatocytes (499), probablement via l'activation du facteur de transcription PPARα, menant ainsi à l'atténuation de l'accumulation des TG dans le foie (500). Considérées dans leur ensemble, ces études indiquent que la BBR est un joueur dans l'homéostasie insulinique, augmentant particulièrement la sensibilité à l'insuline qui, de son côté, est capable de réguler le métabolisme hépatique.

La BBR améliore également la RI chez les souris en activant l'adenosine 5'-monophosphate-activated protein kinase (AMPK) (501) et en inhibant l'expression des gènes impliqués dans la lipogenèse hépatique, incluant ACC, FAS et SCD-1 (502). La BBR contribue également au déclin à la fois de l'expression de SREBP1c et SREBP2 et à la baisse des TG dans le foie (503). Comme les facteurs de transcription SREBPs agissent également en tant que régulateurs des niveaux du cholestérol hépatique en activant les gènes impliqués dans la synthèse du cholestérol (504), l'action de la BBR sur les SREBPs constitue donc un mécanisme potentiel pouvant être exploité dans la NAFLD. En fait, la BBR agit également sur l'expression de la protéine « uncoupling protein 2 » (UCP2) dans le foie de rats avec NAFLD, facilitant ainsi l'oxydation des graisses et la dépense énergétique (505). Finalement, la BBR semble avoir des capacités anti-oxydantes, d'où son potentiel à réduire les ROS dans le foie (506). Les travaux sur des modèles animaux ont validé les données *in vitro* décrites ci-dessus puisque la BBR peut contribuer à la prévention du développement de l'obésité et de la RI chez les rats soumis à une diète riche en lipides (507) ainsi qu'à la prévention de la NAFLD (508, 509). Comme exemples, l'injection de BBR pendant trois semaines à des souris obèses atténue l'hyperlipidémie et abaisse le poids du foie (510), et le traitement de hamsters hyperlipidémiques avec de la BBR réduit fortement l'accumulation des TG dans le foie (511) et empêche la progression de la fibrose hépatique (512).

Chez les patients hypercholestérolémiques, la BBR améliore la cholestérolémie en ciblant surtout les taux de LDL plasmatiques (513). D'autre part, les investigations cliniques ont montré que la BBR contribue à réduire les niveaux des transaminases et à restaurer la fonction

hépatique chez les patients atteints de DT2 (514). La BBR irait jusqu'à réduire la nécrose du foie à un stade avancé de la NAFLD (512). Malgré ces effets prometteurs de la BBR à la fois *in vitro* et *in vivo*, il y a absence de grandes investigations cliniques (515). Par ailleurs, des travaux sont encore nécessaires pour comprendre les mécanismes favorisant l'atténuation de la progression de la NAFLD chez l'humain.

4.4.2 Resvératrol

Le resvératrol (RSV) est un polyphénol pourvu de propriétés antioxydantes, anti-inflammatoires, anti-obésogènes et antidiabétiques.

La première étude montrant les bienfaits du RSV sur la santé métabolique date de 2006 (516, 517). Le gavage de souris, soumises à un régime riche en graisse, avec de faibles doses (10 mg/jour) de RSV, a révélé l'activation de l'AMPK hépatique accompagnée d'une diminution des enzymes lipogéniques (ACC et FAS), du poids du foie et du degré de la stéatose. Des études animales subséquentes avec cet antioxydant ont validé ses effets positifs sur les processus cardiométaboliques associés à la NAFLD. On a pu montrer que le RSV atténue la progression de la NAFLD dans d'autres modèles animaux via la réduction des TG hépatiques, la prévention à la fois de l'obésité, de la dyslipidémie et de la stéatose (518-520). Il a été aussi démontré que le RSV atténue l'inflammation (521) et la sensibilité à l'insuline, par activation de l'AMPK et la régulation de la voie des MAPKs dans les hépatocytes (522, 523). En outre, les analyses métabolomiques, suite à un traitement avec du RSV à forte dose et combiné avec la quercétine, un autre composé polyphénolique, ont montré une restauration importante des voies impliquées dans le métabolisme du glucose et des lipides (glycolyse, β-oxydation), de l'inflammation et de la fonction hépatique chez des souris soumises à une diète riche en lipides (524).
Des expériences similaires avec le RSV sur les rats ou hamsters ont abouti aux mêmes observations. Des effets réparateurs de la NAFLD ont été observés; le RSV agissant sur le poids et l'histologie du foie, (525, 526), sur les TG hépatiques (526, 527), et sur l'accumulation du cholestérol (525, 527), en association avec l'augmentation de l'expression du récepteur LDL et

du transporteur du cholestérol SR-BI au niveau du foie (527).

Cependant, les essais cliniques évaluant les effets de la supplémentation en RSV sur la pathophysiologie de la NAFLD sont peu nombreux et sans grande confirmation des études antérieures sur les animaux. Des patients avec NAFLD supplémentés avec 500 mg de RSV durant 12 semaines exhibaient un amoindrissement de l'inflammation (528) alors que d'autres ayant reçu une dose quotidienne de 150 mg de RSV pendant 30 jours montraient une réduction des transaminases, une atténuation de la stéatose hépatique (observée par résonnance magnétique) et des effets métaboliques bénéfiques (108, 109). Dans une autre étude, l'ALT plasmatique et la teneur intrahépatique en lipides ont été significativement plus faibles après 30 jours de supplémentation (529). Sur la base de ses effets puissants sur le SOx et l'inflammation, ainsi que sa grande biodisponibilité, le RSV est considéré comme une cible thérapeutique pour la prévention des maladies hépatiques.

4.4.3 Silybine/Sylimarine

Plusieurs flavonoïdes ont été étudiés pour le traitement de la NAFLD. Au cours des dernières années, il y a eu un regain d'intérêt en leur faveur. En particulier, la silymarine et la silybine, deux produits naturels dérivés du chardon-Marie (*Silybum marianum*), ont attiré l'attention par l'usage traditionnel de la plante en Grèce antique pour traiter les maladies hépatiques et biliaires (530).

Malgré le peu d'études *in vivo,* des effets antioxydants ont été attribués à ces flavonoïdes. Par ailleurs, une diminution des lipides sériques et une baisse des cytokines inflammatoires simultanément à l'inhibition du facteur NF-κB et à la prévention de la dysfonction mitochondriale ont été rapportées (531-536). Le traitement à la silymarine a été associé à une amélioration des enzymes hépatiques chez des patients avec NAFLD dans une étude contrôlée (531). Dans un essai clinique randomisé de 12 mois, comprenant 138 patients avec NAFLD, le complexe silybine-phosphatidylcholine a induit une diminution des niveaux circulants des

enzymes hépatiques, indépendamment de la RI et de la masse corporelle (90). Même si ces observations sont encourageantes, des efforts supplémentaires doivent être investis pour investiguer le rôle et les mécanismes d'action de la silymarine et de la silybine pour la prévention et le traitement de la NAFLD.

4.5 Pré-Probiotiques

Les prébiotiques sont des fibres alimentaires non digestibles qui stimulent la croissance et modifient l'activité métabolique de la microflore. Les prébiotiques ont également été indiqués pour augmenter l'action des probiotiques.

Dans les études animales, Cani et al. ont montré que des souris génétiquement obèses et supplémentées avec des prébiotiques (oligofructose, un mélange de fibres alimentaires fermentescibles) montraient une amélioration de la perméabilité intestinale et des taux circulants plasmatiques de LPS et de cytokines inflammatoires (TNFα, IL-1B, IL-1, IL-6, et INFy) (238). Cette hypothèse a été testée sur des patients diagnostiqués NAFLD et supplémentés avec l'oligofructose à raison de 16 g/j pendant 8 semaines. L'oligofructose diminue de manière significative les enzymes hépatiques sériques et les niveaux d'insuline (537), suggérant que les prébiotiques sont capables de réguler les dérangements métaboliques dans la NAFLD (**Figure 25**).

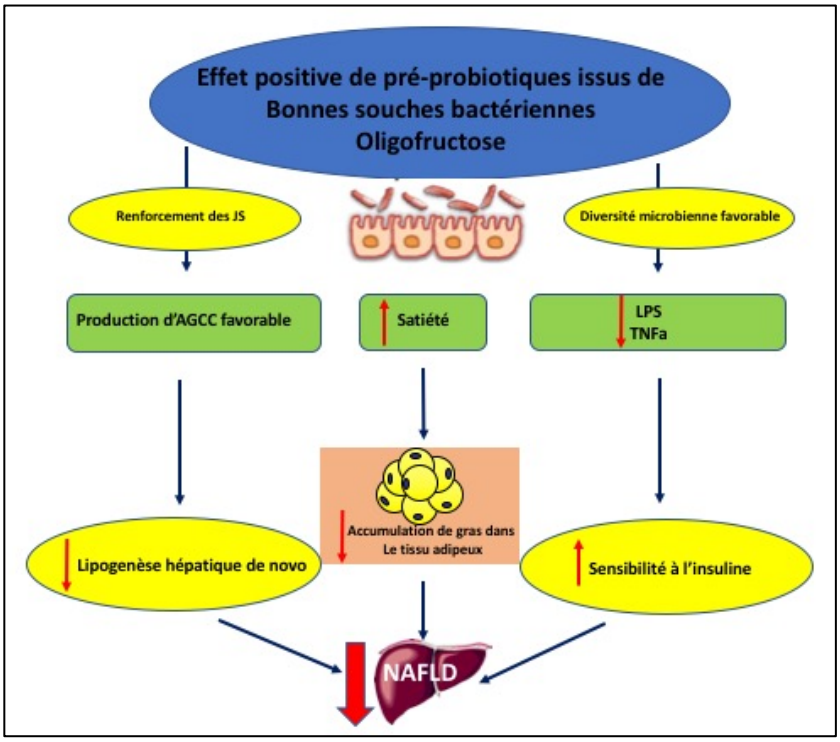

Figure 25. Effet des pré- et probiotiques sur la NAFLD.

Une alimentation riche en fibres contribue à une satiété, une meilleure production d'AGCC, un renforcement de la perméabilité membranaire et aux JS. Ces mécanismes influencent de leur part, un enrichissement en souches bactériennes favorables à un métabolisme lipidique adéquat et une meilleure sensibilité à l'insuline, et par conséquent, une atténuation de la pathogenèse associée à la NAFLD, adaptée de (538).

Similairement, le groupe de Parnell et al. (539) a mené un essai randomisé, contrôlé, en double aveugle et a examiné les effets de l'oligofructose (21 g/j pendant 12 semaines) chez 48 adultes en surpoids et obèses. Les résultats ont montré que l'oligofructose a contribué à la perte de poids et à l'amélioration de la dysrégulation du glucose (539).

Chez les rats avec NAFLD soumis à une diète riche en lipides, le traitement avec le pré-biotique lactulose contribue à la diminution des niveaux d'endotoxines et d'inflammation

hépatique (540), tout en favorisant la croissance de certaines bactéries intestinales telles que le lactobacillus et le bifidobacterium (541).

De ce fait, même si peu d'investigations ont été effectuées, il semble que les pré-biotiques peuvent contribuer à la normalisation de la flore intestinale, à l'amélioration du contrôle glycémique, du cholestérol et de l'accumulation hépatique des TG (237). Avec la validation des données, les prébiotiques peuvent représenter une option thérapeutique pour la NAFLD.

Comme mentionné auparavant, le corps humain compte jusqu'à cent milliards de bactéries dont la majorité se trouve au niveau du gros intestin (265, 542). L'idée que le microbiote intestinal puisse être impliqué dans la pathogénèse de la NAFLD a maintes fois été proposée. En effet, de récentes études expérimentales effectuées chez les animaux et les humains ont démontré que l'interaction entre le foie et la flore intestinale (axe intestin-foie) joue un rôle dans la pathogenèse de la NAFLD (543). D'une part, on observe que la NAFLD est associée à une faible croissance de la flore intestinale et à une augmentation de la perméabilité de l'intestin due à une perturbation de la formation des jonctions serrées (265, 542, 543). D'autre part, des études sur les sujets avec NAFLD rapportent une augmentation de la production d'éthanol et de LPS dans la lumière intestinale et une libération subséquente de cytokines inflammatoire par les cellules de Kupffer, engendrant ainsi une augmentation du SOx hépatique (543, 544). Le foie serait donc sensible à l'exposition des endotoxines et des métabolites actifs dérivés de l'intestin qui lui parviennent par l'intermédiaire du système porte (538, 543). Parmi les cytokines inflammatoires, le TNF-α semble jouer un rôle crucial dans la RI et le recrutement de cellules inflammatoires au foie (265, 539, 543).

Parmi les facteurs qui rendent les êtres humains sensibles à la NAFLD, on cite la fragilité de la perméabilité de l'intestin et les altérations du microbiote intestinal (545). Il a été démontré que les facteurs alimentaires peuvent altérer le profil du microbiote et ce changement permet la translocation des agents microbiens nuisibles (endotoxine) dans la circulation portale, conduisant à une réponse pro-inflammatoire via le TLR4 dans le foie. Cette voie intestin-foie a été confirmée dans plus de 49 études humaines (546) qui ont signalé des degrés importants de

prolifération bactérienne, de perméabilité intestinale, et d'endotoxémie chronique chez les enfants avec NAFLD, et même de leur éventuelle corrélation avec la gravité de la maladie (546). De ce fait, le recours à des probiotiques conduirait à un meilleur équilibre de la flore intestinale. Jusqu'à date, de nombreuses études expérimentales et des essais cliniques ont révélé des effets prometteurs (547).

De plus, il a été montré chez l'humain que la composition du microbiote diffère chez les personnes obèses par rapport aux minces et se traduit par avec une augmentation des firmicutes et une diminution des bacteroidetes (548). Des analyses poussées du microbiote intestinal chez les patients avec NAFLD ont été récemment réalisées, et les individus avec NAFLD exhibent une quantité réduite des bacteroidetes et un nombre élevé d'espèces prevotella et porphyromonas (549).

Dans les études expérimentales animales, il a été clairement montré que les probiotiques réduisent l'inflammation intestinale et améliorent la fonction de la barrière épithéliale chez le rat (550). Dans un autre modèle murin, le traitement par le mélange VSL # 3, un complément alimentaire probiotique, engendre une amélioration de l'histologie du foie, une teneur réduite en acides gras totaux hépatiques, et une diminution sérique de l'enzyme hépatique ALT en association avec une diminution de l'expression génique du TNF-α et l'activité de Jun N-terminal kinase au niveau du foie (551). Xu et al. ont démontré que la supplémentation orale avec la souche bacterienne bifidobacterium atténue l'accumulation des TG hépatiques chez le rat soumis à une diète riche en lipides, comparativement à la souche lactobacillus acidophilus (552).

Plus récemment, Alisi et al. ont effectué une étude clinique randomisée, en double aveugle, avec le VSL3 chez des enfants obèses avec NAFLD. Ils ont constaté qu'une supplémentation de 4 mois améliorait considérablement les anomalies hépatiques dues au dépôt des TG et favorisait la perte de poids d'environ 8,1% (553). Les auteurs ont établi que cette efficacité est dépendante de l'activation de GLP-1 et conclu qu'il serait important d'investiguer ce mécanisme enclenché par le VSL3. Similairement, Loguercio et collègues ont testé le VSL3 (contenant 450 milliards de bactéries dans différentes souches) auprès de patients NAFLD

durant 3 mois pour constater la diminution des marqueurs de la peroxydation lipidique (malondialdéhyde et 4-hydroxynonénal) (554).

En résumé, la perméabilité intestinale et l'altération des jonctions serrées sont présentes dans une grande proportion de patients avec NAFLD et contribuent en grande partie à la sévérité de la pathologie (555). La modulation de la flore intestinale peut représenter une nouvelle façon de traiter ou de prévenir la NAFLD puisque le microbiote intestinal et la mauvaise alimentation contribuent aux perturbations des maladies métaboliques complexes dont fait partie la NAFLD (265, 277). Les probiotiques constituent donc un nouvel agent thérapeutique prometteur pour la NAFLD. Cependant, malgré le nombre de données sur l'efficacité des pro/prébiotiques chez les animaux et de leur utilisation sécuritaire, le manque de données chez l'humain à plusieurs niveaux ne permet pas d'établir des recommandations fermes pour la NAFLD, surtout en fonction des habitudes alimentaires, du mode de vie, de l'âge, du génotype de l'hôte et de l'exposition aux antibiotiques (265).

5. ACIDES GRAS

Les lipides sont classés selon 6 catégories : les AG, les TG, les glycérophospholipides, les stérols/stéroïdes, les sphingolipides et, finalement, les terpénoïdes. Les AG sont des molécules carboxyliques aliphatiques constitués de 4 à 28 atomes de carbone. Les AG se répartissent en trois grandes classes selon le degré de leur saturation : les AG saturés, n'ayant aucun double lien et exhibant la structure moléculaire générale [CH_3-$(CH_2)_n$-COOH)] ; les AG mono insaturés avec une seule double liaison [CH_3-$(CH_2)_X$-CH=CH-$(CH_2)_Y$-COOH] et les AGPI avec plus d'un double lien. Parmi les AGPI, nous distinguons les deux classes majeures (n-6) et (n-3), distinctes sur le plan physiologique et biochimique (**Figure 26**).

La nomenclature des AG se définit par le nombre de carbones (1er chiffre de la formule), le nombre de double lien (indiqué par le second chiffre) et la position du double lien à partir du groupe methyl.

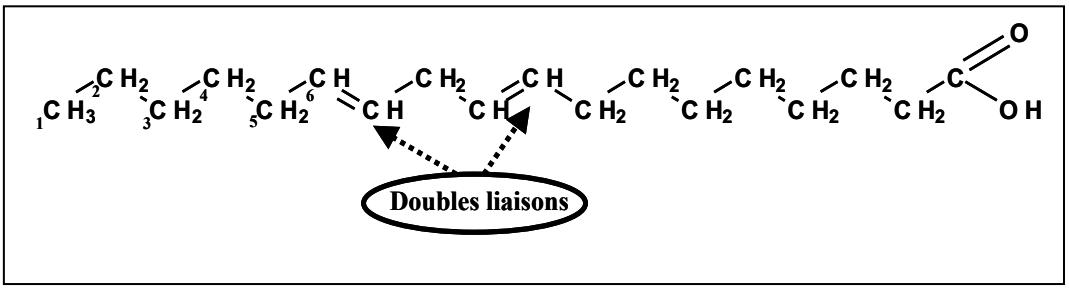

Figure 26A. Structure biochimique d'un acide gras polyinsaturé (n-6).

Un exemple dans cette figure de la formule chimique de l'acide gras alpha-linoléique (18:2n-6). Il est formé de 18 carbones, 2 doubles liens où le 1er double lien se situe au 6ème carbone à partir du methyl (CH_3) (© Spahis).

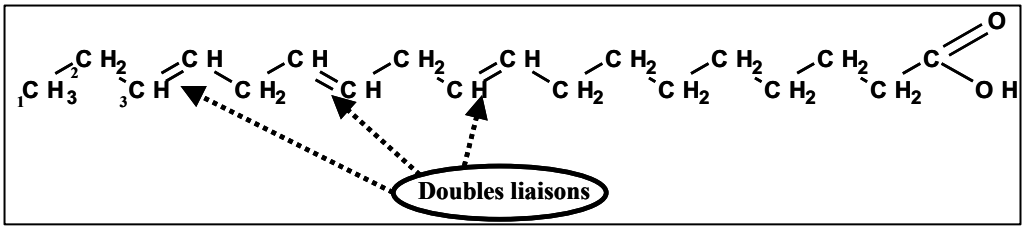

Figure 26B. Structure biochimique d'un acide gras polyinsaturé (n-3).

Un exemple dans cette figure de la formule chimique de l'acide gras alpha-linolénique (18:3n-3). Il est formé de 18 carbones, 3 doubles liens où le 1er double lien se situe au 3ème carbone à partir du methyl (CH_3) (© Spahis).

Les AG jouent plusieurs rôles dépendamment de leur nature : (i) un rôle d'abord structural puisqu'ils servent à la synthèse d'autres lipides comme les phospholipides (PL) et satisfont aux propriétés membranaires, à savoir l'élasticité, la fluidité et la viscosité; (ii) une utilité métabolique étant donné leur potentiel à fournir de l'énergie durant la β-oxydation en plus de moduler plusieurs sentiers métaboliques; (iii) une tâche de messager en vertu de la communication avec le matériel génétique dans le noyau ; et (iv) une figuration de précurseur en raison de leur capacité de se convertir en eicosanoïdes ou hormones telles que les prostaglandines, leucotriènes et thromboxanes.

5.1 Classification des Acides Gras

5.1.1 Acides gras saturés

Ces derniers sont saturés en hydrogène et ne possèdent pas de double liaison carbone-carbone. Ils proviennent essentiellement des lipides d'origine animale. Ils sont les principaux constituants alimentaires responsables de l'augmentation du cholestérol sanguin et peuvent augmenter les risques de MCV (556). Parmi les AG les plus répandus dans la nature, nous trouvons l'acide palmitique, l'acide stéarique, l'acide myristique et l'acide laurique.

5.1.2 Acides gras insaturés

Ce sont des molécules qui comportent une ou plusieurs doubles liaisons carbone-carbone. On parle d'AG mono-insaturés lorsqu'il n'y a qu'une seule double liaison et d'AGPI lorsqu'il y en a plusieurs. La double liaison carbone-carbone-HC=CH- peut déployer les deux formes isomériques *cis* et *trans* dans l'espace, qui modifient la structure tridimensionnelle des AG : la double liaison *cis*, majoritairement trouvée dans la nature, produit un coude dans la chaîne carbonée alors que la double liaison *trans* exhibe une structure étendue. Les AG *trans* (AGT) trouvés dans l'alimentation proviennent de trois sources. La première est essentiellement naturelle, issue de la transformation bactérienne des AG insaturés chez les ruminants (<0,5% de l'apport énergétique total) (557). L'homme les consomme sous forme de produits laitiers (3%) et de viande rouge (4,5 %). La seconde source d'AGT est sous forme d'huiles préparées à haute température, comme dans les fritures, pouvant entraîner un changement moléculaire des AG et leur transformation en « *trans* ». La 3^{e} source, la plus répandue au monde (contribuant 2-4% à l'apport énergétique total) (558) est également issue d'une mutation moléculaire liée à un processus exclusivement industriel: l'hydrogénation catalytique partielle d'huiles végétales.

De nombreuses observations scientifiques ont insisté sur le caractère athérogénique des AGT alimentaires en vue de leur influence positive sur les taux sanguins de LDL, leur impact négatif sur les taux de HDL, leur stimulation des marqueurs sanguins d'inflammation, et leur effet sur la dysfonction endothéliale (559-561). Santé Canada a fait des recommandations en vue de réduire la consommation d'AGT des Canadiens, considérés parmi les consommateurs d'AGT alimentaires les plus élevés au monde (562). Cette recommandation de réduire les risques en choisissant des aliments plus sains, contenant peu d'AGT, seraient encore plus fermes ou restrictives si l'état des connaissances actuelles démontre que la consommation des AGT est associée à une augmentation significative de l'obésité et des maladies cardiométaboliques dont la NAFLD.

Les AG monoinsaturés les plus répandus sont l'acide oléique qui représente 30% des AG apportés par l'alimentation (huile d'olive, huile de colza, amandes etc.). Ses vertus ont été

longuement documentées que ce soit dans des études humaines (563, 564) ou animales (565), par son rôle de protecteur contre l'athérosclérose et l'agrégation plaquettaire.

5.1.3 Acides gras polyinsaturés

5.1.3.1 Acides gras polyinsaturés n-6

Ils sont extrêmement importants dans le fonctionnement des systèmes cardiovasculaires, nerveux et immunitaires, ainsi que dans divers processus allergiques et inflammatoires. Cependant, lorsque l'apport est excessif, les AGPI n-6 deviennent délétères. On note alors la dissipation de l'impact favorable sur l'appareil cardiovasculaire et on assiste à la manifestation de douleurs et de maladies inflammatoires comme l'athérosclérose, l'asthme ou l'arthrite (566, 567).

Dans la série des n-6, seul l'acide linoléique (LA, 18:2n-6) est considéré comme essentiel. Notre organisme possède l'habileté, par élongation et désaturation, de produire les autres AG de la famille n-6, y compris les eicosanoïdes. Notons que le LA est largement présent dans notre alimentation, incluant les huiles de maïs, tournesol, soya, carthame, pépins de raisin, et autres huiles.

Tableau 5: Principaux acides gras polyinsaturés (n-6)

Symbole	Nom commun	Structure chimique	MM (g)
18 :2n-6 (LA)	Linoléique	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$	280.48
18 :3n-6	Gamma-linolénique	$CH_3(CH_2)_3(CH_2CH=CH)_3(CH_2)_4COOH$	278.48
20 :4n-6 (AA)	Arachidonique	$CH_3(CH_2)_4(CH=CHCH_2)_3CH=CH(CH_2)_3COOH$	304.52

L'acide arachidonique (AA, 20:4n-6) est un des dérivés dans la chaîne de conversion de LA. Il se retrouve dans plusieurs aliments tels que le jaune d'œuf et le gras des animaux comme source directe. Sa fonction est primordiale dans plusieurs voies métaboliques (568). L'AA est impliqué dans la cicatrisation, la guérison des blessures et contribue aux mécanismes des réactions allergiques. Néanmoins, il faut noter que la synthèse de l'AA diminue avec l'âge alors que cet AG est considéré comme essentiel chez les personnes âgées (569). Cependant, un surcroit de sa consommation peut se traduire par l'amplification des symptômes de l'arthrite, l'eczéma, le psoriasis et plusieurs maladies auto-immunes et inflammatoires (570-573).

5.1.3.2 Acides gras polyinsaturés n-3

Depuis de nombreuses années, la communauté scientifique s'est penchée sur l'effet bénéfique d'une diète riche en produits marins sur les accidents athérosclérotiques. On a bien remarqué leurs effets bénéfiques sur des populations consommant le poisson en quantités considérables tels que les Inuits ou les Eskimos (574) et les Japonais (575). Les AGPI n-3 sont utilisés dans la production d'AG hautement insaturés et des prostanoides. Ils ont une influence favorable sur les caractéristiques physico-chimiques des membranes cellulaires ainsi que sur divers processus se déroulant dans notre organisme. Notons en particulier leurs effets sur les réactions immunitaires et anti-inflammatoires dans le cancer (576), sur le système cognitif (577, 578), sur la dégénérescence maculaire (579) ou sur l'ostéoporose chez la femme et les personnes âgées (580), pour ne citer que quelques exemples. Plusieurs mécanismes d'action ont été suggérés pour expliquer leur influence sur la régulation de la tension artérielle, l'élasticité des vaisseaux, l'agrégation plaquettaire et l'athérosclérose (581), ainsi que sur les troubles cardiométaboliques (582, 583), sans omettre la NAFLD (467, 584), une section qui sera d'ailleurs développée subséquemment dans ce mémoire.

Parmi les AGPI n-3, seul l'acide alpha-linolénique (ALA, 18:3n-3) est qualifié d'essentiel étant donné le potentiel de l'organisme humain à élaborer les autres AG de la famille des n-3. Il est d'origine strictement végétale et est particulièrement présent dans les huiles de lin, chanvre, canola (colza) et soya. Ses dérivés métabolites, les acides eicosapentaénoïque

(EPA, 20:5n-3) et docosahexaénoïque (DHA, 22:6n-3), peuvent théoriquement être synthétisés à partir du précurseur ALA. Néanmoins, plusieurs travaux scientifiques ont rapporté le faible taux de conversion, ce qui a amené à la recommandation de consommer des aliments riches en EPA et DHA, de source marine, tels que le maquereau de l'Atlantique, le saumon, le hareng, le thon blanc et les sardines. Par conséquent, compte tenu de leur biogenèse limitée, les AGPI n-3 EPA et DHA sont considérés comme essentiels et doivent donc provenir de l'alimentation et principalement des poissons gras (585). Le nombre de doubles liaisons et la longueur de la chaîne fournissent aux AGPI n-3 des propriétés uniques qui les distinguent des autres graisses insaturées (586).

Tableau 6: Principaux acides gras polyinsaturés (n-3)

Symbole	Nom commun	Structure chimique	MM (g)
18:3n-3 (ALA)	Alpha-linolénique	$CH_3(CH_2CH=CH)_3(CH_2)_7COOH$	278.48
20:5n-3 (EPA)	Eicosapentaenoique	$CH_3CH_2(CH=CHCH_2)_4CH=CH(CH_2)_3COOH$	302.52
22:5n-3 (DPA)	Cuplanodonique	$CH_3CH_2CH=CH(CH_2)_2CH=CHCH_2(CH=CH(CH_2)_2)_3COOH$	330.57
22:6n-3 (DHA)	Docosahexaenoique	$CH_3CH_2(CH=CHCH_2)_5CH=CH(CH_2)_2COOH$	328.57

5.1.3.3 Équilibre n-3/n-6 et indice n-3

Les végétaux peuvent transformer le LA, précurseur de la famille des AGPI n-6, en ALA, précurseur des AGPI n-3. Chez l'homme et chez l'animal, ces deux précurseurs, le LA et l'ALA, ne peuvent être fournis que par l'alimentation. De plus, les voies métaboliques assurant la transformation de LA en AA et d'ALA en EPA/DHA utilisent des enzymes communes (**Figure 27)** qui sont mises à contribution pour augmenter le nombre de doubles liaisons (désaturation)

et la longueur de la chaîne carbonée (élongation) en vue de former l'EPA et le DHA à partir du ALA, ou l'AA à partir du LA, entraînant ainsi une compétition entre les deux voies de n-3 et n-6. De ce fait, le déséquilibre d'apport en faveur des n-6, comme c'est actuellement le cas dans la plupart des pays industrialisés, oriente par conséquent le métabolisme vers l'AA qui est plutôt pro-inflammatoire (**Figure 27**).

Par ailleurs, les AGPI n-3 et n-6 précurseurs des eicosanoïdes, soit le LA et l'ALA, se transforment en prostaglandines, thromboxanes et leucotriènes par désaturation alternée (delta-6 et delta-5 désaturases) et allongement de la chaîne par des élongases. Comme exemple, les prostaglandines synthétisées à partir des AGPI n-6 induisent une inflammation, tandis que certaines prostaglandines synthétisées à partir des AGPI n-3 inhibent l'inflammation. L'abondance des n-6 alimentaires inclinent la cyclooxygénase 2 à leur avantage et promeuvent le sentier inflammatoire.

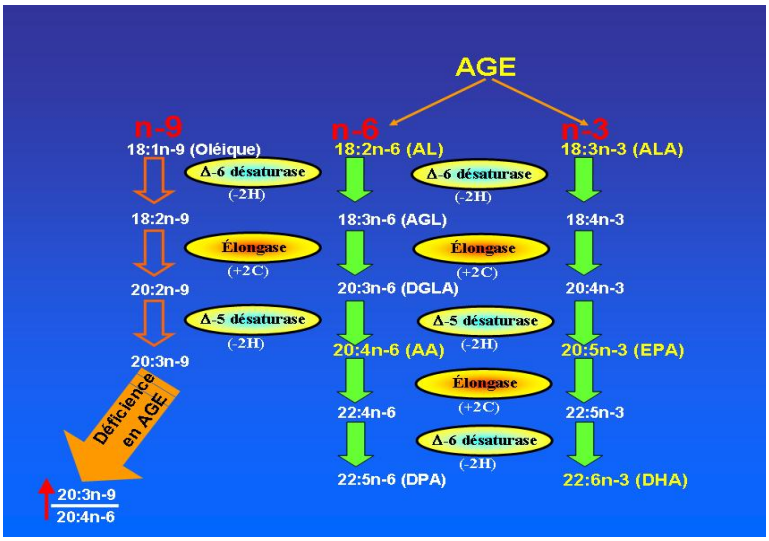

Figure 27. Élongation et désaturation des AGPI (n-6) et (n-3).

En absence des AG : LA et ALA, seule la voie des n-9 reste fonctionnelle produisant ainsi un ratio 20:3n-9/20:4n-6 élevé, un marqueur de déficience en AG essentiels (© Spahis).

L'avènement de l'ère industriel, le développement de l'agriculture céréalière, la production bovine (principale source d'AGPI n-6) et nos habitudes de vie ont fortement contribué à augmenter l'apport en n-6 au détriment des n-3. Dans les pays industrialisés, le rapport n-3/n-6 est estimé à 1/15-1/30. Toutefois, il n'existe aucun consensus universel sur l'apport optimal en n-3 ou l'équilibre adéquat du ratio n-3/n-6. À la base, on s'est inspiré beaucoup des Inuits (587) et des Japonais (588) pour instaurer les recommandations, mais eux aussi présentent un ratio n-3/n-6 différent, à savoir 1/3 et 3/1, respectivement. Par ailleurs, malgré l'évolution des techniques analytiques dans la détermination des compositions et le dosage des AG, la quasi-totalité des tableaux alimentaires sont divergents. Pour ne citer que quelques exemples, certains auteurs se basent sur l'apport énergétique ou calorique, d'autres se concentrent soit sur la teneur en ALA, EPA ou DHA, soit sur le pourcentage des lipides contenus dans les produits marins ou végétaux (589-591).

Toutefois, un consensus scientifique met en évidence la relation étroite entre un ratio n-3/n-6 réduit et la diminution des complications cardiométaboliques et cardiovasculaires. De nombreuses études ont montré qu'un ratio de n-3/n-6 équivalent à 1/4 réduit de 70% la mortalité chez les sujets suspectés de MCV (592, 593). Il est toutefois à noter que malgré les preuves scientifiques sur les bienfaits des AGPI n-3, la compétition de ces derniers avec les AGPI n-6 pour les mêmes enzymes d'élongation ou de désaturation, servant à convertir les acides gras précurseurs en dérivés, et nonobstant les recommandations nutritionnelles des experts à optimiser l'apport alimentaire en n-3, le rapport n-3/n-6 demeure bien bas dans les sociétés nord-américaines.

Des groupes de recherche se sont aussi penchés sur l'empreinte nutritionnelle associée à la prise alimentaire des AGPI n-3 de source marine et le risque cardiovasculaire. Le groupe de Harris a développé l'indice n-3, un outil de mesure, qui fait référence au pourcentage total de EPA+DHA, par rapport aux AG totaux, dans les érythrocytes. L'indice n-3 est ainsi défini par le calcul du niveau de EPA+DHA. Si cet indice est > 8 %, il constitue une très bonne protection cardiométabolique alors que s'il est < 4 %, il est associé à un risque cardiovasculaire (594, 595). Désormais, cet indice est proposé comme un nouveau facteur d'évaluation du risque des MCV.

5.2 Acides Gras Polyinsaturés n-3 et Maladies Cardiométaboliques

Les AGPI n-3 à longue chaîne sont connus pour leurs multiples bienfaits. Leurs propriétés préventives et thérapeutiques, rapportées surtout par des études épidémiologiques, ont été soulignées pour diverses maladies, y compris les désordres cardiométaboliques regroupant l'obésité, le SyM, la NAFLD, le DT2 et les troubles cardiovasculaires (582, 583). Étant donné notre grand intérêt relatif aux nutriments fonctionnels, notamment les AGPI n-3 susceptibles de présenter des avantages pour les sujets porteurs de NAFLD, nous décrirons succinctement les caractéristiques, le métabolisme et l'impact des AGPI n-3 sur les maladies cardiométaboliques, en mettant en relief la NAFLD (467, 584).

L'intérêt pour les AGPI n-3 provient des preuves documentées que les taux de MCV chez les Esquimaux étaient bien inférieurs à ceux de la population danoise, malgré la forte consommation de graisse des Esquimaux (587). L'apport élevé en graisses des Esquimaux était dû à la consommation élevée de viande et de graisse de baleine qui contenait de grandes quantités d'AGPI n-3 (587). L'étude historique de Bang et al. a démontré les effets anticoagulants des AGPI n-3 (596). Depuis lors, il y a eu de nombreuses publications sur les avantages pour la santé cardiovasculaire engendrés par la consommation des AGPI n-3.

Des preuves supplémentaires sur les effets bénéfiques des AGPI n-3 proviennent des études observationnelles informant que les taux de décès cardiaques dans la population japonaise sont de 2,5/1000 comparativement à 17/1000 individus dans la population occidentale (597). Cet avantage extraordinaire est expliqué par le fait que les Japonais consomment en moyenne un repas de poisson par jour qui fournit environ 900 mg d'AGPI n-3 (598). Par contre, lorsque les Japonais déménagent à Hawaï et adoptent une alimentation beaucoup plus faible en AGPI n-3, les facteurs de risque des MCV s'élèvent considérablement, suggérant que l'effet protecteur japonais n'est pas uniquement dû à la génétique (599). De façon intéressante, un apport quotidien de 750 mg d'AGPI n-3 entraine une meilleure protection contre la mortalité ajustée selon l'âge pour les MCV, la coronaropathie et les accidents cérébraux vasculaires dans une étude transversale sur 38 pays (600). De ce fait, des effets cardioprotecteurs sont attribués

aux AGPI n-3, surtout en présence des dérivés EPA et DHA : des bénéfices hypolipidémiants, anti-inflammatoires via la production d'eicosanoïdes, la modulation des canaux ioniques cardiaques et une influence évidente sur l'intégrité membranaire, des impacts anti-thrombotiques et anti-arythmiques, via probablement la génération de bons médiateurs lipidiques, et une amélioration des voies de signalisation cellulaire (601-603). Certains travaux conduits sur des rats démontrent la supériorité du DHA comparativement au EPA à réduire l'incidence et la sévérité des arythmies ventriculaires (604). Chez l'humain, la protection contre l'arythmie cardiaque et la mort subite cardiaque a été confirmée par l'essai GISSI-Prevenzione (840 mg d'AGPI n-3 au total dont 560 mg de DHA) qui a montré une réduction de 20% du nombre total de décès, une réduction de 35% des décès coronariens et une réduction de 45% de la mort subite cardiaque (605). Cependant, des ambiguïtés persistent étant donné les révélations contradictoires des méta-analyses ou de recherches cliniques prospectives visant la collecte des données nutritionnelles: certaines **sont** en appui aux effets favorables (606-612) et d'autres en défaveur (613-615) des AGPI-n3. Ces différences peuvent s'expliquer par les protocoles cliniques variables, la dimension des cohortes, les doses des AGPI n-3, et les statuts pathophysiologiques.

L'effet des AGPI n-3 sur la baisse des TG est le plus rapporté dans la littérature. Leurs mécanismes d'action consistent à réguler à la baisse la production des VLDL hépatiques. D'autre part, les AGPI n-3 sont capables d'abaisser la quantité d'AG libres qui sont livrés au foie, tout en augmentant la β-oxydation des AG dans le foie et même dans d'autres tissus comme le tissu adipeux, le cœur et le muscle (616). Dans le même sens, les AGPI n-3 augmentent la captation des AG (suite à la lipolyse par la lipoprotéine lipase) dans les adipocytes, les cellules musculaires cardiaques et squelettiques, ce qui en plus empêche l'hydrolyse des TG du tissu adipeux tout en en supprimant l'inflammation (616). Finalement, les AGPI n-3 ont la capacité de diminuer l'hypertriglycéridémie postprandiale (617, 618) ainsi que le contenu des CM et des VLDL en TG et la teneur des résidus de CM en cholestérol (619). Des essais cliniques abondent dans le même sens puisque le régime riche en poisson a diminué les concentrations de lipoprotéines riches en TG au cours de 24 semaines de traitement (620), et de même dans une étude d'intervention clinique, à double insu, randomisée et croisée (621).

5.2.1 n-3 et insulinorésistance

La RI est définie comme une condition où les concentrations physiologiques d'insuline ne sont plus capables de maintenir des concentrations normales de glucose sanguin en raison de la moindre captation du glucose par le muscle squelettique, l'augmentation des AG dans le tissu adipeux, l'utilisation musculaire du glucose stimulée par l'insuline diminue, de même que la synthèse musculaire du glycogène et de la néoglucogenèse hépatique (622). La RI est associée à des maladies, incluant l'obésité, le SyM, le DT2, la NAFLD et sa progression en NASH. Plusieurs facteurs sont impliqués dans le développement de la RI, à savoir la prédisposition génétique, l'inactivité physique, les régimes hypercaloriques, l'inflammation, le SOx, l'altération de la signalisation insulinique et la fonction mitochondriale (623-625).

Un traitement avec les AGPI n-3 s'est révélé très efficace pour réduire ou prévenir la RI, tout en supprimant partiellement le processus inflammatoire (626, 627) via la stimulation, par exemple, du récepteur GPR120, connu dans la littérature comme un senseur du DHA (626). D'autres groupes de chercheurs indiquent que l'influence des AGPI n-3 sur la sensibilité périphérique à l'insuline est induite par des modifications de la fonction mitochondriale. En effet, les changements insulino-résistants dans le muscle, induits par la diète riche en lipides, ont été atténués par l'huile de poisson, en agissant sur la phosphorylation des kinases [protéine kinase B (Akt) et de la AMPK], et de l'expression génique des protéines mitochondriales (PGC1α, PPARα, CPT1 et UCP3) (628). Des expériences appuient aussi le déclin de la RI en réponse aux AGPI n-3 par la baisse de l'inflammation via des modifications des microARNs (629).

Cependant, dans une méta-analyse de 31 essais, seulement 9 essais ont montré une amélioration des mesures de la sensibilité à l'insuline suite à une intervention avec les AGPI n-3 avec un effet plus marquant chez les femmes (630).

5.2.2 n-3 et diabète

Plusieurs études de traitement avec les AGPI n-3 démontrent un impact marqué sur le développement du diabète et des marqueurs cardiométaboliques dans des modèles animaux (631-633). Cependant, un bref aperçu des essais cliniques randomisés chez les patients diabétiques a suggéré que l'effet des AGPI n-3 sur le contrôle glycémique est mitigé (634). Certaines études montrent un effet bénéfique et une amélioration de la glycémie, d'autres un effet contraire et **même** une aggravation de la glycémie et, finalement, certains aucun effet des AGPI n-3 (634). Par contre, il est bien probable que de meilleurs résultats auraient été atteints avec des stratégies préventives plutôt que de traitement, basées sur les effets pléiotropiques des AGPI n-3 tels que leur régulation de l'inflammation, de l'immunité innée au niveau des macrophages, et la fonction paracrine ou endocrine des cytokines.

5.2.3 n-3 et NAFLD

Des évidences scientifiques ont montré qu'en plus du fructose, les graisses saturées sont aussi susceptibles de stimuler l'accumulation lipidique hépatique et la progression de la NAFLD, alors que les graisses insaturées peuvent avoir un impact préventif (635, 636). En particulier, les AGPI de la famille des n-3 réduisent considérablement l'accumulation des TG, agissent sur les voies métaboliques associées à la NAFLD et freinent son expansion (637).

Reconnus surtout pour leurs effets cardio-protecteurs (638, 639), les AGPI peuvent se révéler efficaces pour le traitement de la NAFLD. L'AG essentiel α-linolénique et ses dérivés [l'acide éicosapentaénoïque (EPA) et l'acide docohexaenoïque (DHA)] sont les trois principaux AGPI n-3 à chaînes longues (585). On les retrouve principalement dans les poissons gras. Le nombre élevé de leurs doubles liaisons, la longueur de leur chaîne, et la présence de la première double liaison en position 3 confère à ces AGPI des propriétés uniques qui les distinguent des autres gras insaturés (586).

Les mécanismes d'action des AGPI n-3 sur la NAFLD sont nombreux. Les AGPI n-3 affectent plusieurs étapes de la cascade physiopathologique et peuvent empêcher la progression de la NAFLD (**Figure 28**).

Les AGPI n-3 contribuent à (i) la diminution de la RI induite par l'apport calorique excessif et l'accumulation des lipides dans le tissu adipeux; (ii) l'augmentation des concentrations sériques d'adiponectine, préalablement réduites par l'effet de l'obésité qui favorise l'accumulation de graisse dans le foie; (iii) la réduction de la libération des AG libres dans la circulation, qui contribuent généralement à la dyslipidémie en condition d'obésité; (iv) l'augmentation du rapport n-3/n-6 dans les PL de la membrane cellulaire; (v) L'augmentation du nombre de récepteurs de l'insuline sur la membrane hépatique, améliorant ainsi la sensibilité à l'insuline; (vi) la modulation positive de facteurs nucléaires comme le PPARα qui contrôle la transcription de gènes hépatiques impliqués dans le métabolisme des lipides, notamment la β-oxydation mitochondriale, résultant ainsi en une réduction de la synthèse des TG hépatiques; (vii) la modulation négative du facteur de transcription SREBP-1c (qui favorise la synthèse des TG), ce qui entraîne une réduction de l'accumulation des TG hépatiques; (viii) un effet inhibiteur sur le SOx mitochondrial et la synthèse du ROS; (ix) une réduction du stress du RE, prévenant ainsi la lipotoxicité hépatique; (x) la prévention de l'augmentation de la synthèse des eicosanoïdes pro-inflammatoires dans les hépatocytes et de la transcription des cytokines pro-inflammatoires via leurs dérivés n-3 bioactifs: protectines et résolvines; et finalement, (xi) la réduction de la fibrose hépatique et la progression de la maladie vers le NASH en agissant sur la réduction des niveaux de ROS et la suppression de la production de l'inflammation (585).

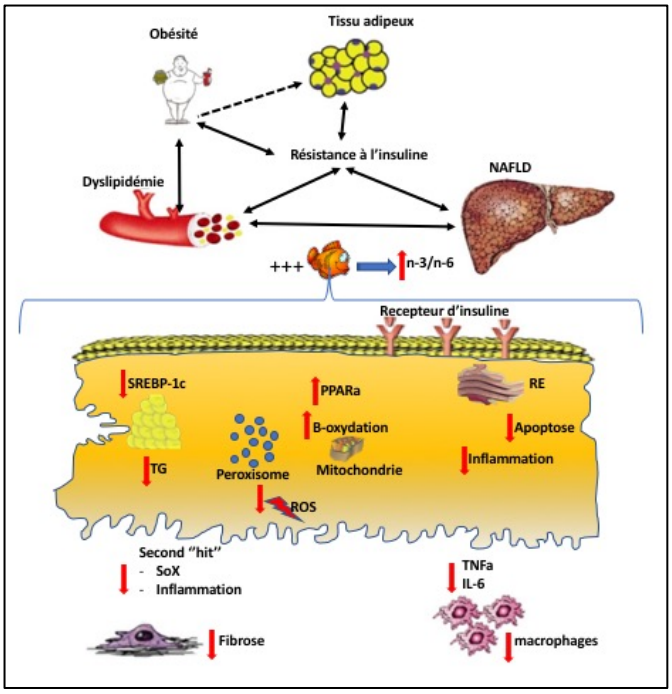

Figure 28. Mécanismes d'action des AGPI n-3 sur la NAFLD.

Les actions proposées sont indiquées par des flèches ↑ et ↓ pour augmentation et diminution, respectivement, adaptée de (585).

L'accumulation des TGs, qui est la principale caractéristique de la pathologie NAFLD, est fortement associée au développement de la RI (640). Actuellement, la communauté scientifique continue à explorer si l'augmentation des niveaux de TG constitue une condition préalable pour le développement de la RI (641). Les AGPI n-3 agissent sur la synthèse des lipides en inhibant l'expression des facteurs de transcription comme la SREBP-1, impliquée dans la production d'AG et l'accumulation de TG hépatiques (642) via la liaison avec PPARα (585).

PPARα est un récepteur nucléaire exprimé principalement dans le foie. Les AGPI n-3 sont des ligands naturels et régulateurs des récepteurs de PPARα. Grâce à l'activation de PPARα, ils réduisent les lipides plasmatiques et augmentent la β-oxydation mitochondriale au niveau hépatique (643). Dans ce contexte, des effets bénéfiques de l'EPA ont été rapportés sur la

sensibilité hépatique à l'insuline de souris soumises à une diète riche en lipides (644). De même, un traitement avec l'EPA a supprimé la lipogenèse et la stéatose hépatique chez les souris nourries avec un régime riche en graisses et en sucrose (645).

Comme il a été suffisamment documenté, les AGPI n-3 ont des propriétés bioactives et sont bénéfiques pour le traitement de l'inflammation du foie associée à l'accumulation de graisses hépatiques (638). Dans la NAFLD, il a été montré que les AGPI (EPA et DHA) diminuent la production d'IL-6 et de TNF-α (646). Ils inhibent l'activation de la voie du NF-κB, un chef d'orchestre de l'inflammation (463, 646). En effet, les AGPI n-3 préviennent la phosphorylation et la dégradation d'IκB-α, empêchant ainsi la translocation de NF-κB au noyau et la production de cytokines pro-inflammatoires.

La production d'eicosanoïdes pro-inflammatoires hépatiques, principalement par le biais des AGPI n-6, augmente le statut insulino-résistant et inflammatoire dans la physiopathologie de la NAFLD (647). De ce fait, les AGPI n-3 peuvent agir en tant que médiateurs anti-inflammatoires endogènes en modulant la voie de signalisation de l'insuline entre la membrane et le noyau des hépatocytes via leurs dérivés (résolvines, protectines), modifiant ainsi la transcription des cytokines pro-inflammatoires (648). De même, les AGPI n-3 sont impliqués dans le contrôle de l'homéostasie du glucose et affectent en particulier le développement de la RI et de la progression de la NAFLD (647), en régulant négativement l'activité de ChREBP, un facteur de transcription contribuant en partie à la NAFLD et à la RI (649). Dans des modèles expérimentaux, le blocage de ChREBP a abouti à une sensibilité accrue de l'insuline et à la réduction de la stéatose (649).

Depuis les cinq dernières années, l'avancement de la recherche a permis la découverte des effets bénéfiques des résolvines, maresines et protectines, ces médiateurs lipidiques dérivés des AGPI n-3 (DHA et EPA) qui peuvent fournir une protection pour le foie, en réduisant l'activité du processus inflammatoire hépatique (650). González-Périz et al ont déterminé les effets de l'administration d'une diète enrichie en DHA à des souris ob/ob, un modèle expérimental de RI et de NAFLD induites par l'obésité (648). Ils ont observé une amplification de la formation des résolvines et des protectines accompagnée par l'inhibition de la formation

de médiateurs inflammatoires dérivés des AGPI n-6. Ce groupe de chercheurs a noté que ces dérivés stimulent la sensibilité de l'insuline et accroissent l'expression de l'adiponectine (648).

Spécifiquement chez l'humain, il a été clairement documenté qu'une accumulation d'AG au niveau du foie, incite à une régulation positive de SREBP-1c (un facteur de transcription clé de la lipogenèse) et une régulation négative de PPARα (un facteur de transcription clé dans l'oxydation des AG), en association avec un déclin des AGPI n-3 chez des patients obèses en présence d'une RI (651, 652). Des méta-analyses d'essais cliniques ont été également réalisées pour déterminer si les AGPI n-3 avaient une influence bénéfique sur la NAFLD. La première, employant des essais cliniques non contrôlées, a bien confirmé cette hypothèse (653). Une seconde méta-analyse a recensé 13 études cliniques contrôlées et randomisées et a systématiquement examiné les résultats regroupant 266 patients supplémentés avec les AGPI n-3 et 402 patients non soumis à un régime d'AGPI n-3 (654). Clairement, les AGPI n-3 ont abaissé les taux sériques d'ALT et ont amélioré la fonction hépatique chez les patients avec NAFLD comparativement aux contrôles.

Les effets des AGPI n-3 ont également été évalués chez les jeunes patients diagnostiqués avec NAFLD en raison de leurs caractéristiques hypotriglycéridémiques et anti-inflammatoires. Le traitement des enfants avec le DHA a réduit les troubles métaboliques ainsi que des TG hépatiques (655). Cependant, d'autres essais cliniques avec le DHA n'ont pas montré d'amélioration au niveau des enzymes hépatiques et du poids corporel (462).

Récemment, le groupe de Nobili a évalué l'effet de différentes doses (250 mg et 500 mg/jour) du DHA sur des enfants avec la NAFLD (461). Une diminution significative du contenu lipidique du foie a été notée chez les deux groupes supplémentés sans effets secondaires notables, suggérant que les doses administrées sont sans danger et efficaces. Ils ont aussi noté une amélioration significative du profil lipidique et de la RI. D'autre part, une dose d'EPA de 2700 mg/jour a amélioré significativement les concentrations des transaminases hépatiques, d'AG libres et de cholestérol chez des sujets avec NAFLD (656). Les AGPI n-3 réduiraient alors la stéatose, rétablissant presque l'homéostasie lipidique hépatique (585). Par ailleurs, le traitement quotidien de patients atteints de NAFLD avec de l'huile de poisson pendant 6 mois a

amélioré le profil lipidique des patients, diminué la libération de cytokines pro-inflammatoires au niveau du foie, tout en inhibant le déséquilibre redox caractérisé par une augmentation de la peroxydation lipidique et l'activité des radicaux libres (657).

En dépit des résultats rapportés, les études présentent plusieurs limites : l'impossibilité de répéter les résultats chez les enfants à cause des difficultés à prélever des biopsies en raison de contraintes éthiques, le manque de suivi, l'absence de randomisation, l'absence de groupes témoins, et le faible nombre d'échantillons (461, 656). Néanmoins, une supplémentation en AGPI est une approche prometteuse dans le traitement des patients NAFLD car les AGPI n-3 affectent différents points de la cascade physiopathologique dans le foie. Les données provenant d'essais cliniques humains ou d'études animales indiquent clairement que la supplémentation ou la consommation de poisson pourrait prévenir ou réduire la NAFLD et les facteurs cardiométaboliques qui lui sont associés (638, 658). De plus, il n'y a aucune étude clinique randomisée qui a décelé le risque potentiel d'une hémorragie interne suite à la consommation excessive des AGPI n-3 qui pourrait ainsi les incriminer ou contredire leur bénéfice métabolique (659). En ce qui a trait à la quantité à prescrire, l'hétérogénéité des interventions ne permet pas d'établir une dose optimale minimum ni les doses individuelles pour les patients NAFLD, mais les avantages sont observés avec un apport de plus de 0,83 g/jour de supplémentation en AGPI n-3. Il faut noter que l'*American Heart Association* et l'*American Diabetes Association* recommandent un apport moyen journalier chez le sujet sain de 100 mg d'EPA+DHA ou au moins deux portions de poisson gras par semaine, tandis que l'OMS recommande une consommation moyenne d'environ 500 mg/jour d'EPA+DHA. Les gouvernements de divers pays (Royaume-Uni, France, Australie, etc.) recommandent 140-600 mg/jour d'EPA+DHA, ce qui correspond à 30-40 g/jour de consommation de poisson (647, 660). Des recherches complémentaires sont nécessaires pour définir les groupes de patients appropriés, pour quantifier l'ampleur de la réduction de la graisse du foie, et, enfin, pour établir la dose optimale et la durée de traitement avec les AGPI n-3. À l'heure actuelle, il est suggéré que la dose soit personnalisée dans la NAFLD en fonction de l'évaluation clinique et la présence ou l'absence de comorbidités cardiaques ou autres complications (661).

Malgré les avancées sur les avenues thérapeutiques nutritionnelles pour la prise en charge de la NAFLD et de ses complications cardiométaboliques et physiopathologiques, indépendamment de l'obésité, la recherche clinique n'a pas été exhaustive pour permettre d'établir les doses optimales et de connaître la biodisponibilité des nutriments fonctionnels ou bioactifs. C'est suite à des travaux de grande qualité qu'il sera possible de renforcer les recommandations tout en proposant l'adoption d'un meilleur style de vie chez les patients atteints de la NAFLD, axé sur un environnement nutritionnel favorable. Pour l'instant, les recommandations peuvent se résumer à la restriction de l'apport calorique de moins de 30 kcal/kg/j, avec un faible apport en gras saturés et en sucres simples, une supplémentation en antioxydants (400-800 UI/j de vitamine E, 1000 UI/j de vitamine D), en polyphenols et, en AGPLI-3 (1 g/jour) ou au moins la consommation de poissons 2 fois/semaine. Finalement, pour favoriser le maintien de cette hygiène de vie et le suivi des patients, il serait souhaitable de regrouper tous les intervenants de la santé en plus des chercheurs, comme équipes multidisciplinaires, pour concentrer les efforts et intervenir d'une manière plus efficace et rationnelle sur les facteurs de risque métaboliques associés à la NAFLD.

HYPOTHÈSES ET OBJECTIFS DE RECHERCHE

L'obésité est certes devenue l'un des plus grands défis en santé publique, contribuant à l'augmentation du risque de nombreuses maladies chroniques telles que le DT2, la NAFLD, l'hypertension, les MCV et autres comorbidités (6). Il est largement admis que notre mode de vie contribue à une augmentation de l'obésité, mais il est également clair qu'une composante génétique importante est derrière le risque de développer un phénotype obèse (37). Toutefois, notre compréhension des interactions entre la génétique et l'environnement nutritionnel demeure ambigüe. C'est dans ce cadre que s'inscrit mon projet scientifique de doctorat qui, je l'espère, jettera un peu de lumière sur cet aspect.

L'épidémie de l'obésité soulève des préoccupations cliniques, économiques et sociétales sur la santé. Cependant, les stratégies préventives et thérapeutiques efficaces font actuellement défaut, principalement en raison de l'incompréhension des mécanismes moléculaires sous-jacents. Les approches moléculaires ont été certainement d'un grand support pour définir plusieurs mutations et variances génétiques. Cependant, la communauté scientifique s'est vite rendue compte que, suite à l'identification de gènes endocriniens et neurologiques étiologiques, l'obésité n'est certainement pas seulement une maladie endogène. Le premier objectif de ce travail de doctorat est justement de démontrer, *in vivo,* que des gènes codant pour les protéines intestinales clés associées au transport des lipides, comme c'est le cas du Sar1b GTPase, peuvent interagir avec l'environnement nutritionnel pour produire l'obésité et des désordres métaboliques.

De plus, pour montrer la gravité de l'obésité associée aux complications cardiométaboliques, nous décrivons dans le second objectif, le degré de la NAFLD (léger, modéré et sévère) chez des sujets adultes ayant subi une chirurgie bariatrique. L'avantage d'avoir à notre disposition des échantillons de foie humain nous permet d'approfondir les mécanismes de la lipogenèse, la gluconéogenèse et la dysfonction mitochondriale dans un état de dérangement métabolique et une NAFLD, tout en mettant en évidence les interactions protéine-protéine en utilisant la protéomique comme outil d'analyse.

Finalement, s'il nous semble clair que des lignes directrices nutritionnelles sont requises pour réduire l'obésité et ses comorbidités, les aliments fonctionnels demeurent assez ignorés et ne sont pas exploités à leur pleine capacité comme moyen d'intervention pour les désordres cardiométaboliques. Par conséquent, le troisième objectif de notre programme scientifique est d'évaluer l'efficacité de la supplémentation en AGPI n-3 et sa tolérance par des enfants/adolescents diagnostiqués de la NAFLD.

Les objectifs spécifiques de ce programme scientifiques sont donc de :

1- *Définir si un milieu nutritionnel défavorable agit en synergie avec un gène relié au transport intestinal des lipides pour prédisposer à l'obésité et accroitre les risques cardiométaboliques sous-jacents à l'obésité;*

2- *Élucider les mécanismes de la NAFLD en scrutant des processus intrahépatiques dans des spécimens de foies provenant de sujets obèses morbides immédiatement après la chirurgie bariatrique;*

3- *Déterminer si une intervention avec un nutriment fonctionnel, les AGPI n-3, améliore les conditions de la NAFLD, une des co-morbidités majeures associée à l'obésité.*

RÉSULTATS

Les résultats, émanant de mon projet de recherche doctoral, sont présentés sous forme d'articles scientifiques dans ce chapitre et répondent à chaque objectif énuméré dans la présente thèse. Ils se répartissent de la façon suivante;

Objectif #1 : Définir si un milieu nutritionnel défavorable agit en synergie avec un gène relié au transport intestinal des lipides pour prédisposer à l'obésité et accroitre les risques cardiométaboliques sous-jacents à l'obésité.

Deux articles scientifiques et une revue de synthèse sont reliés à cet objectif;

- *Article 1 : « Sar1b transgenic male mice are more susceptible to high-fat diet-induced obesity, insulin insensitivity and intestinal chylomicron overproduction ».*
- *Article 2 : «Tissue distribution and regulation of the small Sar1b GTpase in mice»*
- *Revue 1: «The Epigenetic Machinery in Vascular Dysfunction and Hypertension».*

Objectif #2 : Élucider les mécanismes de la NAFLD en scrutant des processus intrahépatiques dans des spécimens de foies provenant de sujets obèses morbides immédiatement après la chirurgie bariatrique.

Un article scientifique et une revue de synthèse sont reliés à cet objectif;

- *Article 3 : «Uncovering the hepatic metabolic signature of non-alcoholic fatty liver disease via direct investigation and proteomic analysis of morbid obese patient»*
- *Revue 2: «Metabolic Syndrome as a Multifaceted Risk Factor for Oxidative Stress»*

Objectif #3 : Déterminer si une intervention avec un nutriment fonctionnel, les AGPI n-3, améliore les conditions de la NAFLD, une des co-morbidités majeures associée à l'obésité.

Deux articles scientifiques et une revue de synthèse sont reliés cet objectif;

- *Article 4: «Plasma fatty acid composition in French-Canadian children with non-alcoholic fatty liver disease: Effect of n-3 PUFA supplementation»*
- *Article 5: «Non-alcoholic fatty liver disease severity and metabolic complications in obese children: impact of Omega-3 fatty acids»*
- *Revue 3: « Oxidative Stress as a Critical Factor in Non-alcoholic Fatty Liver Disease Pathogenesis»*

Article 1

« Sar1b transgenic male mice are more susceptible to high-fat diet-induced obesity, insulin insensitivity and intestinal chylomicron overproduction »

Levy E, **Spahis S**, Garofalo C, Marcil V, Montoudis A, Sinnet D, Sanchez R, Peretti N, Beaulieu JF, Sane A. *J Nutr Biochem*. 2014 May;25(5):540-8. doi: 10.1016/j.jnutbio.2014.01.004.

CONTRIBUTION DES AUTEURS

Levy E a contribué au concept et à la supervision de l'étude, aux ressources financières et matérielles pour la réalisation de l'étude. Dr Levy E a participé à la rédaction, l'analyse critique des résultats et la révision du manuscrit.

Spahis S a été impliquée dans l'acquisition des données, leur analyse et leur interprétation. Elle a conçu les figures, contribué à la rédaction, configuration, soumission et révision du manuscrit sous la supervision du Directeur de recherche, Dr Levy E.

Garofalo C, Montoudis A et Sané A ont procédé au maintien et au suivi de la colonie des animaux, à la collecte des tissus et aux expériences spécifiques dans le laboratoire.

Marcil V, Sinnett D, Sanchez R, Peretti N et Beaulieu JF ont révisé le manuscrit.

SAR1B TRANSGENIC MALE MICE ARE MORE SUSCEPTIBLE TO HIGH-FAT DIET-INDUCED OBESITY, INSULIN INSENSITIVITY AND INTESTINAL CHYLOMICRON OVERPRODUCTION

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Running Head: *Sar1b and cardiometabolic complications*

Keywords: Sar1B GTPase**,** fat absorption, insulin resistance, chylomicron production, fatty acids, lipids

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ABSTRACT

In the intracellular secretory network, nascent proteins are shuttled from the endoplasmic reticulum to the Golgi by transport vesicles requiring Sar1b, a small GTPase. Mutations in this key enzyme impair intestinal lipid transport and cause chylomicron retention disease. The main aim of this study was to assess whether Sar1b overexpression under a hypercaloric diet accelerated lipid production and chylomicron (CM) secretion, thereby inducing cardiometabolic abnormalities. To this end, we generated transgenic mice overexpressing human Sar1b ($Sar1b^{+/+}$) using pBROAD3-mcs that features the ubiquitous mouse ROSA26 promoter. In response to a high-fat diet (HFD), $Sar1b^{+/+}$ mice displayed significantly increased body weight and adiposity compared with $Sar1b^{+/+}$ mice under the same regimen or with wild-type (WT) mice exposed to chow or HFD. Furthermore, $Sar1b^{+/+}$ mice were prone to liver steatosis as revealed by significantly elevated hepatic triglycerides (TG) and cholesterol in comparison with WT animals. They also exhibited augmented levels of plasma TG along with alterations in fatty acid composition. Concomitantly, they showed susceptibility to develop insulin insensitivity and they responded abnormally to oral glucose tolerance test. Finally, $Sar1b^{+/+}$ mice that have been treated with Triton WR-1330 (to inhibit TG catabolism) and orotic acid (to block secretion of VLDL by the liver) responded more efficiently to fat meal tests as reflected by the rise in plasma TG and CM concentrations, indicating exaggerated intestinal fat absorption. These results suggest that $Sar1b^{+/+}$ under HFD can elicit cardiometabolic traits as revealed by incremental weight gain, fat deposition, dyslipidemia, hepatic steatosis, insulin insensitivity and intestinal fat absorption.

INTRODUCTION

The gut is indispensable for the transport of alimentary fat, the most calorically dense nutrient, in the form of chylomicrons (CM). The formation of these triglyceride (TG)-rich lipoproteins within the enterocyte is a multistep pathway, which includes the uptake and translocation of digestive lipolytic products from the brush border membrane to the endoplasmic reticulum (ER), lipid esterification and synthesis/posttranslational modification of different apolipoproteins (apo), as well as the assembly of lipid and apo components into lipoprotein particles (1–3). Evidently, the alteration of endogenous key proteins, such as apo B-48 and microsomal triglyceride transfer protein, could have an impact on intestinal fat transport and circulating lipoprotein concentrations and metabolism (4–8).

Our recent studies and other contributions by various groups have provided compelling evidence that Sar1b GTPase also plays a pivotal role in rallying coat protein complexes II (COPII) to form a shell around the vesicles that transport CM cargo in the secretory pathway (9,10). At each vesicle formation step, secretory CM cargo proteins must be selectively and efficiently sorted into transport vesicles, but gene mutations of *SARA2* on chromosome 5q31, coding for Sar1b, obviate COPII coat recruitment and CM movement from the ER to the Golgi apparatus, thereby impeding CM exocytosis (11–13). Thus, patients with *SARA2* gene aberrations present with chylomicron retention disease and severe intestinal fat malabsorption along with many complications such as failure to thrive, diarrhea, essential fatty acid deficiency, decline of liposoluble vitamins, and ophthalmologic and neurological abnormalities (14,15).

Our group has also reported that discrete *SARA2* genotypes may modify lipid and saturated fatty (FA) acid profile in chylomicron retention disease (11,12). In addition, as the Sar1b protein has not been extensively investigated in relation to intestinal lipid transport and regulation, we have recently overexpressed Sar1b in Caco-2/15 cells and can for the first time document that the stable forcing of Sar1b is tightly associated with the amplification of intestinal fat absorption (16). Therefore, it appears that Sar1b is determinant for the routing/movement of apo B-48-containing CM from the ER to the Golgi apparatus, as well as for CM assembly and output from enterocytes. However, an intriguing question is whether its abundant expression *in vivo* enables more efficient intestinal fat transport, which in turn may increase cardiometabolic risk factors. In the present study, to tackle this interesting and important issue, we have engineered a transgenic mouse that

overexpressed Sar1b (Sar1b$^{+/+}$) that was found to develop obesity, insulin insensitivity, hepatic and plasma lipid abnormalities and CM hyperproduction when fed a high-fat diet (HFD).

MATERIALS AND METHODS

Generation of Sar1b Transgenic Mice

pCMV6-XL4 vector (OriGene Technologies, Rockville, MD) harboring full length cDNA of *SAR2* was digested with *Not*I to excise a 1960 bp fragment. This fragment was subsequently cloned into pBROAD3-mcs vector (InvivoGen, San Diego, CA) restricted with *Not*I to create pBROAD3-SARA2. pBROAD3 features the ubiquitous mouse ROSA26 promoter. The high CpG content promoter directs *in vivo* expression of the transgene in most tissue types, including cells of the developing germ line and hematopoietic lineage. The strength of the ROSA26 promoter is ascribed to the 10 potential Sp1 sites found within the CpG island extending from the proximal promoter to the first half of intron 1, the highest number of Sp1 sites ever recorded in any promoter (17). Chicken ß-globin gene insulator was subcloned upstream from the ROSA26 promoter at the *Nde*I site to enhance transgene expression. Cloning the ß-globin gene insulator created pBROAD3-β-Sara2 vector. This 2-kb insulator fragment was derived from PCR amplification of chicken DNA with insulator-specific primers containing the *Nde*I site (primer 1: CAT ATG GCG GCC GCT CTA GACT; primer 2: CAT ATG GGA TCC GTC GAC GC). The capability of this construct to allow *SARA2* expression in enterocytes was tested by transient transfection of Caco-2 cells. The DNA transgene vector, pBROAD-ß-SARA2, was then injected into fertilized eggs from mice at the *Réseau de Recherche en Transgenèse du Québec*. Genomic DNA was prepared from founder mice and their offspring tail DNA and screened for the presence of *SARA2* DNA by PCR amplification, using specific primers (primer 1: ATG TCC TTC ATA TTT GGA TT; primer 2: ATC AAT GTA CTG TGC CAT CC).

Animals and Diets

Starting at three weeks of age, homozygous male Sar1b$^{+/+}$ and wild-type male mice (WT) were housed in a temperature-controlled room at 24°C with a 12-h light-dark cycle and fed a standard laboratory chow diet (ND; 2018 Teklad Global, 18% protein rodent diet, Harlan Laboratories, Indianapolis, IN) or a HFD (BioServ F3282, 60% fat Calories, BioServ, Frenchtown, NJ) and double distilled water *ad libitum* for 12 weeks. Body weight was recorded weekly from 3 to 15 weeks of age. Animal experiments were conducted according to the Canadian Council on Animal Care guidelines for the care and use of experimental animals and were approved by the Institutional Animal Care Committee of the Sainte-Justine UHC Research Center.

Body Fat Pad Weight and Organ Weight

After 12 weeks of feeding, mice were fasted for overnight, weighed and anesthetized by intraperitoneal injection of a mixture of Ketamine/Xylazine/Acepromazine (100/10/1 mg/kg), and isoflurane inhalant. After sacrifice, individual organs and tissues were isolated, weighed, snap-frozen, and stored at -80°C for experiments.

Densitometric Animal Fat Determination

Dual Energy X-ray Absorptiometry (DEXA) technology was used with Lunar PIXImus2 (GE Medical Systems, Madison, WI). The entire animal was exposed to a cone-shaped beam of both high- and low-energy X-rays for 3 min. A high-resolution digital picture (0.18 x 0.18mm) was taken of an image of the X-rays hitting a luminescent panel. The ratio of attenuation of the high and low energies made it possible to separate bone from tissue and, from within the tissue samples, lean and fat tissue.

Hepatic and intestinal Lipids

Hepatic and jejunal tissues were homogenized in T-PER Reagent for lipid measurement. Total cholesterol and TG were determined using cholesterol CHOD-PAP and Trig/GB assays, respectively, from Roche/Hitachi (Indianapolis, IN). For phospholipid measurements, liver tissue was homogenized in 0.9% NaCl isotonic solution (Baxter, Toronto, ON). To 10 µl of homogenate 400 µl of perchloric acid were added and incubated for 3 h at 160°C. After incubation, 4 ml H_2O, 400 µl of 2.5% ammonium molybdate and 200 µl of 0.1% 2.4-Diaminophenol Dihydrochloride in 20% bisulfite were added and incubated for 7 min at 100°C. Phosphates in phospholipid were read at 830 nm. Standard curve was performed with serial dilutions of a 32-mM KH_2PO_4 solution. Protein concentrations were determined by colorimetric Bio-Rad Protein Assay Dye Reagent Concentrate.

Biochemical Analyses

After 12 weeks of feeding, mice were fasted overnight and sacrificed. Blood was collected for insulin and lipid assays. Plasma TG and cholesterol levels were measured enzymatically (Boehringer-Mannheim, Mannheim, Germany). Blood glucose levels were determined using a OneTouch Ultra Meter (lifeScan, Milpitas, CA) and plasma insulin was measured using a rat/mouse insulin ELISA kit (Millipore, Billerica, MA). Plasma fatty acid composition was determined by gas chromatography as described previously (18).

Insulin Sensitivity

Insulin sensitivity was determined using the HOMA-IR index. The score was calculated using the formula: (Insulinemia (mIU/L) x Glycemia (mmol/L)) / 22.5.

Oral Glucose Tolerance Test (OGTT)

Glucose tolerance was assessed by oral glucose administration. After a 15-h fast, the mice were weighed and glucose (2g/kg of body weight) was administered orally. Glucose concentrations in the retro-orbital venous plexus were measured.

Intestinal Fat Absorption and Transport

Transgenic and control mice under HFD with or without orotic acid (1% gavage for 14 d before sacrifice) were fasted for 12 h. Animals were then given 200 µl Microlipids (Nestlé Healthcare Nutrition, Minneapolis, MN) containing 50% fat emulsion through a gavage syringe. One hour later, Triton WR 1339 diluted in saline (300 mg/ml) and warmed to 37°C was injected slowly (400 mg/kg of body weight) via a catheter inserted into the carotid artery. Anesthesia with ketamine (80 mg/kg of body weight) was maintained throughout the experimental period. Blood was collected from the tail vein at 0, 2, 4 and 6 h after Triton WR 1339 administration. Blood TG levels were determined enzymatically in the plasma, as described above. CM were isolated by ultracentrifugation as described previously (19,20) and TGs in CM were quantified enzymatically.

Western blot analysis

Sections of jejunum and liver were homogenized in ice-cold T-PER Tissue Protein Extraction Reagent (Thermo Scientific, Rockford, IL). Protease inhibitors were added to tissues prior to homogenization. Supernatant proteins (20 µg) were denatured at 95˚C for 5 min in a sample buffer containing SDS and DTT (Thermo Scientific, Rockford, IL), separated on a 15% sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) and electroblotted onto Hybond-C extra nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ) in 25 mM Tris and 192 mM glycine. Nonspecific binding sites of the membranes were blocked with Tris-buffered saline [20 mM Tris-HCl (pH 7.5) plus 137 mM NaCl] containing 0.1% Tween 20 and 5% non fat dry milk for 60 min at room temperature. The blots were then incubated overnight at 4˚C in blocking solution with the primary antibodies directed against the targeted proteins Sar1b (1:20000) (kindly provided by Dr Randy Schekman, Department of Molecular and Cell Biology, University of California at Berkeley, Berkeley, CA) or β-actin (1:250000) (Sigma-Aldrich Canada). The relative amount of primary antibody was revealed with a species-specific horseradish peroxidase-

conjugated secondary antibody, e.g. anti-mouse IgG-POD/anti-rabbit IgG-POD (Roche Diagnostics, Indianapolis) (1:20000). Blots were developed and the protein mass was quantified using an HP Scanjet scanner equipped with a transparency adapter and the UN-SCAN-IT gel 6.1 software.

RNA extraction and quantitative RT-PCR (RT-qPCR)

Intestinal and hepatic samples were homogenized in TRIzol reagent (Ambion by Life Technologies Corporation, Carlsbad, CA) and total RNA was extracted. RNA concentration and quality were tested by absorbance at 260 nm while the absorbance ratio of 260/280 (using a Nanodrop® ND-1000 Spectrophotometer from Thermo Scientific, Wilmington, DE) and RNA analysis by agarose gel electrophoresis served to assess integrity. Complementary DNA was obtained by reverse transcripting 1 µg of RNA with the qScript cNDA Supermix (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer's instructions. Negative controls without enzyme were also prepared.

The TaqMan® Express Human Endogenous Control Plate (Life Technologies) was used to select the most stable reference gene amongst organs according to the manufacturer's instructions. Using this technique, polymerase (RNA) II (DNA directed) polypeptide AI (Polr2a) was chosen as an endogenous control to normalize the different cDNA sample amounts. RT-qPCRs were performed in 96-wells microtiter plates using Taqman® Fast Advanced Master Mix (Life Technologies, Foster City, CA) in an ABI Prism® 7900 Sequence Detection System. The RT-qPCRs were carried in 96-well plates with a final volume of 10 µl per well. A volume of 5 µl Taqman® Fast Advanced Master Mix (1 X) was added to a well containing 1 µl of Custom Taqman® Gene Expression Assay (Life Technologies) (1 X) for Sar1b or for Polr2a comprising 1 µl of cDNA previously diluted 1:5 and 3 µl of diethylpyrocarbonate H_2O. Taqman® hydrolysis probes used were Mm01294633_m1 for Sar1b and Mn00839493_m1 for Polr2a (Life Technologies). Subsequently, negative controls without cDNA were prepared. The amplification reaction was carried out using 40 cycles and was performed in duplicate for each gene in the same plate under the following conditions: 95°C 20 s, 95°C 1 s and 60°C for 20 s. The relative mRNA fold-changes between the animal groups were calculated using the $2^{-\Delta\Delta CT}$ method (21). The analyses and statistics were performed using the Expression Suite Software Version 1.0 (Life Technologies) and the Prism 5.01 software.

Statistical Analysis

The data were analysed with Prism 5.01 (GraphPad Software) and are expressed as means ± SEM. Statistical analysis was performed using either Student's *t* test or one-way ANOVA with a post-hoc Bonferroni multiple comparison test. $P<0.05$ was considered significant.

RESULTS

Overexpression Analysis

The first set of experiments was designed to validate Sar1b overexpression in the genetic manipulated animals. To this end, intestinal and hepatic tissues were tested. As noted in **Figure 1A,** increased mRNA and protein expression was noted in the gut (3-fold and 6-fold) and liver (8-fold and 6.5-fold), respectively, in Sar1b$^{+/+}$ compared to WT mice. Of note, all the findings of the present study were obtained only on this line of transgenic mice that exhibited 6-fold protein overexpression in the intestine and the liver since the other lines displayed lower values. Importantly, the animals were viable throughout adulthood and they did not exhibit abnormalities in fertility rate. Additionally, litter sizes at birth were comparable between the WT and Sar1b$^{+/+}$ groups, but the mice of the latter were apparently fatter (**Fig. 1B**).

Body Weight, Weight Gain and Food Intake

Weight gain was recorded in animals exposed to chow and HFD diets during 12 weeks. On the normal diet (ND), Sar1b$^{+/+}$ mice showed an apparent weight gain from 8 to 12 weeks compared with WT mice and this irregularity was more pronounced on an HFD as observed in the body weight gap between the WT (Δ=66.7) and the Sar1B$^{+/+}$ (Δ = 54.6) groups of mice (**Fig. 1C**). The mean of area under curve show also significant ($p<0.05$) differences between WT (ND: 277.00 ± 13.46; HFD: 349 ± 18) and Sar1B$^{+/+}$ (ND: 353.90 ± 24.99; HFD: 424.60 ± 11). Clearly, at the end of the 12-week high-fat regimen, Sar1b$^{+/+}$ HFD mice demonstrated a significant weight increase compared with the WT or Sar1b$^{+/+}$ ND mice (**Fig. 1D**).

Food intake was examined at least twice a week for 3 months during the feeding period. Food intake was around 3 g/day/mice for all different experimental groups: on the regular diet, WT and Sar1B$^{+/+}$mice displayed an average of (3.30 ± 0.13) and (3.09 ± 0.24) g/day/mice, respectively, whereas on HFD, they exhibited an average food intake of (3.30 ± 0.27) and (3.17 ± 0.45) g/day/mice, respectively.

Body Fat Pad Weight and Organ Weight

To determine the effect of Sar1b overexpression on fat development, Sar1b$^{+/+}$ and WT littermates were placed on an HFD immediately after weaning (3 weeks old) for a 12-week diet-period. As shown in **Table 1**, the Sar1b$^{+/+}$ mice exhibited a high weight mass in adipose tissue, liver and intestine. This observation was significantly apparent in adipose tissue and liver, particularly in the presence of an HFD. Additionally, the perirenal adipose tissue was characterized by

significantly higher fat deposition in Sar1b$^{+/+}$ HFD compared to Sar1b$^{+/+}$ ND mice with a trend of increase compared with WT HFD mice, while epididymal tissue was significantly higher in Sar1b$^{+/+}$ compared to WT HFD mice. Mesenteric adipose tissue showed no changes between the groups (**Fig. 2A**). However, as expected from these observations, using DEXA, total adipose mass accumulation was found to be significantly elevated in Sar1b$^{+/+}$ HFD compared to all animal groups (**Fig. 2B**). Therefore, Sar1b$^{+/+}$ mice were more susceptible to diet-induced obesity than control animals.

Hepatic and intestinal Lipids

Since changes in adipose tissue mass are frequently associated with alterations in whole body lipid homeostasis, we therefore measured lipid amounts in the liver. As shown in **Figure 3A**, Sar1b overexpression led to a significant rise in TG and cholesterol, with only a parallel trend in phospholipids, particularly under a hypercaloric diet, even if the WT HFD mice exhibited the same profile. Of note, no significant differences were observed in TG and cholesterol in the jejunum among the groups of Sar1b$^{+/+}$ and WT mice under the two dietary regimens as illustrated in **Figure 4**.

Plasma Lipids

Marked differences were detected in the lipid profiles of WT and Sar1b$^{+/+}$ mice under the HFD. As expected, an HFD caused an augmentation in plasma TG in WT mice compared with control animals on ND (**Fig. 5**). However, after 12 weeks of high-fat feeding, the Sar1b$^{+/+}$ animals experienced a more marked rise in fasting plasma TG and cholesterol concentrations.

Plasma Fatty Acid Composition

Given the relationship between abnormal FA composition and cardiometabolic disorders, particular attention was paid to the impact of Sar1b overexpression on FA distribution in animals exposed to an ND and HFD. While no distinguishable pattern was noticed in WT mice, various alterations were recorded in Sar1b$^{+/+}$ mice (**Table 2**). However, HFD administration caused a significant alteration in total and derived polyunsaturated n-3 FA family composition in Sar1b$^{+/+}$ HFD mice (**Table 2**), resulting in a decline in the polyunsaturated FA/saturated ratio. Interestingly, even under an ND diet, Sar1b$^{+/+}$mice exhibited differences in FA distribution, which emphasizes the role of Sar1b in FA metabolism.

Glycemia and Insulinemia

The effects of ND and HFD on glucose/insulin homeostasis were measured in animal groups. The HOMA-IR index was calculated as well (**Fig. 6**). While an increase in fasting plasma glucose (**Fig. 6A**) and insulin (**Fig. 6B**) levels was noticed in WT HFD compared to WT ND, their increment in Sar1b$^{+/+}$ mice was more substantial and reached statistical significance. As a result, the HOMA-IR index showed a significant increase in these Sar1b$^{+/+}$ animals (**Fig. 6C**), thereby indicating less insulin sensitivity. The insulin resistance feature was confirmed in Sar1b$^{+/+}$ animals with OGTT since they displayed a delayed glucose clearance and increased area under the curve especially on the HFD (**Fig. 6D**).

Intestinal Lipid Absorption and Transport

Since mutations in the *SAR1B* gene result in defects in intestinal fat absorption, we tested the corollary that *Sar1b* overexpression could induce CM secretion. Two precautions were taken: CM measurements were performed in the presence of Triton WR 1339 in order to block their degradation by lipoprotein lipase (**Fig. 7A and 7B**) and in the presence of orotic acid (**Fig.7 C and 7D**), an inhibitor of VLDL secretion from the liver, to prevent their contamination in the circulation. The oral fat tolerance test showed a significantly increased (~3-fold) output of TG in Sar1b$^{+/+}$ mice in both conditions (e.g. Triton WR 1339 and orotic acid), which was confirmed by the calculated area under curve (**Fig. 7A and 7C**). Finally, the measurement of TG content in CM supported the markedly enhanced intestinal fat transport in Sar1b$^{+/+}$ mice (**Fig. 7B and 7D**). Importantly, assessment of fecal total lipids in stools (mg/3d) did not disclose differences among the different experimental groups: the values for WT and Sar1B$^{+/+}$mice on the regular diet were (55.3 ± 3.0) and (52.1 ± 4.4), respectively, and on the HFD (57.6 ± 5.7) and (58.2 ± 4.8), respectively.

DISCUSSION

Our initial studies were the first to shed light on key biochemical, clinical and metabolic features in chylomicron retention disease mediated by Sar1b mutations. In fact, this autosomal recessive disorder was characterized by fat malabsorption, hypocholesterolemia, essential FA deficiency and fat-soluble vitamin depletion. More particularly, after a fatty meal, plasma TG did not increase and CM could not be identified in the blood circulation, whereas the enterocytes contained particles, morphologically similar to CM, indicating a defect in the mechanism of CM exocytosis. In the present study, a quite opposite portrait is observed following Sar1b overexpression. Overall, fed with HFD, the transgenic mice ($Sar1b^{+/+}$) developed an increase in body weight, adiposity, hepatic steatosis, plasma lipids as well as an alteration in polyunsaturated n-3 FAs, insulin insensitivity and CM output. Therefore, in addition to its well-known role in intracellular COPII formation and protein trafficking, Sar1b may regulate certain functions related to glucose and lipid homeostasis. Complex pathways are needed to transfer cargo between lipid-bilayer-enclosed organelles. In particular, proteins synthesized in the ER are packaged into COPII-coated vesicles involving Sar1b GTPase (22–24). When activated by Sec12, Sar1b exposes an N-terminal amphipathic helix, which embeds in the membrane and recruits the Sec23/Sec24 and Sec13/Sec31 complexes (25). Additional functions for Sar1b have progressively been unraveled and include cargo sorting, COPII coat dynamics and completion of the final fission (26). Only recently has the Sar1b GTPase requirement been established for intracellular CM transport as a result of mutations identified in the encoding *SARA2* gene (11,12,27), which is consistent with previous data demonstrating that GTP binding and hydrolysis are required for cargo transport, including CM, from the ER to the Golgi apparatus (28). For the first time, our *in vivo* experiments have discovered its close implication in speeding up intestinal lipid transport. Although the regulatory mechanisms controlling its activity and expression are almost completely unknown, we propose that, in situations where CM-TG flux is stimulated by dietary fat load, Sar1b (mediating COPII formation) should be enhanced in order to accommodate CM cargos in cooperation with key proteins governing intracellular assembly in TG-rich lipoproteins as documented recently in our laboratory (16).

An important new finding of this study is the demonstration that $Sar1b^{+/+}$ mice, under an HFD, developed metabolic aberrations, including body and adipose weight gain, hepatic steatosis, elevation in circulating lipids and insulin insensitivity. Very often, chronic consumption under an

HFD is accompanied by these cardiometabolic traits (29–31), but the presence of Sar1b expression abundance was shown to cause more significant complications. The mechanisms underlying this intriguing relationship are currently unclear. However, we can mention the causative role of lipids. Indeed, the longer exposure to enhanced intestinal fat absorption associated with Sar1b profusion could lead to greater TG accumulation in insulin-responsive tissues with age and a greater effect on insulin insensitivity. Such phenomena may especially impair hepatic lipid homeostasis, thereby prompting liver steatosis.

Our experiments could not demonstrate significant differences in food consumption or fecal output between $Sar1B^{+/+}$ and WT mice. Nevertheless, the oral fat meal test showed that Sar1b overexpression in mice under HFD enhanced intestinal fat absorption efficiency. Therefore, the exaggerated postprandial lipids may amplify adipose tissue, which may rise body weight in $Sar1b^{+/+}$ mice. Interestingly, there were differences in the size of various adipose tissues as illustrated in Figure 2. According to our preliminary data (data not shown), analysis of Sar1b expression revealed minimal mRNA content, and without differences in the different adipose tissues as a function of the two dietary regimens. Nevertheless, ongoing studies should confirm these findings and will highlight their functional impact.

FAs normally provide an alternative fuel source to glucose for energy during periods of fasting. Biochemical studies have indicated a direct relationship between concentrations of circulating FA and insulin resistance (32). Interestingly, Sar1b forcing in transgenic mice exposed to an HFD resulted in high plasma FA, which may impede glucose uptake by several tissues, thereby provoking insulin resistance. Some studies have targeted the impact of mitochondria dysfunction under HFD in the regulation of glucose homeostasis (33) as well as FA-β-oxidation (34), while others have focused on the role of long-term HFD feeding in intestinal and hepatic lipid accumulation (35–37). Additional studies are necessary to provide the molecular basis for the observed physiological differences between WT and $Sar1b^{+/+}$ mice.

It is important to note that when we initiated the present studies, we first examined the differences between mice of different gender. We did not find significant differences between the male and female group for the various parameters tested herein. We, therefore, decided to use just males in order to lessen the data variability.

In conclusion, we have generated the first transgenic mouse model for Sar1b research and uncovered a novel role for Sar1b in augmenting body weight, lipid storage, liver steatosis, insulin

insensitivity and intestinal fat absorption. Our findings will lead to a better understanding of the molecular and physiological functions of Sar1b, a key protein in intracellular cargo trafficking and cellular lipid homeostasis, at least in the small intestine.

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Table 1: Effect of high-fat feeding on weight tissues in Sar1b$^{+/+}$ and wild type male mice

Weight (g)	WT	Sar1b$^{+/+}$	WT	Sar1b$^{+/+}$
	ND		HFD	
Adipose Tissue (perirenal, epididymal, mesenteric)	3.58 ± 0.39	3.94 ± 0.42	3.71 ± 0.28	4.94 ± 0.34$^{\#}$
Heart	0.15 ± 0.01	0.16 ± 0.02	0.18 ± 0.01	0.16 ± 0.01
Lung	0.17 ± 0.01	0.21 ± 0.02	0.18 ± 0.01	0.22 ± 0.03
Liver	1.90 ± 0.16	1.93 ± 0.26	2.39 ± 0.17	2.86 ± 0.21$^{\#}$
Spleen	0.14 ± 0.03	0.13 ± 0.01	0.14 ± 0.02	0.14 ± 0.01
Kidney	0.43 ± 0.04	0.43 ± 0.05	0.45 ± 0.03	0.41 ± 0.03
Stomach	0.25 ± 0.02	0.20 ± 0.01	0.22 ± 0.02	0.18 ± 0.03
Intestine	1.07 ± 0.08	1.26 ± 0.01	1.12 ± 0.08	1.14 ± 0.06

At 3-weeks of age, male mice were fed with a normal (ND) or high fat diet (HFD) for 12 weeks. After sacrifice, tissues were collected and weighted as described in Materials and Methods. Values are mean ± SEM for n = 10/group. #P<0.05 vs. Sar1b+/+ ND.
WT: wild type; ND: normal diet; HFD: high fat diet.

Table 2: Effect of high-fat feeding on fatty acid composition and ratio values in Sar1b$^{+/+}$ and wild type male mice

Fatty acids (%)	WT ND	WT HFD	Sar1b$^{+/+}$ ND	Sar1b$^{+/+}$ HFD
16:0	25.09 ± 0.36	25.30 ± 1.15	22.10 ± 0.18*	25.82 ± 0.70
18:0	20.51 ± 0.33	21.20 ± 2.19	14.30 ± 1.99*	17.05 ± 0.65
18:1n-9	8.15 ± 0.30	10.28 ± 0.30	8.62 ± 0.91	11.02 ± 0.54
18:2n-6	15.2 ± 0.32	14.15 ± 0.57	16.47 ± 1.43	12.80 ± 1.22
18:3n-6	0.15 ± 0.01	0.17 ± 0.00	0.25 ± 0.01	0.25 ± 0.02
18:3n-3:ALA	0.25 ± 0.04	0.10 ± 0.01	0.25 ± 0.05	0.09 ± 0.02$^{\#}$
20:0	0.32 ± 0.01	0.33 ± 0.05	0.25 ± 0.06	0.22 ± 0.00
20:1n-9	0.30 ± 0.02	0.30 ± 0.02	0.33 ± 0.04	0.25 ± 0.01
20:2n-6	0.16 ± 0.01	0.20 ± 0.02	0.15 ± 0.00	0.17 ± 0.01
20:3n-6	1.03 ± 0.09	0.85 ± 0.13	1.74 ± 0.30*	1.12 ± 0.03$^{\#}$
20:3n-9	1.07 ± 0.01	1.09 ± 0.01	1.11 ± 0.02	1.09 ± 0.00
20:4n-6: AA	16.01 ± 0.27	15.75 ± 1.58	21.75 ± 2.32*	20.31 ± 0.43
20:5n-3 EPA	0.34 ± 0.02	0.15 ± 0.02	0.38 ± 0.05	0.17 ± 0.01$^{\#\#}$
22:0	0.25 ± 0.02	0.27 ± 0.01	0.25 ± 0.01	0.25 ± 0.02
22:1n-9	1.40 ± 0.08	1.45 ± 0.32	2.26 ± 1.90	0.95 ± 0.05
22:2n-6	0.03 ± 0.01	0.06 ± 0.03	0.05 ± 0.03	0.06 ± 0.04
22:4n-6	0.03 ± 0.00	0.03 ± 0.01	0.09 ± 0.02*	0.09 ± 0.03
22:5n-3	0.14 ± 0.02	0.15 ± 0.06	0.23 ± 0.02*	0.08 ± 0.02$^{\#}$
22:6n-3: DHA	3.80 ± 0.11	3.21 ± 0.03	5.26 ± 0.29**	3.71 ± 0.18$^{\#\#}$
24:0	0.05 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
24:1n-9	0.26 ± 0.03	0.27 ± 0.01	0.41 ± 0.07*	0.41 ± 0.03
Total of FA and Ratios				
Total n-3	4.70 ± 0.14	3.69 ± 0.02	6.21 ± 0.33**	4.14 ± 0.23$^{\#\#\#}$
Total n-6	33.01 ± 0.40	31.32 ± 1.74	40.85 ± 3.48	34.90 ± 0.94
Total n-7	2.46 ± 0.05	2.01 ± 0.29	2.87 ± 0.09	2.18 ± 0.07
Total n-9	10.18 ± 0.27	12.35 ± 0.10	11.72 ± 1.23	12.72 ± 0.54
Saturated FA	49.20 ± 0.51	50.07 ± 1.88	38.33 ± 2.66*	45.67 ± 1.48
Mono-unsaturated FA	13.11 ± 0.21	14.76 ± 0.24	14.70 ± 1.23	15.20 ± 0.42
PUFA	37.44 ± 0.51	34.94 ± 1.71	46.79 ± 3.76	38.96 ± 1.14
PUFA/Saturated	0.76 ± 0.02	0.70 ± 0.06	1.24 ± 0.17*	0.86 ± 0.05
EFA (LA+ALA)/Non-	0.18 ± 0.00	0.17 ± 0.01	0.20 ± 0.02	0.15 ± 0.02
EPA/DHA	0.09 ± 0.00	0.05 ± 0.00	0.07 ± 0.01	0.05 ± 0.00
DHA/AA	0.24 ± 0.01	0.21 ± 0.02	0.25 ± 0.02	0.18 ± 0.01
ALA/EPA	0.81 ± 0.16	0.68 ± 0.11	0.67 ± 0.12	0.50 ± 0.07
ALA/LA	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
n-6/n-3	6.96 ± 0.15	8.46 ± 0.50	6.49 ± 0.27	8.42 ± 0.26
Δ6 20:3n-6/18:2n-6	0.07 ± 0.00	0.06 ± 0.01	0.11 ± 0.02*	0.09 ± 0.01
Δ9 18:1n-9/18:0	0.40 ± 0.02	0.50 ± 0.07	0.64 ± 0.14	0.65 ± 0.05
Δ7 16:1n-7/16:0	0.04 ± 0.00	0.03 ± 0.00	0.05 ± 0.00	0.03 ± 0.00

At 3-weeks of age, male mice were fed with a normal (ND) or high fat diet (HFD) for 12 weeks and the percentage of fatty acid composition was determined by gas chromatography as described in Materials and Methods.
Values are mean ± SEM for n = 10/group. *P<0.05 vs. WT; $^{\#}P$<0.05, $^{\#\#}P$<0.001 vs. Sar1b$^{+/+}$ ND.
WT: wild type; ND: normal diet; HFD: high fat diet.
AA: arachidonic acid, ALA: alpha-linolenic acid, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, LA: linoleic acid, PUFA: polyunsaturated fatty acids, FA: fatty acid; EFA: essential fatty acids.

REFERENCES

(1) Xiao C, Lewis GF. Regulation of chylomicron production in humans. Biochim Biophys Acta. 2012;1821:736-46.

(2) Pan X, Hussain MM. Gut triglyceride production. Biochim Biophys Acta. 2012;1821:727-35.

(3) Kindel T, Lee DM, Tso P. The mechanism of the formation and secretion of chylomicrons. Atheroscler Suppl. 2010;11:11-6.

(4) Levy E, Stan S, Delvin E, Menard D, Shoulders C, Garofalo C et al. Localization of microsomal triglyceride transfer protein in the Golgi: possible role in the assembly of chylomicrons. J Biol Chem. 2002;277:16470-7.

(5) Levy E, Stan S, Garofalo C, Delvin EE, Seidman EG, Menard D. Immunolocalization, ontogeny, and regulation of microsomal triglyceride transfer protein in human fetal intestine. Am J Physiol Gastrointest Liver Physiol. 2001;280:G563-G571.

(6) Slight I, Bendayan M, Malo C, Delvin E, Lambert M, Levy E. Identification of microsomal triglyceride transfer protein in intestinal brush-border membrane. Exp Cell Res. 2004;300:11-22.

(7) Raabe M, Veniant MM, Sullivan MA, Zlot CH, Bjorkegren J, Nielsen LB et al. Analysis of the role of microsomal triglyceride transfer protein in the liver of tissue-specific knockout mice. J Clin Invest. 1999;103:1287-98.

(8) Wetterau JR, Gregg RE, Harrity TW, Arbeeny C, Cap M, Connolly F et al. An MTP inhibitor that normalizes atherogenic lipoprotein levels in WHHL rabbits. Science. 1998;282:751-4.

(9) Jones B, Jones EL, Bonney SA, Patel HN, Mensenkamp AR, Eichenbaum-Voline S et al. Mutations in a Sar1 GTPase of COPII vesicles are associated with lipid absorption disorders. Nat Genet. 2003;34:29-31.

(10) Siddiqi S, Mansbach CM. Phosphorylation of Sar1b protein releases liver fatty acid-binding protein from multiprotein complex in intestinal cytosol enabling it to bind to endoplasmic reticulum (ER) and bud the pre-chylomicron transport vesicle. J Biol Chem. 2012;287:10178-88.

(11) Peretti N, Roy CC, Sassolas A, Deslandres C, Drouin E, Rasquin A et al. Chylomicron retention disease: a long term study of two cohorts. Mol Genet Metab. 2009;97:136-42.

(12) Peretti N, Sassolas A, Roy CC, Deslandres C, Charcosset M, Castagnetti J et al. Guidelines for the diagnosis and management of chylomicron retention disease based on a review of the literature and the experience of two centers. Orphanet J Rare Dis. 2010;5:24.

(13) Roy CC, Levy E, Green PH, Sniderman A, Letarte J, Buts JP et al. Malabsorption, hypocholesterolemia, and fat-filled enterocytes with increased intestinal apoprotein B. Chylomicron retention disease. Gastroenterology. 1987;92:390-9.

(14) Levy E, Marcel Y, Deckelbaum RJ, Milne R, Lepage G, Seidman E et al. Intestinal apoB synthesis, lipids, and lipoproteins in chylomicron retention disease. J Lipid Res. 1987;28:1263-74.

(15) Silvain M, Bligny D, Aparicio T, Laforet P, Grodet A, Peretti N et al. Anderson's disease (chylomicron retention disease): a new mutation in the SARA2 gene associated with muscular and cardiac abnormalities. Clin Genet. 2008;74:546-52.

(16) Levy E, Harmel E, Laville M, Sanchez R, Emonnot L, Sinnett D et al. Expression of Sar1b enhances chylomicron assembly and key components of the coat protein complex II system driving vesicle budding. Arterioscler Thromb Vasc Biol. 2011;31:2692-9.

(17) Kisseberth WC, Brettingen NT, Lohse JK, Sandgren EP. Ubiquitous expression of marker transgenes in mice and rats. Dev Biol. 1999;214:128-38.

(18) Spahis S, Vanasse M, Belanger SA, Ghadirian P, Grenier E, Levy E. Lipid profile, fatty acid composition and pro- and anti-oxidant status in pediatric patients with attention-deficit/hyperactivity disorder. Prostaglandins Leukot Essent Fatty Acids. 2008;79:47-53.

(19) Levy E, Menard D, Delvin E, Stan S, Mitchell G, Lambert M et al. The polymorphism at codon 54 of the FABP2 gene increases fat absorption in human intestinal explants. J Biol Chem. 2001;276:39679-84.

(20) Levy E, Thibault L, Menard D. Intestinal lipids and lipoproteins in the human fetus: modulation by epidermal growth factor. J Lipid Res. 1992;33:1607-17.

(21) Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25:402-8.

(22) Lee MC, Miller EA, Goldberg J, Orci L, Schekman R. Bi-directional protein transport between the ER and Golgi. Annu Rev Cell Dev Biol. 2004;20:87-123.

(23) Jensen D, Schekman R. COPII-mediated vesicle formation at a glance. J Cell Sci. 2011;124:1-4.

(24) Gurkan C, Stagg SM, Lapointe P, Balch WE. The COPII cage: unifying principles of vesicle coat assembly. Nat Rev Mol Cell Biol. 2006;7:727-38.

(25) Lee MC, Miller EA. Molecular mechanisms of COPII vesicle formation. Semin Cell Dev Biol. 2007;18:424-34.

(26) Sato K, Nakano A. Mechanisms of COPII vesicle formation and protein sorting. FEBS Lett. 2007.

(27) Okada T, Miyashita M, Fukuhara J, Sugitani M, Ueno T, Samson-Bouma ME et al. Anderson's disease/chylomicron retention disease in a Japanese patient with uniparental disomy 7 and a normal SAR1B gene protein coding sequence. Orphanet J Rare Dis. 2011;6:78.

(28) Siddiqi SA, Gorelick FS, Mahan JT, Mansbach CM. COPII proteins are required for Golgi fusion but not for endoplasmic reticulum budding of the pre-chylomicron transport vesicle. J Cell Sci. 2003;116:415-27.

(29) Rajala MW, Scherer PE. Minireview: The adipocyte--at the crossroads of energy homeostasis, inflammation, and atherosclerosis. Endocrinology. 2003;144:3765-73.

(30) Qureshi K, Abrams GA. Metabolic liver disease of obesity and role of adipose tissue in the pathogenesis of nonalcoholic fatty liver disease. World J Gastroenterol. 2007;13:3540-53.

(31) Bouloumie A, Curat CA, Sengenes C, Lolmede K, Miranville A, Busse R. Role of macrophage tissue infiltration in metabolic diseases. Curr Opin Clin Nutr Metab Care. 2005;8:347-54.

(32) Boden G, Chen X, Ruiz J, White JV, Rossetti L. Mechanisms of fatty acid-induced inhibition of glucose uptake. J Clin Invest. 1994;93:2438-46.

(33) Kleiner S, Mepani RJ, Laznik D, Ye L, Jurczak MJ, Jornayvaz FR et al. Development of insulin resistance in mice lacking PGC-1alpha in adipose tissues. Proc Natl Acad Sci U S A. 2012;109:9635-40.

(34) Nyman LR, Tian L, Hamm DA, Schoeb TR, Gower BA, Nagy TR et al. Long term effects of high fat or high carbohydrate diets on glucose tolerance in mice with heterozygous carnitine palmitoyltransferase-1a (CPT-1a) deficiency: Diet influences on CPT1a deficient mice. Nutr Diabetes. 2011;1:e14.

(35) Ciapaite J, van den Broek NM, Te BH, Nicolay K, Jeneson JA, Houten SM et al. Differential effects of short- and long-term high-fat diet feeding on hepatic fatty acid metabolism in rats. Biochim Biophys Acta. 2011;1811:441-51.

(36) Kim S, Sohn I, Ahn JI, Lee KH, Lee YS, Lee YS. Hepatic gene expression profiles in a long-term high-fat diet-induced obesity mouse model. Gene. 2004;340:99-109.

(37) Desmarchelier C, Dahlhoff C, Keller S, Sailer M, Jahreis G, Daniel H. C57Bl/6 N mice on a western diet display reduced intestinal and hepatic cholesterol levels despite a plasma hypercholesterolemia. BMC Genomics. 2012;13:84.

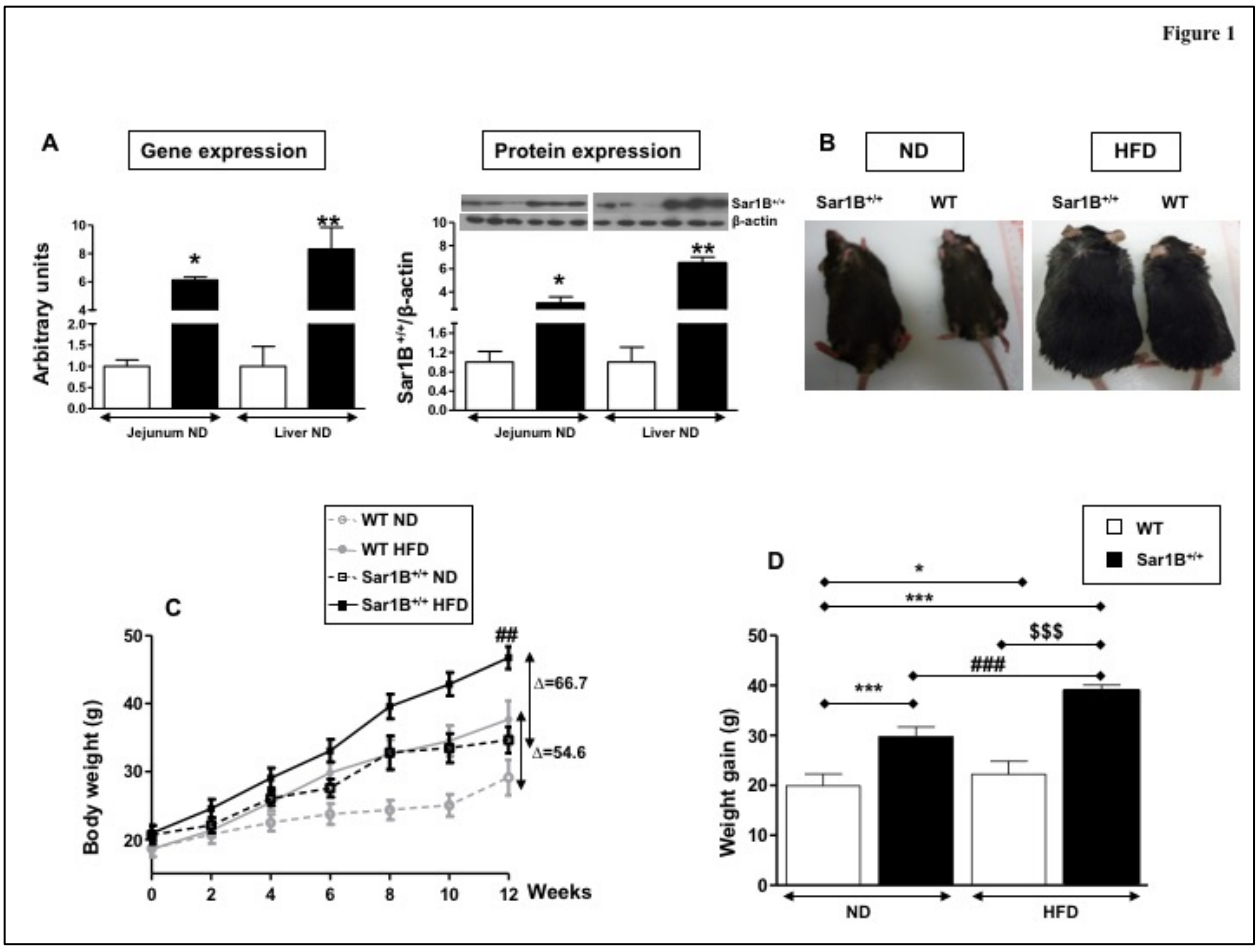

Figure 1. Sar1b expression, growth curves and body weight gains of male mice under normal and high-fat diets.

Differential gene and protein expression levels of Sar1b in the jejunum and liver of wild type (WT) and transgenic mice was assessed by RT-qPCR and Western Blot as described in the Material and Methods. (A). Results represent the means ± SEM of 5 animals per group. To assess weight gain, mice at 3 weeks of age were fed with a normal (ND) or high-fat diet (HFD) for 12 weeks and body weights were measured each week after mice were fed the special diet (B). The mean body weight gains of Sar1b$^{+/+}$ and WT mice were reported after 12 weeks of feeding (C). Differences (Δ) were calculated between the WT and Sar1b$^{+/+}$ mice to evaluate the effect of HFD on weight progression. Values are means ± SEM for n = 8-18/group. *$P < 0.05$, **$P < 0.001$ ***$P < 0.0001$ vs. WT; $^{\#\#}P < 0.001$, $^{\#\#\#}P < 0.0001$ vs. Sar1b$^{+/+}$ ND; $^{\$\$\$}P < 0.0001$ vs. WT HFD.

WT: wild type; ND: normal diet; HFD: high-fat diet.

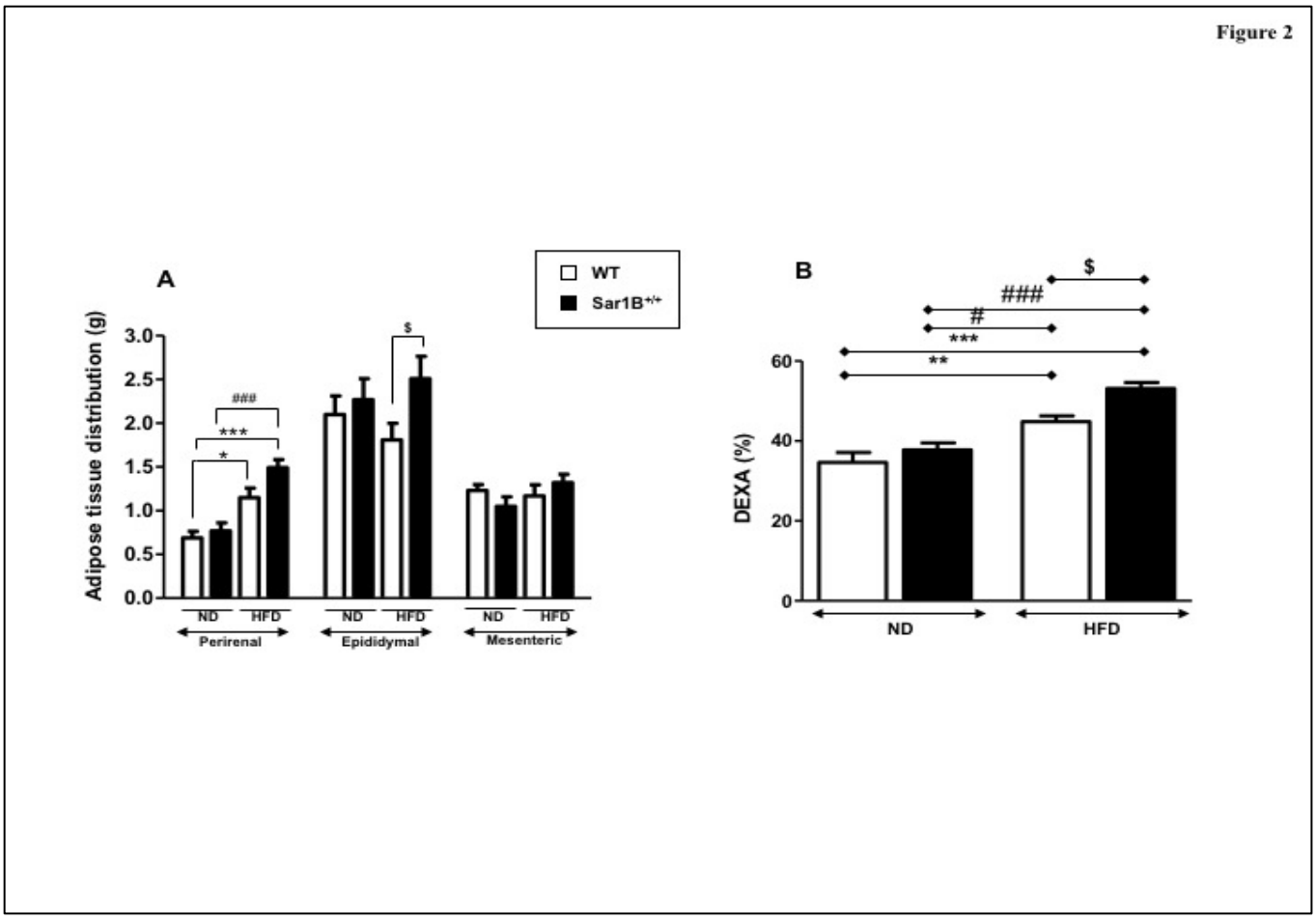

Figure 2. Effect of high-fat feeding on adipose mass accumulation in Sar1b$^{+/+}$ and control male mice.

Mice at 3 weeks of age were fed with a normal (ND) or high-fat diet (HFD) for 12 weeks and the different masses of adipose tissue distribution were evaluated by their weight quantification (A) and using dual energy X-ray absorptiometry (DEXA) for total adipose mass accumulation (B). Values are means ± SEM for n = 10/group. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$ vs. WT; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.0001$ vs. Sar1b$^{+/+}$ ND; $^{\$}P < 0.05$ vs. WT HFD.

WT: wild type; ND: normal diet; HFD: high-fat diet.

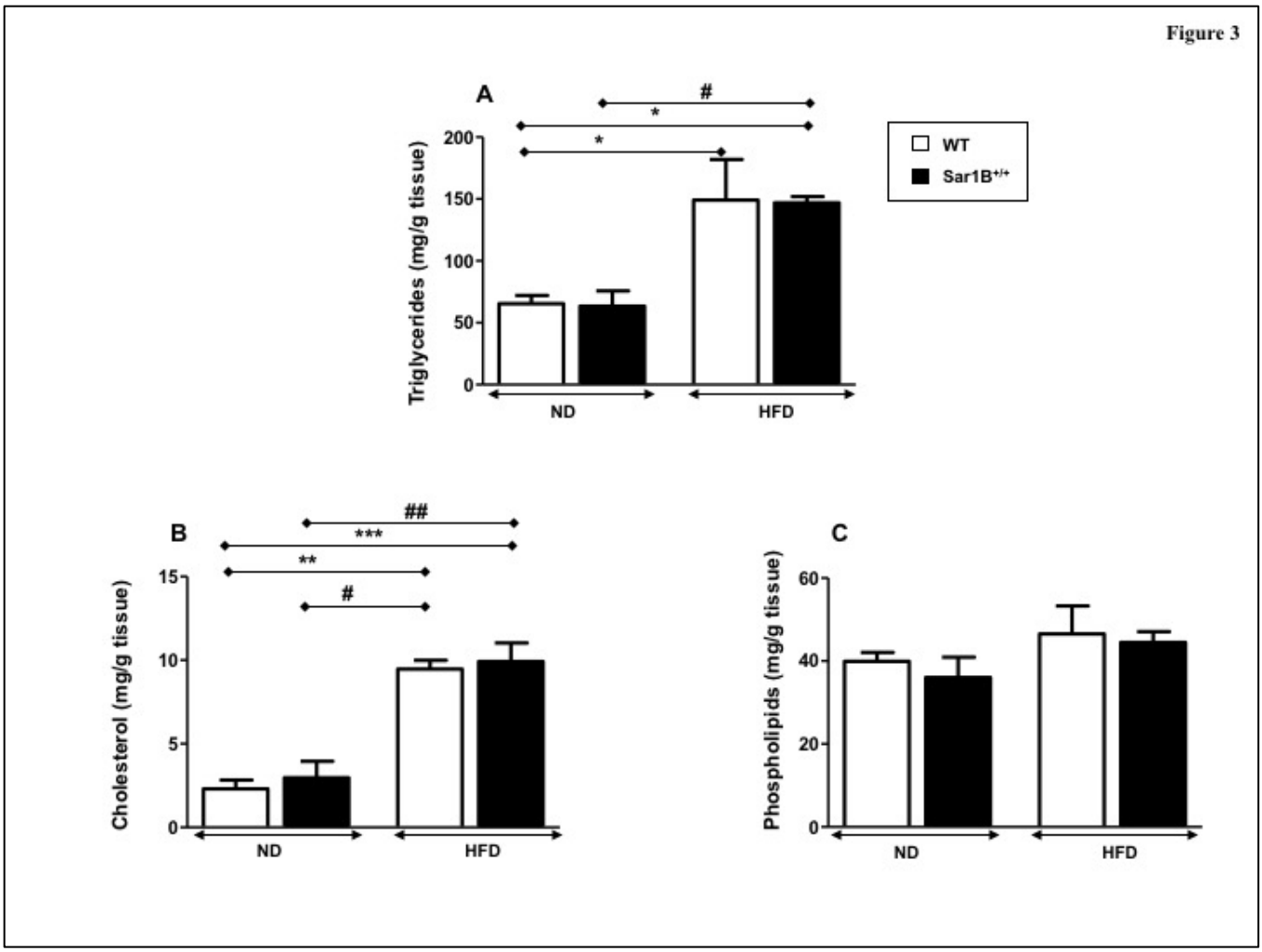

Figure 3. **Effect of high-fat feeding on lipid accumulation in Sar1b$^{+/+}$ and control male mice in the liver**.

Mice at 3 weeks of age were fed with a normal (ND) or a high-fat diet (HFD) for 12 weeks. After sacrifice, liver was collected and homogenized. Lipids were extracted by chloroform/methanol (2:1, vol/vol). After isolation by thin layer chromatography, triglycerides (A), cholesterol (B) and phospholipids (C) fractions were quantitated as described in Materials and Methods.

Values are means ± SEM for n = 10/group. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$ vs. WT; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ vs. Sar1b$^{+/+}$ ND

WT: wild type; ND: normal diet; HFD: high-fat diet.

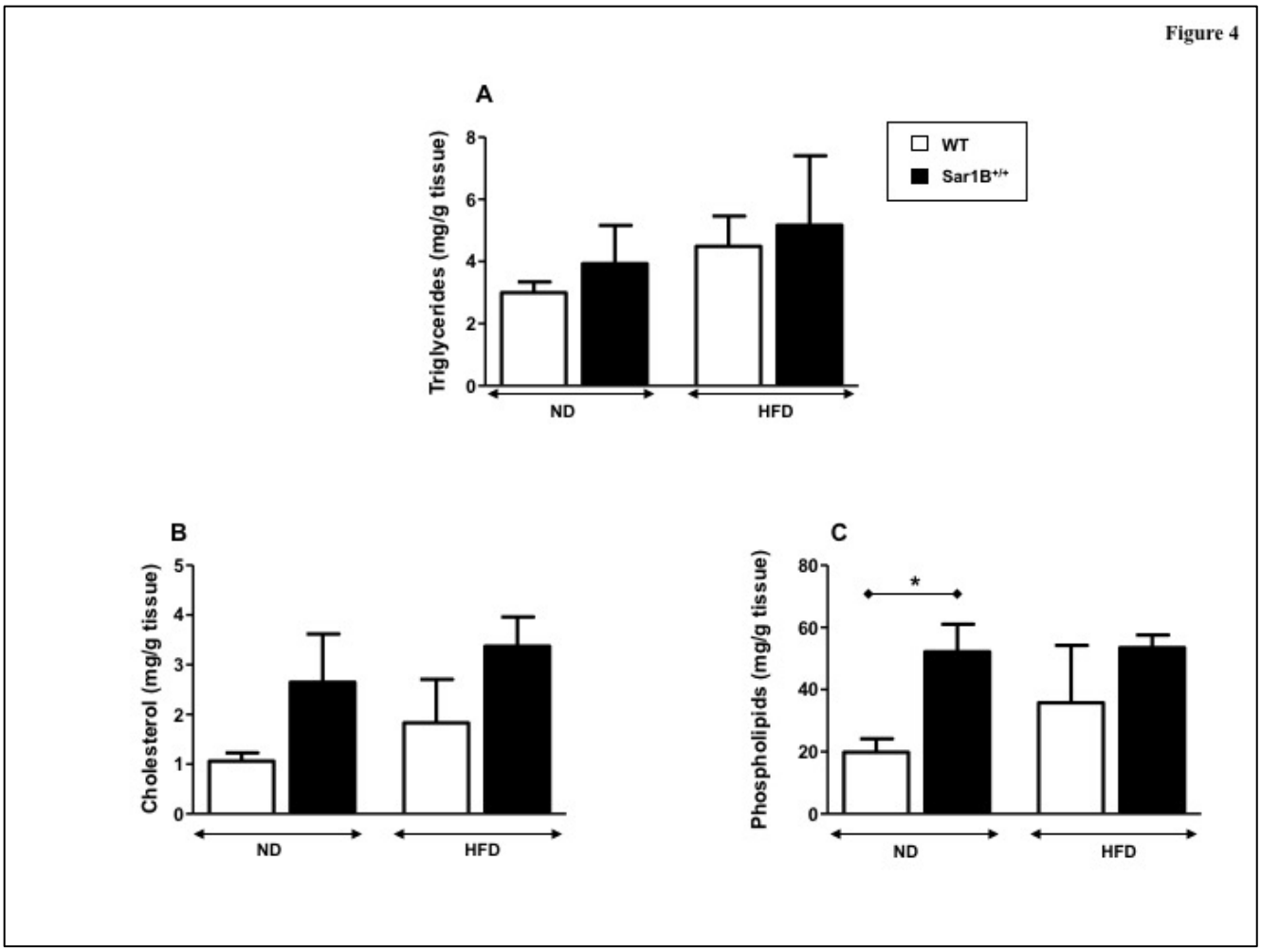

Figure 4. **Effect of high-fat feeding on lipid accumulation in Sar1b$^{+/+}$ and control male mice in the intestine**.

Mice at 3 weeks of age were fed with a normal (ND) or a high-fat diet (HFD) for 12 weeks. After sacrifice, jejunum was collected and homogenized. Lipids were extracted by chloroform/methanol (2:1, vol/vol). After isolation by thin layer chromatography, triglycerides (A), cholesterol (B) and phospholipids (C) fractions were quantitated as described in Materials and Methods.

Values are means ± SEM for n = 10/group. *$P < 0.05$ vs. WT

WT: wild type; ND: normal diet; HFD: high-fat diet.

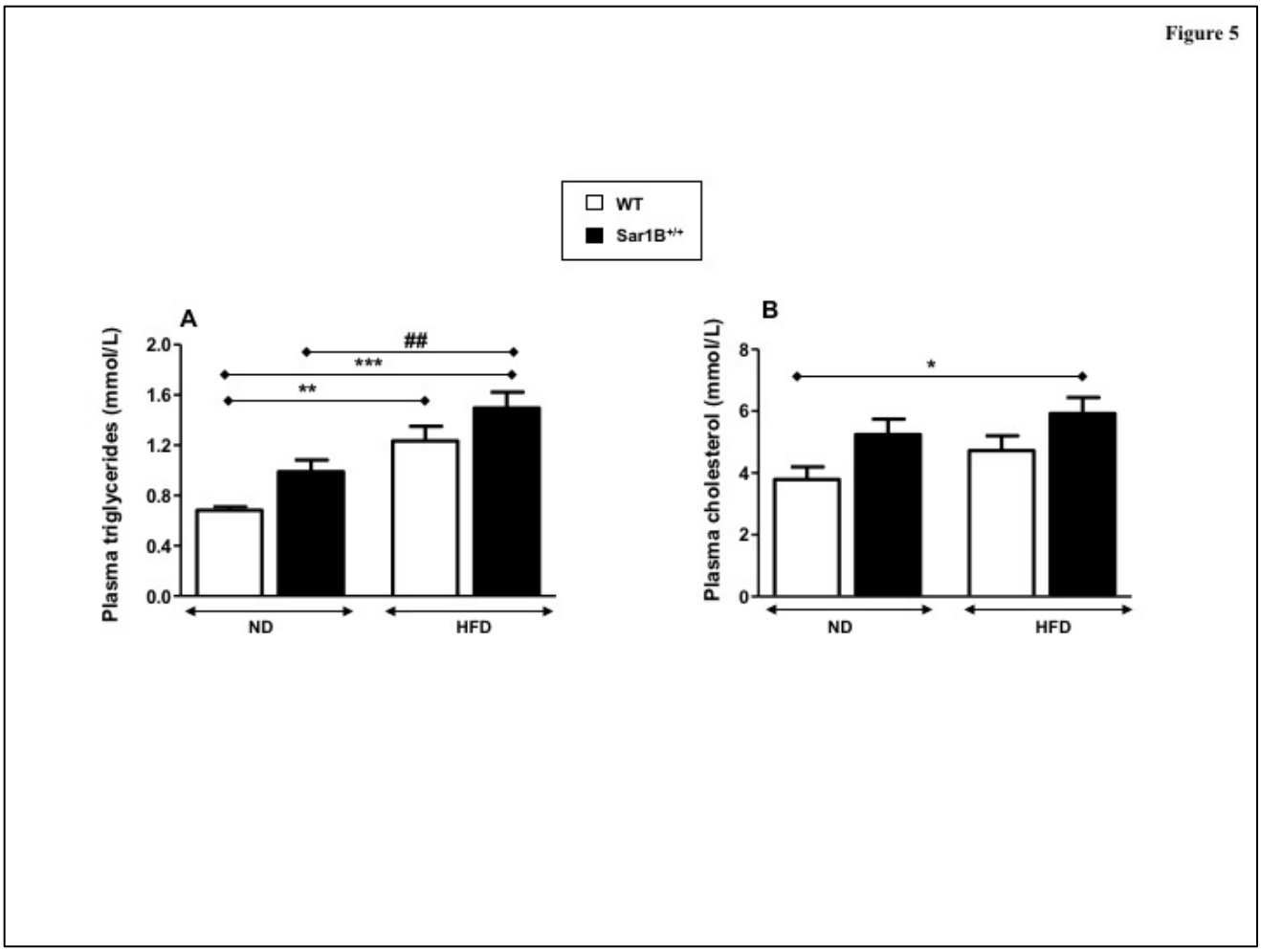

Figure 5. **Effect of high-fat feeding on plasma lipids in Sar1b$^{+/+}$ and control male mice**.

Mice at 3-weeks of age were fed with a normal (ND) or a high-fat diet (HFD) for 12 weeks. After overnight fasting and before sacrifice, blood was collected and circulating triglycerides (A) and cholesterol (B) were measured enzymatically as described in Materials and Methods. Values are means ± SEM for n = 10/group. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$ vs. WT; $^{\#\#}P < 0.001$, $^{\#\#}P < 0.001$ vs. Sar1b$^{+/+}$ ND

WT: wild type; ND: normal diet; HFD: high-fat diet.

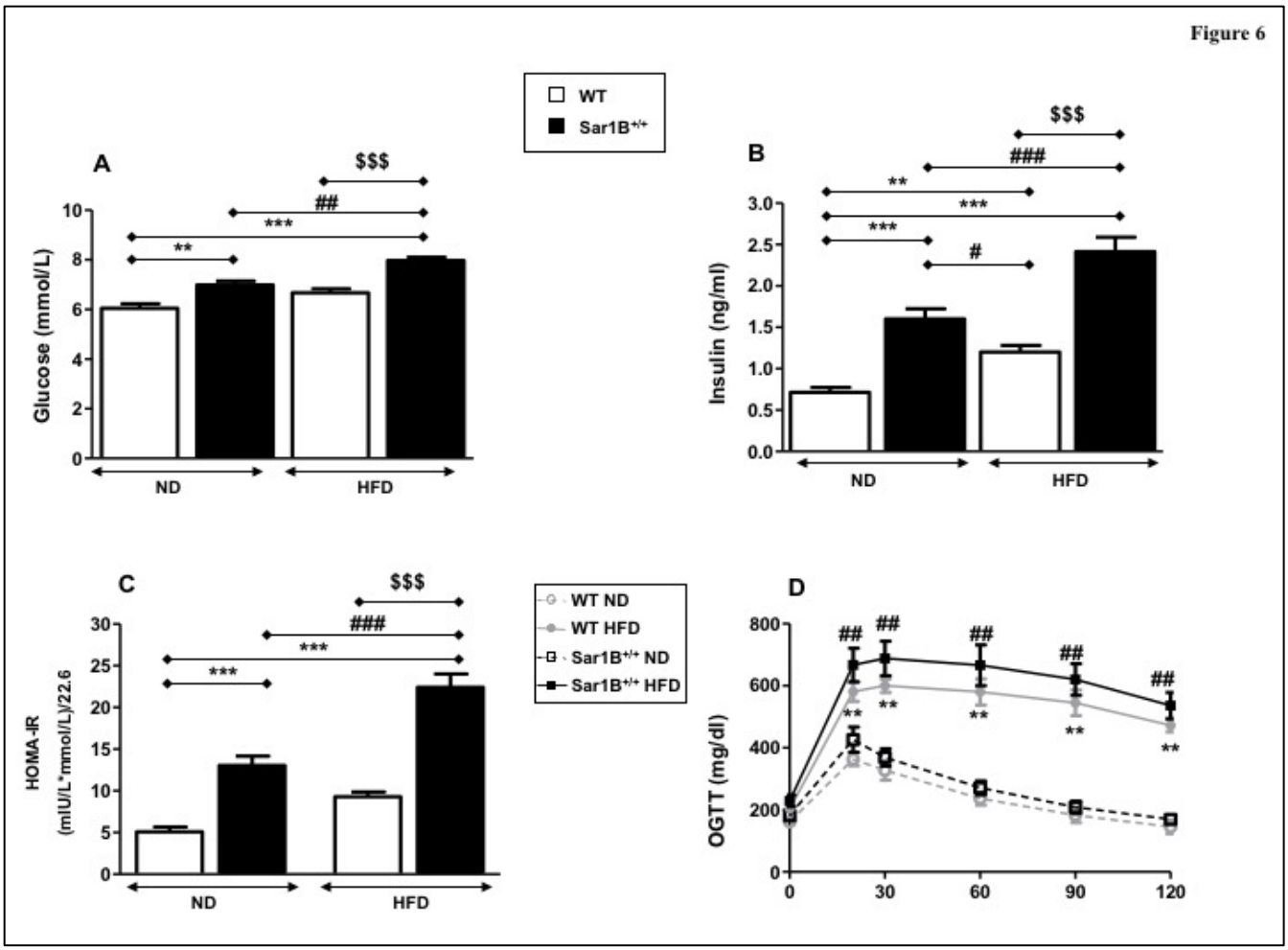

Figure 6. Effect of high-fat feeding on glucose homeostasis and insulin sensitivity in Sar1b$^{+/+}$ and control male mice.

Mice at 3 weeks of age were fed with a normal (ND) or a high-fat diet (HFD) for 12 weeks. After overnight fasting and before sacrifice, blood was collected and glucose was measured enzymatically (A). Plasma insulin levels (B) were determined by radioimmunoassay using a specific commercial kit, as described in Materials and Methods. To evaluate glucose/insulin homeostasis, the HOMA-IR index was calculated (C) and oral glucose test tolerance (OGTT) from 0-120 mn was assessed (D). Values are means ± SEM for n = 10/group. $^{**}P < 0.001$, $^{***}P < 0.0001$ vs. WT; $^{\#\#}P < 0.001$, $^{\#\#\#}P < 0.0001$ vs. Sar1b$^{+/+}$ ND; $^{\$\$\$}P < 0.0001$ vs. WT HFD.

WT: wild type; ND: normal diet; HFD: high-fat diet.

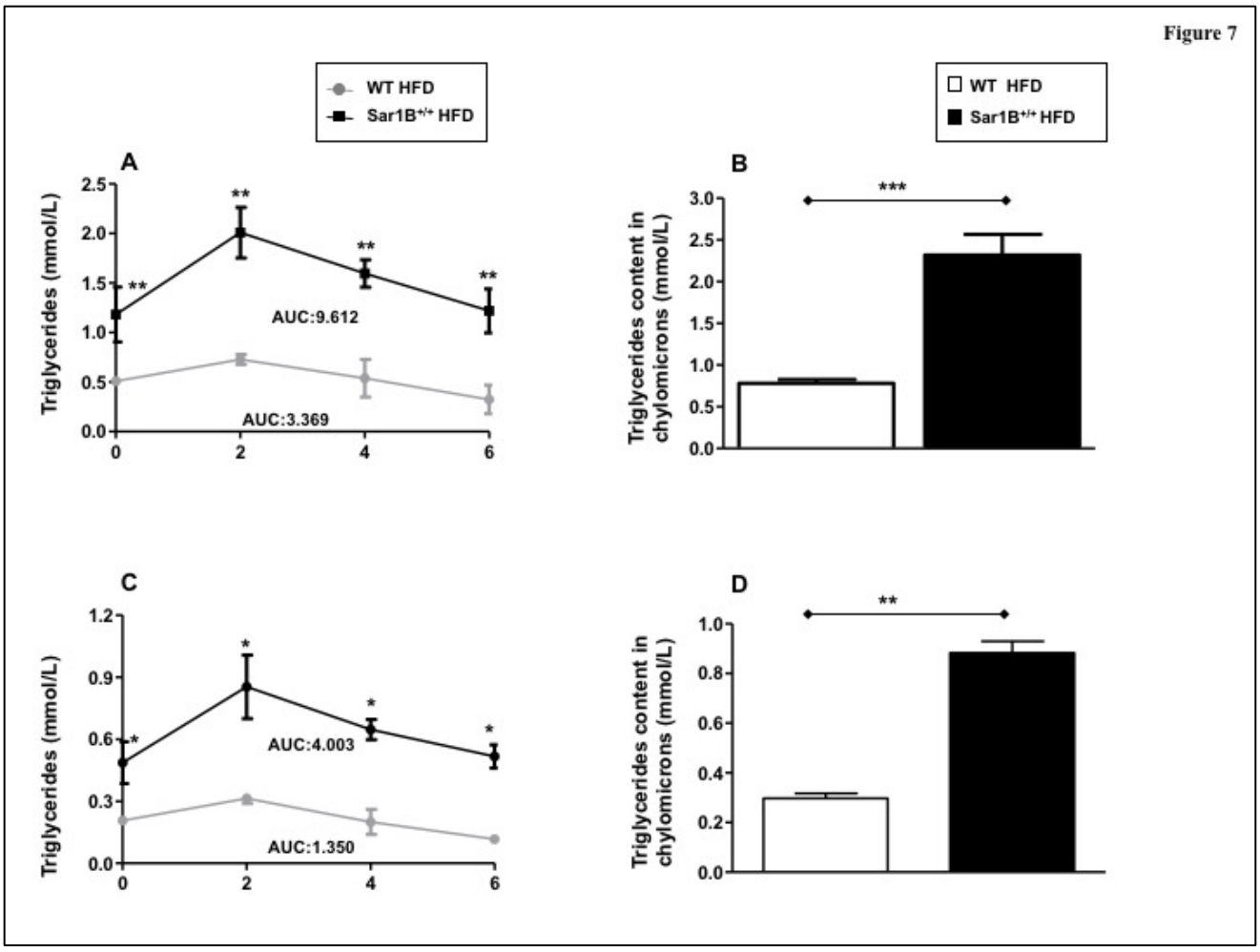

Figure 7 Effect of high-fat feeding on triglyceride-rich secretion in Sar1b$^{+/+}$ and control male mice.

Mice at 3 weeks of age were fed with a normal (ND) or high-fat diet (HFD) for 12 weeks. After overnight fasting, HFD mice were subjected to a fat meal test consisting of the oral administration of 200 µl of microlipids. Blood was collected at 2, 4 and 6 h and plasma triglycerides (A, C) were measured enzymatically, using a specific kit. The production rate of chylomicron components (B, D) was assessed after the ultracentrifugation of plasma triglycerides from postprandial animals treated with Triton WR-1339, in the presence or absence of orotic acid as described in Materials and Methods.

Values are means ± SEM for n = 10/group. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$ vs. WT HFD

WT: wild type; HFD: high-fat diet.

Article 2

«Tissue distribution and regulation of the small Sar1b GTpase in mice»

Marcil V, Seidman E, Sinnett D, Sanchez R, **Spahis S**, Sané A, Levy E. *Cell Physiol Biochem*. 2014;33(6):1815-26. doi: 10.1159/000362960.

CONTRIBUTION DES AUTEURS

Marcil V a contribué aux expériences, à l'analyse, l'interprétation des données et à la rédaction du manuscrit.

Sinnett D, Sanchez R et Seidman E ont révisé le manuscrit.

S. Spahis a été impliquée dans la conception des Figures, la rédaction, configuration, soumission et révision du manuscrit sous la supervision du Directeur de recherche, Dr Levy E.

Sané A a participé au maintien et suivi de la colonie, à la collecte des tissus et aux expériences spécifiques dans le laboratoire.

Levy E a contribué au concept et à la supervision de l'étude, aux ressources financières et matérielles pour la réalisation de l'étude. E. Levy a participé à la rédaction, l'analyse critique des résultats et la révision du manuscrit.

TISSUE DISTRIBUTION AND REGULATION OF THE SMALL SAR1B GTPASE IN MICE

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ABSTRACT

Background: Sar1b GTPase (Sar1b) represents an obligatory component of COPII vesicles that bud from the endoplasmic reticulum to transport proteins to the Golgi apparatus. Sar1b is involved in cargo selection and export, and it also promotes curvature and tubulation during vesicle formation. Its genetic mutations lead to a severe disorder known as chylomicron retention disease. Despite growing knowledge on Sar1b, little is known about its tissue distribution and regulation. *Aim and Methods*: To determine the Sar1b tissue distribution and modulation by a high-fat diet and gene forcing using transgenic mice in comparison to wild-type mice. *Results:* Evaluation of *Sar1b* mRNA by qRT-PCR in mice revealed the skeletal muscle as the tissue with the highest *Sar1b* expression, followed by the heart and liver, the organs composing the digestive tract, the brain and finally the lung and the adipose tissue. Sar1b protein expression levels follow a similar pattern among the organs, except for its lower expression in the heart. In an attempt to approach the regulation of Sar1b, we studied in these mice the effect of Sar1b transgenesis and insulin resistance induced by a 12-week high-fat diet on *Sar1b* gene expression. While the high-fat diet did not exert any significant alterations, Sar1b transgenic mice displayed higher gene expression in the liver, ileum, jejunum, proximal and distal colon compared to wild-type mice. *Conclusions:* Our findings support the importance of Sar1b in organs involved in lipid transport and/or calcium trafficking such as the liver, intestine, skeletal muscle and heart.

INTRODUCTION

In eukaryote cells, intracellular delivery to downstream compartments requires that newly synthesized and properly folded proteins are transported from the endoplasmic reticulum (ER) to the Golgi network via coat protein complex (COP) II vesicles (1-3). As revealed by numerous studies (4), COPII polymerizes on the membrane surface, captures cargo and SNARE molecules, and deforms the membrane to sculpt out vesicles (5). Importantly, COPII vesicle biogenesis is initiated by the activation of a small Sar1 GTP-binding protein through Sec12, a guanine nucleotide exchange factor (6, 7). The presence of Sar1b-GTPase (Sar1b) stimulates the recruitment of the inner coat components Sec23 and Sec24, which are responsible for capturing cargo proteins into the nascent vesicles (8, 9). Then, the Sec13/31 heterotetramer is recruited to form the outer coat and stabilize the cargo-bound pre-budding complexes (2, 7, 10).

The Sar1 protein has two paralogs, Sar1a and Sar1b. In humans, the *SAR1B* gene (*SARA2*, HGNC: 10535; MIM *607690), not *SAR1A*, was identified as the specific gene undergoing molecular defects present in Anderson's disease or chylomicron (CM) retention disease, a rare autosomal recessive disorder characterized by an inability of the enterocytes to export dietary lipids via CMs. To date, 16 *SAR1B* mutations have been described in patients with CM retention disease (11-19). No mutation in the *SAR1A* gene has been detected in patients with this specific disease. Accordingly, it has been suggested that, in enterocytes, the Sar1b paralogue would be used specifically for pre-CM mobilization by transport vesicles while the Sar1a paralogue would be employed for the transport of small soluble and membrane bound proteins by transport vesicles (15).

Patients with CM retention disease present with steatorrhea, chronic diarrhea and failure to thrive, as well as low plasma lipid and liposoluble vitamin levels, causing neurological impairment (20-23). It appears that Sar1b is involved in multiple aspects of the cargo sorting process, e.g. the regulation of COPII coat dynamics and membrane deformation during vesicle formation, which dictates routing of numerous proteins and triglyceride-rich lipoproteins, including CM in the small intestine and very-low-density lipoproteins in the liver (11, 24). Additional convincing evidence has recently been obtained by genetic manipulation that clearly demonstrated that Sar1b overexpression mediates stimulation of intestinal lipid transport, which was likely induced by the efficient assembly of CMs (25).

Despite the importance of Sar1b in COPII vesicle elaboration, there is an important gap of knowledge on the status and regulation of Sar1b in different organs. In this study, we assessed the tissue distribution and regulation by a high-fat diet and gene forcing of *Sar1b* gene expression in wild-type (WT) and transgenic (TG, $Sar1b^{+/+}$) mice.

MATERIAL AND METHODS

Generation of Sar1b transgenic mice

The pCMV6-XL4 vector (OriGene Technologies, Rockville, MD), harbouring full length cDNA of *SAR1B*, was digested with *Not*I to excise a 1960 bp fragment. This fragment was subsequently cloned into a pBROAD3-mcs vector (InvivoGen, San Diego, CA) restricted with *Not*I to create pBROAD3-*SAR1B*. pBROAD3 features the ubiquitous mouse ROSA26 promoter, a high CpG content promoter that directs *in vivo* expression of the transgene (26). Chicken β-globin gene insulator was subcloned upstream the ROSA26 promoter at the *Nde*I site to enhance transgene expression. Cloning of the β-globin gene insulator created pBROAD3-β-*SAR1B* vector. This 2 kb insulator fragment was derived from PCR amplification of chicken DNA with insulator specific primers containing *Nde*I site (primer 1: CATATGGCGGCCGCTCTAGACT; primer 2: CATATGGGATCCGTCGACGC). The capability of this construct to allow *SAR1B* expression in enterocytes was tested by transient transfection of Caco-2/15 cells. The DNA transgene vector, pBROAD-β-*SAR1B*, was then injected in fertilized eggs from 129/Sv mice at the Réseau de Recherche en Transgenèse du Québec facility. Genomic DNA was prepared from founder mice and their offspring tail and screened for the presence of *SAR1B* DNA by PCR amplification, using specific primers (primer 1: ATGTCCTTCATATTTGGATT; primer 2: ATCAATGTACTGTGCCATCC). Experiments were performed in male mice of mixed 129/Sv x C57BL6 backgrounds.

Animals and diets

Three weeks aged TG for Sar1b and WT mice were housed in a temperature-controlled room at 24°C with a 12-h light-dark cycle and maintained on a standard laboratory chow normal diet (2018 Teklad Global 18% protein rodent diet, Harlan Laboratories, Indianapolis, IN) or a high-fat diet (BioServ F3282 60% fat Calories, BioServ, Frenchtown, NJ) and double distilled water *ad libitum* for 12 weeks. Animal experiments were conducted according to the Canadian Council on Animal Care guidelines for the care and use of experimental animals and were approved by the institutional animal care committee of Sainte-Justine Research Center.

Biochemical Analyses

After 12 weeks of feeding, mice were fasted overnight, weighed and sacrificed. Blood was collected for insulin and lipid assays. Plasma triglycerides and cholesterol levels were measured enzymatically (Boehringer-Mannheim, Mannheim, Germany). Blood glucose levels were

determined using a OneTouch Ultra Meter (LifeScan, Milpitas, CA) and plasma insulin was measured using a rat/mouse insulin ELISA kit (Millipore, Billerica, MA). Plasma fatty acid composition was determined by gas chromatography as described previously (25). Insulin sensitivity was determined using the HOMA-IR index. The score was calculated using the formula: (Insulinemia (mIU/L) x Glycemia (mmol/L) / 22.5.

Cell Culture for Antibody Specificity

To test the specificity of our Sar1a/b antibody, we cultured Caco-2/15 cells for 14 days post-confluence as previously described (27). We also established Caco-2/15 cell line overexpressing Sar1b and green fluorescent protein (GFP). To do so, the blunt-end PCR fragment encoding *SAR1B* gene was amplified from the human cDNA clone SC114457 (Origene Technologies, Rockville, MD) with ATGTCCTTCATATTTG as forward primer and CAGATCCTCTTCTGAGATGAGTTTTTGTTCATCAATGTTACTGTGCCAT containing *myc* epitope as reverse primer and TOPO Cloned into pLenti6/V5-D-TOPO (Invitrogen Corp., Carlsbad, CA 92008) to create an expression construct. The pLenti6/V5-D-TOPO expression plasmid of *SAR1B* was then transfected into Caco-2/15 cells with GenJet In Vitro Transfection Reagent (SignaGen Laboratories, Ijamsville, MD) according to the supplier's instructions. Generation of stable cell line was achieved using Blasticidin selection at a concentration of 1 μg/ml. Control Mock cells were obtained by transfection with pLentiV5/GFPtag (kindly provided by Dr. Jean-François Beaulieu (Université de Sherbrooke) harbouring the same features as the pLenti6/V5-D-TOPO system.

Western Blot Analysis

Sections of different organs from a total of 12 mice (3 mice/group for the 4 groups: WT-normal diet; WT-high-fat diet; TG-normal diet; TG-high-fat diet) were homogenized in ice-cold T-PER Tissue Protein Extraction Reagent (Thermo Scientific, Rockford, IL). Protease inhibitors were added to tissues prior to homogenization. As for Caco-2/15 cells, they were homogenized and adequately prepared for Western blotting as described previously (27). The Bradford assay was used to estimate protein concentration. Supernatant proteins (20 μg) were denatured at 95˚C for 5 min in a sample buffer containing sodium dodecyl sulfate (SDS) and dithiothreitol (Thermo Scientific, Rockford, IL), separated on a 15% SDS-polyacrylamide gel electropheresis (SDS-PAGE) and electroblotted onto Hybond-C extra nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ) in 25 mM Tris and 192 mM glycine. Nonspecific binding sites of

the membranes were blocked with Tris-buffered saline [20 mM Tris-HCl (pH 7.5) plus 137 mM NaCl] containing 0.1% Tween 20 and 5% non fat dry milk for 60 min at room temperature. The blots were then incubated overnight at 4˚C in blocking solution with the primary antibodies directed against the targeted proteins Sar1b/a (1:20,000) (kindly provided by Dr Randy Schekman, Department of Molecular and Cell Biology, University of California at Berkeley, Berkeley, CA), β-actin 1:250,000 (Sigma Aldrich) and myc (1:250) (provided by Dr Mounib Elchebly, Sainte-Justine Hospital Research Center, Montreal, Canada). The relative amount of primary antibody was revealed with a species-specific horseradish peroxidase-conjugated secondary antibody, e.g. anti-mouse IgG-POD/anti-rabbit IgG-POD (Roche Diagnostics, Indianapolis) (1:20,000). Blots were developed and the protein mass was quantified using an HP Scanjet scanner equipped with a transparency adapter and the UN-SCAN-IT gel 6.1 software.

RNA Extraction and Reverse Transcription

Tissue specimens obtained from a total of 24 mice (6 mice/group for the 4 groups: WT-normal diet; WT-high-fat diet; TG-normal diet; TG-high-fat diet) were immediately conserved in RNA-Later according to the manufacturer's protocol. They were homogenized in TRIzol reagent (Ambion by Life Technologies Corporation, Carlsbad, CA) and total RNA was extracted. RNA concentration and quality were tested by absorbance at 260 nm while the absorbance ratio of 260/280 (using a Nanodrop® ND-1000 Spectrophotometer from Thermo Scientific, Wilmington, DE) and RNA analysis by agarose gel electrophoresis served to assess integrity. Complementary DNA was obtained by reverse transcripting 1 µg of RNA with the qScript cNDA Supermix (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer's instructions. Negative controls without enzyme were also prepared.

Quantitative PCR

The TaqMan® Express Human Endogenous Control Plate (Applied Biosystems) was used to select the most stable reference gene amongst organs according to the manufacturer's instructions. Using this technique, polymerase (RNA) II (DNA directed) polypeptide AI (*Polr2a*) was chosen as an endogenous control to normalize the different cDNA sample amounts. Quantitative PCRs (qPCR) were performed in 96-wells microtiter plates using Taqman® Fast Advanced Master Mix (Applied Biosystems, Foster City, CA) in an Applied Biosystems Step One Plus™ Real-Time PCR System (Applied Biosystems). The qPCRs were carried in 96-well plates with a final volume of 10 µl per well. A volume of 5 µl Taqman® Fast Advanced Master Mix (1 X) was added to a well

containing 1 µl of Custom Taqman® Gene Expression Assay (Applied Biosystems) (1 X) for *Sar1b* or for *Polr2a* comprising 1 µl of cDNA previously diluted 1:5 and 3 µl of diethylpyrocarbonate H_2O. Taqman® hydrolysis probes used were Mm01294633_m1 for *Sar1b* and Mn00839493_m1 for *Polr2a* (Applied Biosystems). Subsequently, negative controls without cDNA were prepared. The amplification reaction was carried out using 40 cycles and was performed in duplicate for each gene in the same plate under the following conditions: 95°C 20 s, 95°C 1 s and 60°C for 20 s.

Analyses

The relative mRNA fold-changes (relative quantification, RQ) between the animal groups were calculated using the $2^{-\Delta\Delta Ct}$ method (28). For each sample, the first ΔCt was calculated using the corresponding endogenous control. The second ΔCt was calculated using the defined control group. For the comparison of *Sar1b* gene expression in the different organs, the average ΔCt of the lung group was used as reference. Similarly, the average ΔCt was employed for the comparison of *Sar1b* gene expression between WT and TG mice under the normal chow and high-fat diets, using the WT/normal diet mice as reference. Exponential values were then transformed in linear values and expressed as relative fold change in gene expression. The analyses and statistics were performed using the Expression Suite Software Version 1.0 (Applied Biosystems) and the Prism 5 for Mac Software Version 5.0d (GraphPad Software Inc., La Jolla, CA). Data are expressed as means ± SEM. For statistical analysis, one-way ANOVA followed by Bonferroni's multiple comparison test or t-tests were used and a P value < 0.05 was considered significant.

RESULTS AND DISCUSSION

At first, we characterized the metabolic and lipid profile of mice from each group at sacrifice, following a 12-week diet. Results are presented in **Table 1**. To determine whether the overexpression of Sar1b influenced diet-induced obesity, male TG mice were maintained with *ad libitum* access to a pelleted high-fat diet. Then anthropometric and biochemical parameters were assessed. Although high-fat diet increased body weight in TG and WT mice, the mean body weight of TG carriers was slightly higher compared to corresponding WT controls. Similarly, the high-fat diet led to a rise in glucose and insulin in WT and TG mice, but the increase was more substantial in the Sar1b$^{+/+}$ mice. Noteworthy, differences in these variables in WT and TG mice were observed even on the regular chow diet, which depicts the separate influence of the two factors, i.e. transgenesis and diet. As a result from the glucose and insulin modifications, the HOMA-IR index showed a more significant increase in the Sar1b$^{+/+}$ mice vs. controls, thereby indicating reduced insulin sensitivity. Finally, our data show that the high-fat diet caused an augmentation in plasma triglycerides and cholesterol levels in WT and TG mice, but their increment was more substantial in Sar1b$^{+/+}$ mice. Hence, as expected, the HFD induced a state of insulin resistance and dyslipidemia in the animals, and these effects were exacerbated by Sar1b transgenesis. A study, recently published by our group, has described the complete metabolic profile of Sar1b$^{+/+}$ mice under control and high-fat diets and corroborates the present findings (29). When fed a HFD, Sar1b$^{+/+}$ mice were found to develop obesity, insulin insensitivity, hepatic and plasma lipid abnormalities and CM hyperproduction. This increased triglyceride-rich lipoprotein production elicited by Sar1b$^{+/+}$ mice is in line with our other previous results obtained in Caco-2/15 cells, demonstrating that Sar1b overexpression can enhance intestinal lipoprotein trafficking and sorting (25). According to the prevailing concept, intestinal CM assembly requires the association of apo B-48 with lipids, which is mediated by microsomal triglyceride transfer protein in the rough ER, resulting in a lipid-poor, small apo B-48-containing particles that serve as precursors of mature CM. The intracellular traffic of apo B-48-containing CM is dependent on the COPII coat complex, which buds vesicles from the ER membrane to transport newly synthesized proteins to the Golgi apparatus. COPII vesicle biogenesis and assembly necessitates Sar1b to trigger a sequence of recruitment events that assemble the COPII coat from its cytosolic components and ultimately drives vesicle budding (3). Our TG mouse model shows that overexpressing Sar1b leads to

increased insulin resistance and dyslipidemia, phenomena that were amplified by the high-fat diet. Although the mechanisms controlling these metabolic changes are still nebulous and deserve further exploration, we propose that the increased intestinal fat absorption associated with Sar1b profusion in Sar1b$^{+/+}$ mice leads to to greater triglyceride accumulation in the blood circulation and insulin-responsive tissues (29), which would impede glucose uptake and provoke insulin resistance.

We have conducted experiments to determine the specificity of our Sar1b/a antibody in WT and TG animals. First, in Caco-2/15 cells, the Western blots reveal two visible bands, previously described as Sar1b (the upper band) and Sar1a (the lower band) (30) (**Figure 1A**). The experiments were repeated in Caco-2/15 cells in which a pLenti6/V5-D-TOPO expression plasmid of *SAR1B* was transfected and, as a result, Sar1b was overexpressed. In this step, not only the blots were incubated with the anti-Sar1b/a antibody, but also with an anti-myc antibody, permitting to distinguish between the endogenous Sar1 and the transfected Sar1b. As illustrated in **Figure 1B**, even if the double band (endogenous Sar1a and Sar1b) is still visible, the exogenous form of Sar1b represented 222% of the Sar1b/a band. However, in mice, the band corresponding to the Sar1a protein was barely visible. In fact, the band was rarely observed since Sar1b was overexpressed compared to Sar1a. **Figure 2** shows an experiment where the Sar1a and Sar1b bands could be distinguished in the liver of TG animals. These results showed that the densitometric ratio of Sar1b was over 1,500% of Sar1a. Hence, we concluded that, although our antibody could recognize Sar1a and Sar1b, the important difference in protein expression, especially in mice, allowed us to study the regulation of Sar1b.

Next, we defined Sar1b protein and mRNA expression in different organs in mice. As shown in **Figure 3A**, the organ with the highest mRNA expression of *Sar1b* was the skeletal muscle, followed by the heart and the liver. The organs composing the digestive system and the brain had similar levels of expression. Finally, we found relatively low gene expression in the lung and perirenal adipose tissue. The skeletal muscle was the only organ for which post-hoc analyses revealed a significant difference with the others. The measurement of Sar1b at the protein level demonstrated a similar pattern among the organs, except for its lower expression in the heart (**Figure 3B**). Western blots showed that the liver was the organ that mostly expressed Sar1b and confirmed the high expression in the skeletal muscle, followed by a roughly equivalent expression

in the organs of the digestive tract. The gene and protein expression profile of Sar1b in the duodenum, jejunum and ileum, parts of the digestive system, was relatively equivalent, which corresponds to their respective function in lipid and bile acid handling. It is also important to specify that, since the pattern of Sar1b protein distribution was similar between WT and TG mice under chow and high-fat diet, all the tissue samples from the different groups were analyzed on the same Western blot for comparison needs.

Limited information is available concerning the distribution of Sar1b in various organs despite its crucial function in intracellular trafficking. Using the state-of-the-art technique for mRNA analysis, e.g. qPCR, we documented a preponderance of *Sar1b* mRNA content in the skeletal muscle. The surprisingly abundant expression of *Sar1b* in the muscle suggests highly specialized role of Sar1b in this particular tissue, including the regulation of calcium (Ca^{2+}) trafficking among multiple Ca^{2+} storage organelles, for instance the sarcoplasmic reticulum and the ER. In fact, the involvement of Sar1b in Ca^{2+} trafficking in skeletal muscle was demonstrated a decade ago when calsequestrin, an acidic, low-affinity, high-capacity Ca^{2+}-binding glycoprotein that plays a crucial role in Ca^{2+} routing to sarcoplasmic reticulum, was found sensitive to the Sar1-H79G mutant, resulting in its accumulation in COPII vesicles in rat skeletal muscle fibers (31). Later, it was demonstrated that the molecular mechanism underlying the human Ca^{2+} receptor exit from the ER to the cell surface requires Sar1b and is dependent on COPII vesicles (32). Likewise, the apoptosis-linked gene 2, a Ca^{2+}-binding protein that serves as a Ca^{2+} sensor, is dependant of the COPII vesicle formation process and components (33). Hence, the role of Sar1b in Ca^{2+} trafficking can explain its high level of expression not only in the skeletal muscle, but also in the heart, in view of Ca^{2+} signaling and trafficking in cardiomyocytes (34, 35). Accordingly, it was reported that patients with chylomicron retention disease suffer from myolysis, cardiac abnormalities and elevated creatine kinase levels (16).

Next, we evaluated the effect of insulino-resistance induced by high-fat diet and the impact of Sar1b transgenesis on *Sar1b* gene expression in different tissues. The provision of a 12-week high-fat diet to mice did not result in significant changes in mRNA *Sar1b* expression in the various tissues when compared to a normal diet in neither WT nor TG mice (**Figure 4**). This is divergent from a previous study documenting *Sar1b* up-regulation by fructose diet and olive oil in Syrian golden hamsters (36), suggesting that the regulation of *Sar1b* expression by diet and insulin resistance may be species-dependent. Also, *in vitro* studies have suggested that Sar1b can be

regulated by lipids, as demonstrated by the regulation of COPII vesicle formation by lipid composition of membranes (37). Similarly, depletion of sterols or inhibition of sterol synthesis reduce the mobility of membrane cargo proteins within the ER and inhibit the dynamic interactions of COPII subunits with ER exit sites (38, 39). Previous work from our laboratory demonstrated that, in Caco-2/15 cells, Sar1b overexpression promotes intestinal lipid transport in a process involving the COPII network (25). In the present experimental conditions, the high-fat diet did not effectively regulate *Sar1b* expression, which allows us to speculate that diet could regulate *Sar1b* turnover without altering mRNA, thereby increasing recycling activity of COPII vesicle.

Our results also revealed that TG mice displayed enhanced mRNA level of *Sar1b* in several organs (**Figure 4**). Significant enrichment was noted in the liver, ileum, jejunum, proximal colon and distal colon while there was also a higher, albeit not statistically significant, expression in the skeletal muscle, heart and lung. Interestingly, the enrichment was observed in the tissues endowed with functional roles in Ca^{2+} and lipid transport. In line with our results showing disparate enrichment between organs, various reports have emphasized that various TG mouse models equipped, genetically manipulated in different genes expressed variable amounts of mRNA in peripheral tissues (40). More specifically, the pCAG promoter in the Rosa26 locus suffers from mosaic transgene expression among organs (41) and the responder gene expression was moderate and highly variable between and within different tissues (42).

We have evaluated the effect of transgenesis on Sar1b protein expression in the the liver and jejunum, the two key organs involved in lipid transport and lipoprotein assembly. **Figure 5A** shows that Sar1b in these two organs is elevated in TG compared to WT mice. In fact, we found 446% ($P<0.004$) and 388% ($P<0.022$) increases in Sar1b expression in the liver and jejunum, respectively. This corresponds to the elevation in gene expression in these organs presented in **Figure 4**. Compared to WT animals, transgenesis led to elevated gene expression (459%, $P<0.0015$) in the liver and in the jejunum (90%), although the differences in the latter were not statistically significant. Using standard RT-PCR method, we have also compared the endogenous and exogenous *Sar1b* gene expression in mice. **Figure 5B** reveals that the exogenous gene expression in transgenic animals represents approximately 70% of the total expression in the liver and in jejunum.

A limit of the present work is that it was been performed in mice, which does not necessarily reflect the expression profile in humans. However, previous investigation has shown that the presence of Sar1b in the digestive tract is consistent in different species: it was found in the human duodenum (15), in the human Caco-2/15 cell model (25), in the small intestine of hamsters (36) and in rat enterocytes (43). Further studies are needed to evaluate the distribution and expression profile of Sar1b in the digestive tract and in other tissues.

CONCLUSIONS

In conclusion, Sar1b in our mouse model was not regulated by insulin resistance or by high-fat content. However, the results obtained from tissue distribution pattern and regulation by transgenesis support the importance of Sar1b in organs involved in lipid transport such as the liver, jejunum, ileum and colon, which highlight its crucial role in organs transporting lipids. Interestingly our findings also support a significant role for Sar1b in organs necessitating major Ca^{2+} trafficking, namely the skeletal muscle and the heart.

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Table 1. Metabolic characteristics of mice at sacrifice

	Wild-type Normal Diet	Wild-type High-fat Diet	Transgenic Normal Diet	Transgenic High-fat Diet
Weight (g)	29.35 ± 2.14	34.90 ± 2.13	37.67 ± 2.74*	50.70 ± 0.72***,##
Blood glucose (mmol/L)	6.00 ± 0.20	6.70 ± 0.20	7.00 ± 0.20**	7.80 ± 0.10***,##
Insulin (ng/mL)	0.71 ± 0.06	1.20 ± 0.08**	1.60 ± 0.12***	2.41 ± 0.17***,###
HOMA-IR (mIU/L*mmol/L)/22.5	5.00 ± 0.60	9.30 ± 0.60	13.0 0 ± 1.10***	22.40 ± 1.60***,###
Triglycerides (mmol/L)	0.68 ± 0.07	1.23 ± 0.12**	1.00 ± 0.09	1.50 ± 0.13##
Total cholesterol (mmol/L)	3.80 ± 0.40	4.70 ± 0.50	5.20 ± 0.50	5.90 ± 0.50*

Mice were sacrificed after being fed a normal or high-fat diet for 12 weeks. Results represent the means ± SEM of 6 animals per group. One-way ANOVA were performed (P<0.0001) followed by Tuckey's multiple comparison tests. *P<0.05; **P<0.01; ***P<0.001 vs. Wild-type, normal diet mice. #P<0.05; ##P<0.01; ###P<0.001 vs. Transgenic, normal diet mice.

REFERENCES

(1) Antonny B, Schekman R. ER export: public transportation by the COPII coach. Current opinion in cell biology. 2001;13(4):438-43.

(2) Barlowe C. Signals for COPII-dependent export from the ER: what's the ticket out? Trends Cell Biol. 2003;13(6):295-300.

(3) Barlowe C, Orci L, Yeung T, Hosobuchi M, Hamamoto S, Salama N, et al. COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. Cell. 1994;77(6):895-907.

(4) Gillon AD, Latham CF, Miller EA. Vesicle-mediated ER export of proteins and lipids. Biochimica et biophysica acta. 2012;1821(8):1040-9.

(5) Kirchhausen T. Three ways to make a vesicle. Nature reviews Molecular cell biology. 2000;1(3):187-98.

(6) Barlowe C, Schekman R. SEC12 encodes a guanine-nucleotide-exchange factor essential for transport vesicle budding from the ER. Nature. 1993;365(6444):347-9.

(7) Futai E, Hamamoto S, Orci L, Schekman R. GTP/GDP exchange by Sec12p enables COPII vesicle bud formation on synthetic liposomes. The EMBO journal. 2004;23(21):4146-55.

(8) Bi X, Corpina RA, Goldberg J. Structure of the Sec23/24-Sar1 pre-budding complex of the COPII vesicle coat. Nature. 2002;419(6904):271-7.

(9) Matsuoka K, Orci L, Amherdt M, Bednarek SY, Hamamoto S, Schekman R, et al. COPII-coated vesicle formation reconstituted with purified coat proteins and chemically defined liposomes. Cell. 1998;93(2):263-75.

(10) Aridor M, Traub LM. Cargo selection in vesicular transport: the making and breaking of a coat. Traffic. 2002;3(8):537-46.

(11) Jones B, Jones EL, Bonney SA, Patel HN, Mensenkamp AR, Eichenbaum-Voline S, et al. Mutations in a Sar1 GTPase of COPII vesicles are associated with lipid absorption disorders. Nature genetics. 2003;34(1):29-31.

(12) Peretti N, Sassolas A, Roy CC, Deslandres C, Charcosset M, Castagnetti J, et al. Guidelines for the diagnosis and management of chylomicron retention disease based on a review of the literature and the experience of two centers. Orphanet journal of rare diseases. 2010;5:24.

(13) Charcosset M, Sassolas A, Peretti N, Roy CC, Deslandres C, Sinnett D, et al. Anderson or chylomicron retention disease: molecular impact of five mutations in the SAR1B gene on the structure and the functionality of Sar1b protein. Mol Genet Metab. 2008;93(1):74-84.

(14) Peretti N, Roy CC, Sassolas A, Deslandres C, Drouin E, Rasquin A, et al. Chylomicron retention disease: a long term study of two cohorts. Mol Genet Metab. 2009;97(2):136-42.

(15) Georges A, Bonneau J, Bonnefont-Rousselot D, Champigneulle J, Rabes JP, Abifadel M, et al. Molecular analysis and intestinal expression of SAR1 genes and proteins in Anderson's disease (Chylomicron retention disease). Orphanet journal of rare diseases. 2011;6:1.

(16) Silvain M, Bligny D, Aparicio T, Laforet P, Grodet A, Peretti N, et al. Anderson's disease (chylomicron retention disease): a new mutation in the SARA2 gene associated with muscular and cardiac abnormalities. Clinical genetics. 2008;74(6):546-52.

(17) Annesi G, Aguglia U, Tarantino P, Annesi F, De Marco EV, Civitelli D, et al. SIL1 and SARA2 mutations in Marinesco-Sjogren and chylomicron retention diseases. Clinical genetics. 2007;71(3):288-9.

(18) Treepongkaruna S, Chongviriyaphan N, Suthutvoravut U, Charoenpipop D, Choubtum L, Wattanasirichaigoon D. Novel missense mutations of SAR1B gene in an infant with chylomicron retention disease. Journal of pediatric gastroenterology and nutrition. 2009;48(3):370-3.

(19) Cefalu AB, Calvo PL, Noto D, Baldi M, Valenti V, Lerro P, et al. Variable phenotypic expression of chylomicron retention disease in a kindred carrying a mutation of the Sara2 gene. Metabolism: clinical and experimental. 2010;59(4):463-7.

(20) Levy E, Marcel Y, Deckelbaum RJ, Milne R, Lepage G, Seidman E, et al. Intestinal apoB synthesis, lipids, and lipoproteins in chylomicron retention disease. Journal of lipid research. 1987;28(11):1263-74.

(21) Levy E, Chouraqui JP, Roy CC. Steatorrhea and disorders of chylomicron synthesis and secretion. Pediatr Clin North Am. 1988;35(1):53-67.

(22) Roy CC, Levy E, Green PH, Sniderman A, Letarte J, Buts JP, et al. Malabsorption, hypocholesterolemia, and fat-filled enterocytes with increased intestinal apoprotein B. Chylomicron retention disease. Gastroenterology. 1987;92(2):390-9.

(23) Strich D, Goldstein R, Phillips A, Shemer R, Goldberg Y, Razin A, et al. Anderson's disease: no linkage to the apo B locus. Journal of pediatric gastroenterology and nutrition. 1993;16(3):257-64.

(24) Gusarova V, Caplan AJ, Brodsky JL, Fisher EA. Apoprotein B degradation is promoted by the molecular chaperones hsp90 and hsp70. The Journal of biological chemistry. 2001;276(27):24891-900.

(25) Levy E, Harmel E, Laville M, Sanchez R, Emonnot L, Sinnett D, et al. Expression of Sar1b enhances chylomicron assembly and key components of the coat protein complex II system driving vesicle budding. Arteriosclerosis, thrombosis, and vascular biology. 2011;31(11):2692-9.

(26) Kisseberth WC, Brettingen NT, Lohse JK, Sandgren EP. Ubiquitous expression of marker transgenes in mice and rats. Dev Biol. 1999;214(1):128-38.

(27) Levy E, Menard D, Suc I, Delvin E, Marcil V, Brissette L, et al. Ontogeny, immunolocalisation, distribution and function of SR-BI in the human intestine. J Cell Sci. 2004;117(Pt 2):327-37.

(28) Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.

(29) Levy E, Spahis S, Garofalo C, Marcil V, Montoudis A, Sinnet D, et al. Sar1b transgenic male mice are more susceptible to high-fat diet-induced obesity, insulin insensitivity and intestinal chylomicron overproduction. The Journal of nutritional biochemistry. 2014;25(5):540-8.

(30) Fromme JC, Ravazzola M, Hamamoto S, Al-Balwi M, Eyaid W, Boyadjiev SA, et al. The genetic basis of a craniofacial disease provides insight into COPII coat assembly. Developmental cell. 2007;13(5):623-34.

(31) Nori A, Bortoloso E, Frasson F, Valle G, Volpe P. Vesicle budding from endoplasmic reticulum is involved in calsequestrin routing to sarcoplasmic reticulum of skeletal muscles. The Biochemical journal. 2004;379(Pt 2):505-12.

(32) Zhuang X, Chowdhury S, Northup JK, Ray K. Sar1-dependent trafficking of the human calcium receptor to the cell surface. Biochemical and biophysical research communications. 2010;396(4):874-80.

(33) Yamasaki A, Tani K, Yamamoto A, Kitamura N, Komada M. The Ca2+-binding protein ALG-2 is recruited to endoplasmic reticulum exit sites by Sec31A and stabilizes the localization of Sec31A. Molecular biology of the cell. 2006;17(11):4876-87.

(34) Guo A, Cala SE, Song LS. Calsequestrin accumulation in rough endoplasmic reticulum promotes perinuclear Ca2+ release. The Journal of biological chemistry. 2012;287(20):16670-80.

(35) Higazi DR, Fearnley CJ, Drawnel FM, Talasila A, Corps EM, Ritter O, et al. Endothelin-1-stimulated InsP3-induced Ca2+ release is a nexus for hypertrophic signaling in cardiac myocytes. Molecular cell. 2009;33(4):472-82.

(36) Wong DM, Webb JP, Malinowski PM, Xu E, Macri J, Adeli K. Proteomic profiling of intestinal prechylomicron transport vesicle (PCTV)-associated proteins in an animal model of insulin resistance (94 char). J Proteomics. 2010;73(7):1291-305.

(37) Matsuoka K, Morimitsu Y, Uchida K, Schekman R. Coat assembly directs v-SNARE concentration into synthetic COPII vesicles. Molecular cell. 1998;2(5):703-8.

(38) Ridsdale A, Denis M, Gougeon PY, Ngsee JK, Presley JF, Zha X. Cholesterol is required for efficient endoplasmic reticulum-to-Golgi transport of secretory membrane proteins. Molecular biology of the cell. 2006;17(4):1593-605.

(39) Runz H, Miura K, Weiss M, Pepperkok R. Sterols regulate ER-export dynamics of secretory cargo protein ts-O45-G. The EMBO journal. 2006;25(13):2953-65.

(40) Ma X, Robin C, Ottersbach K, Dzierzak E. The Ly-6A (Sca-1) GFP transgene is expressed in all adult mouse hematopoietic stem cells. Stem Cells. 2002;20(6):514-21.

(41) Nyabi O, Naessens M, Haigh K, Gembarska A, Goossens S, Maetens M, et al. Efficient mouse transgenesis using Gateway-compatible ROSA26 locus targeting vectors and F1 hybrid ES cells. Nucleic acids research. 2009;37(7):e55.

(42) Wortge S, Eshkind L, Cabezas-Wallscheid N, Lakaye B, Kim J, Heck R, et al. Tetracycline-controlled transgene activation using the ROSA26-iM2-GFP knock-in mouse strain permits GFP monitoring of DOX-regulated transgene-expression. BMC Dev Biol. 2010;10:95.

(43) Siddiqi SA, Mahan J, Siddiqi S, Gorelick FS, Mansbach CM, 2nd. Vesicle-associated membrane protein 7 is expressed in intestinal ER. J Cell Sci. 2006;119(Pt 5):943-50.

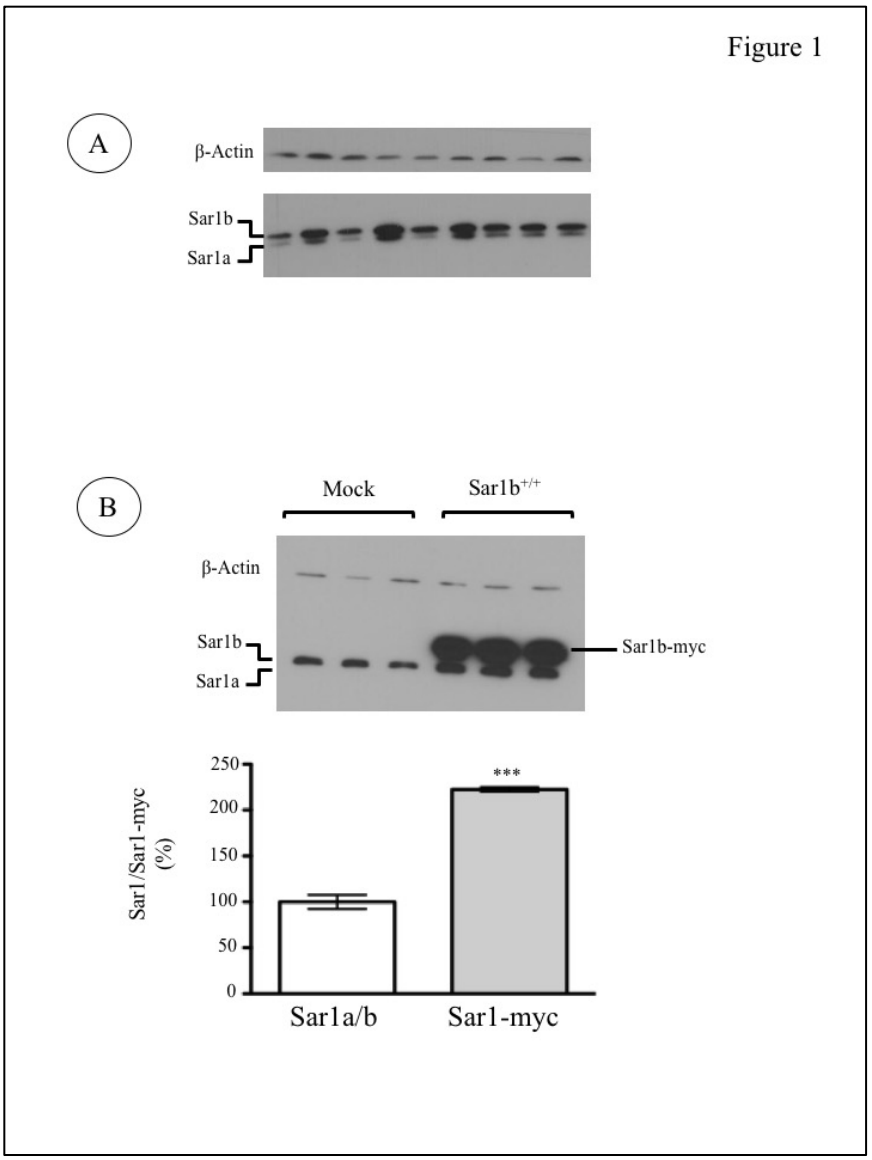

Figure 1. **Protein expression Sar1 in Caco-2/15 cells.**

(**A**) Proteins from cell homogenates were extracted and protein expression was measured by Western blot as described in the Material and Methods section. A representative blot is shown. (**B**) After transfection with GFP and Sar1b constructs, Caco-2/15 cells were allowed to differentiate for 14 days before being tested for Sar1b protein expression by Western blot using anti-Sar1 and anti-myc antibodies. Results represent the means ± SEM of 3 experiments and were calculated as densitometric ratios of Sar1b/a to Sar1b-myc and are illustrated as % of Sar1a/b. A representative blot is shown. ***P<0.0001 vs. SAR1A/B.

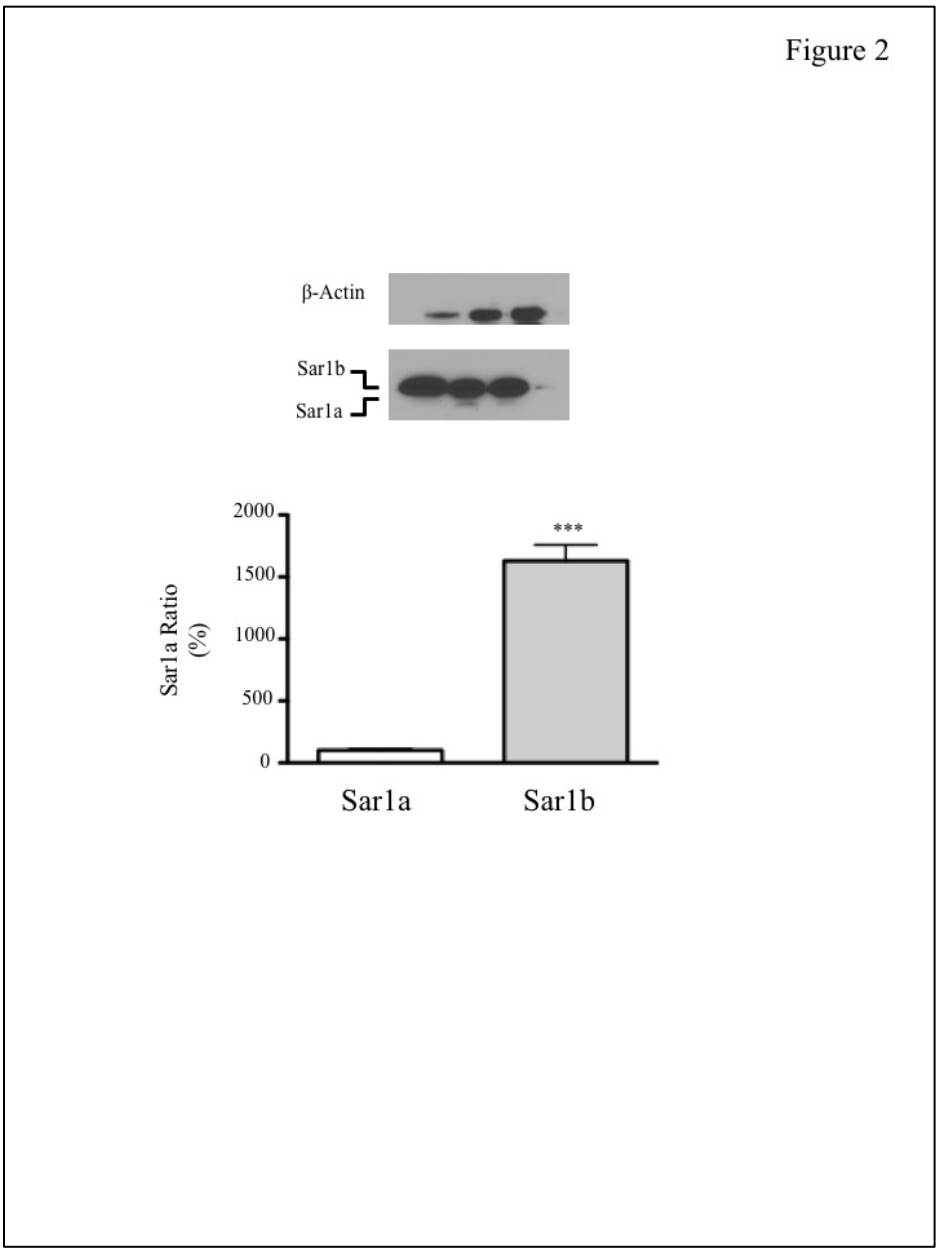

Figure 2. **Protein expression Sar1a and Sar1b in transgenic mice.**

(**A**) Proteins from liver were extracted and protein expression was measured by Western blot as described in the Material and Methods section. A representative blot is shown. Results represent the means ± SEM of 3 experiments. Results were calculated as densitometric ratios of Sar1a to Sar1b and are illustrated as % of Sar1a. A representative blot is shown. ***P<0.0003 vs. SAR1A.

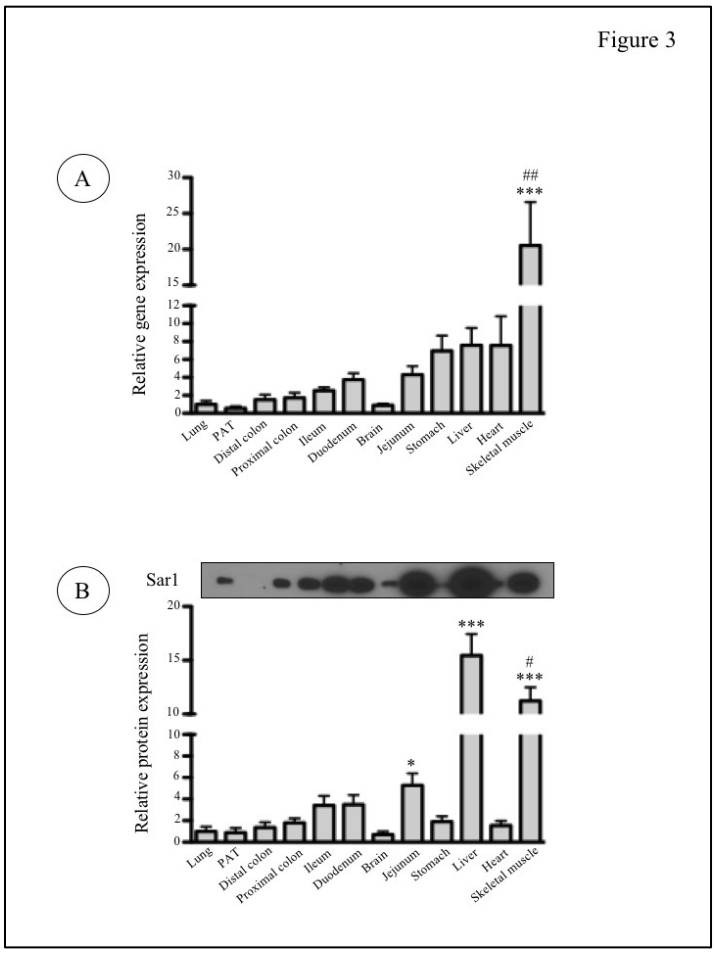

Figure 3. **Differential gene and protein expression levels of Sar1b in organs in transgenic mice.**

RNA (A) and protein (B) from tissues were extracted as described in the Material and Methods section. Gene and protein expression were measured by RT-qPCR and Western blot, respectively. The mean relative expression was calculated relative to lung. Results represent the means ± SEM of 24 (gene) and 12 (proteins) animals. One-way ANOVA were performed ($P<0.0001$) followed by Tuckey's multiple comparison tests. A. ***$P<0.001$ vs. lung, PAT, distal colon, proximal colon, ileum, duodenum, brain and jejunum; $^{\#\#}P<0.01$ vs. stomach, liver and heart. B. *$P<0.05$ vs. lung, PAT and brain; ***$P<0.001$ vs. lung, PAT, distal colon, proximal colon, ileum, duodenum, brain, jejunum, stomach and heart; $^{\#}P<0.05$ vs. liver. PAT: perirenal adipose tissue. A representative blot is shown.

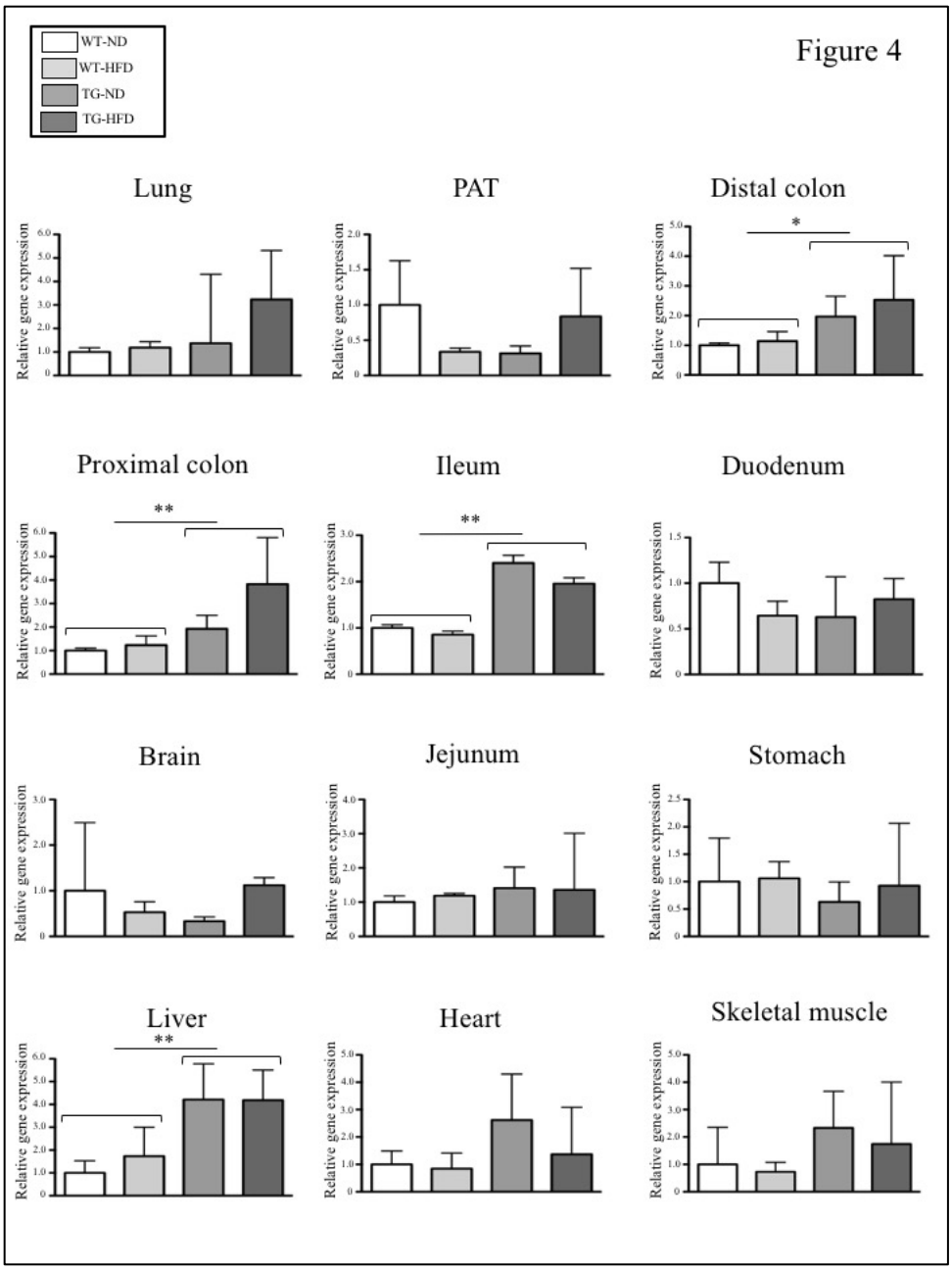

Figure 4. **Effect of diets and transgenesis on the differential gene expression levels of *Sar1b* in organs in mice.**

Wild-type and transgenic Sar1b mice were fed a standard laboratory chow or high-fat diet for a 12-week period. RNA from tissues was extracted as described in the Material and Methods section. Gene expression was measured by RT-qPCR and mean relative expression was calculated for each

organ to compare gene expression relative to wild-type mice fed a normal diet. Results represent the means ± SEM of 6 animals per group. T-tests were performed. WT-ND: wild-type mice, normal diet; WT-HFD: Wild-type mice, high-fat diet; TG-ND: Sar1b+/+ transgenic mice, normal diet; TG-HFD: Sar1b+/+ transgenic mice, high-fat diet; PAT: perirenal adipose tissue. *$P<0.05$; **$P<0.01$ vs. wild-type mice; $^{\#}P<0.05$ vs. wild-type/normal diet mice.

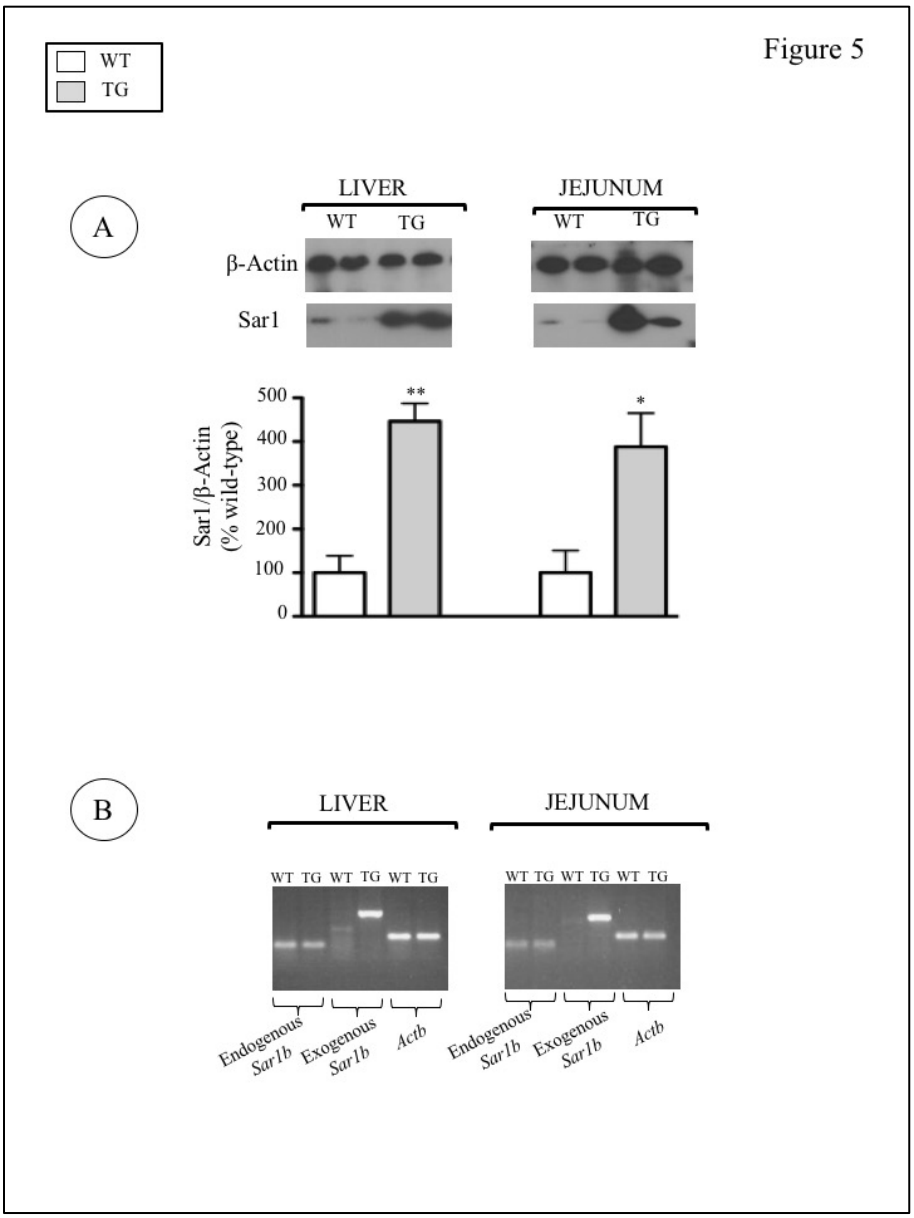

Figure 5. **Effect of transgenesis on Sar1b protein and gene expression in liver and jejunum.** Differential levels of Sar1b protein (A) and mRNA (B) expression in the liver and the jejunum of wild-type (WT) and transgenic (TG) mice was assessed by Western Blot and by RT-PCR as described in the Material and Methods. **A.** Results represent the means ± SEM of 3 animals per group. Results were calculated as densitometric ratios of Sar1b to β-actin and are illustrated as % of WT mice. **P<0.004; *P<0.022 vs. WT mice.

Article 3

«Uncovering the hepatic metabolic signature of non-alcoholic fatty liver disease via direct investigation and proteomic analysis of morbid obese patient»

Schohraya Spahis, Eric Bonneil, André Tchernof, Alain Sane, Carole Garofalo, Edgard Delvin, Emile Levy. *J Clin Invest* 2018 (To be submitted).

CONTRIBUTION DES AUTEURS

Spahis S a été impliquée dans la conception et la planification de l'étude; l'acquisition des données, leur analyse et leur interprétation. SS a conçu et configuré les Figures. Elle a contribué à la rédaction, soumission et révision du manuscrit sous la supervision du Directeur de recherche, Dr Levy E.

Bonneil E a contribué à l'analyse et à l'interprétation des données de la protéomique.

Tchernof A a participé à la collecte et la gestion de la banque des tissues.

Sané A et Garofalo C ont effectué les expériences sur les tissus.

Delvin E a révisé le manuscrit.

Levy E a contribué au concept et à la supervision de l'étude, aux ressources financières et matérielles pour la réalisation de l'étude. Dr Levy E a participé à la rédaction, l'analyse critique des résultats et la révision du manuscrit.

Uncovering the hepatic metabolic signature of non-alcoholic fatty liver disease via direct investigation and proteomic analysis of morbid obese patient liver

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Running Head: Hepatic metabolic pathways in NAFLD

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ABSTRACT

Background & Objective: Non-alcoholic fatty liver disease (NAFLD) is of growing interest in view of its alarming prevalence, severe complications, lack of effective medication and poor long-term prognosis. As its pathophysiology is incompletely understood, this study was designed to evaluate NAFLD intrinsic mechanisms.

Approaches and Results: Liver specimens from 35 morbidly obese patients undergoing biliopancreatic diversion were assigned to 3 groups matched for age, weight and body mass index (BMI), according to hepatic transaminases and steatosis degree. Insulin resistance (IR) and dyslipidemia were associated with high liver triglyceride and sphingomyelin content and with low phosphatidylcholine mass in patients with moderate (Mo-NAFLD) and severe (Se-NAFLD) steatosis compared to subjects with minimal steatosis (Mi-NAFLD). The liver of the Se-NAFLD group displayed disturbed fatty acid composition, characterized by a lower proportion of polyunsaturated fatty acids derived from n-3 and n-6 families, leading to decreased n-3/n-6 ratio and omega 3 index (18:1n-9/18:0 & 16:00/18:2n-6) and to enhanced lipogenesis indices. Liver tissue from Se-NAFLD patients was endowed with oxidative stress and inflammation, while exhibiting biomarker upregulation of lipogenesis (pACC, FAS, FABP4) and gluconeogenesis (GLUT2, PEPCK), as well as downregulation of the key proteins of β-oxidation (CPT-1, ACADL). Accordingly, changes were noted in powerful regulatory transcription factors (SREBP-1c, PPARs) and proteomics profiling, as reflected by alterations in proteins related to fatty acid transport and metabolism, as well as protein-protein interactions. ***Conclusions***: The present work is the first to show that NAFLD coexists only in patients who are morbidly obese and possess features of metabolic syndrome, thereby referring to metabolically abnormal obese patients. Then, it emphasizes the liver abnormalities in lipid homeostasis, IR, dyslipidemia, pro-oxidant/antioxidant balance, inflammatory pathways, and proteomics profiling in association with the degree of steatosis.

Keywords: Obesity, Liver; Steatosis; Lipogenesis; Gluconeogenesis; Insulin resistance; inflammation; oxidative stress; proteomic profile, fatty acid composition

ABBREVIATION LIST

ACADL	Acyl-Coa dehydrogenase long chain
ACC	Acetyl-CoA Carboxylase
BMI	Body mass index
CD36	Cluster of differentiation 36
CPT1	Carnitine palmitoyltransferase-1
FA	Fatty acids
FABP	Fatty acid-binding protein
FAS	Fatty acid synthase
FATP	Fatty acid transport protein
FC	Free cholesterol
FXR	Farnesoid X receptor
G6P	Glucose-6-phosphatase
GLUT	Glucose transporter
H_2O_2	Hydrogen peroxide
HDL-C	High-density lipoprotein cholesterol
iNOS	Inducible nitric oxide synthase
LDL-C	Low-density lipoprotein cholesterol
LXR	Liver X receptor
NAFLD	Non-alcoholic fatty liver disease
NASH	Steatohepatitis
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEPCK	Phosphoenolpyruvate carboxykinase
PGE2	Prostaglandin E2
PI	Phosphatidylinositol
PL	Phospholipids
PPAR	Peroxisome proliferator-activated receptor
PS	phosphatidylserine
SP	Sphingomyelin
SREBP1-c	Sterol regulatory element binding transcription factor 1-c

T2D	Type 2 diabetes
TC	Total cholesterol
TG	Triglyceride
TNFa	Tumor necrosis factor-alpha

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is now considered as the most common non-infectious chronic liver disease in concert with the global epidemic of obesity, metabolic syndrome (MetS), type 2 diabetes (T2D) and other cardiometabolic disorders (1-3). It is characterized by increased lipid hepatic accumulation and the presence of ≥ 5% steatosis appears as a consequence of sedentary life-style, over-feeding and genetic predisposition (4). However, the specific mechanisms of its development and progression into steatohepatitis (NASH), fibrosis and hepatocellular carcinoma remain incompletely understood (5, 6). In view of several associative or epidemiological investigations in humans, and mostly various animal studies, many groups ascribed NAFLD to the occurrence of insulin resistance (IR), dyslipidemia, oxidative stress (OxS), inflammation, and apoptosis (7, 8), but the specific pathological mechanisms are unclear to date.

Although obesity and MetS constitute the preponderant risk factors in the development of NAFLD, the natural history of NAFLD is not well established (9, 10). The abundant literature highlights the striking inter-individual divergences in NAFLD (11) and its complications (12, 13). Furthermore, comorbidities are frequent due to silent NAFLD progression and diagnostic difficulties given the invasiveness of liver biopsy and limitations in non-invasive tools . On top of that, there is no approved drug for the treatment of NAFLD/NASH except lifestyle changes (14). Obviously, the underlying mechanisms of NAFLD should be completely elucidated in order to discover drug targets and surrogate measures and develop novel and effective strategies for treatment.

Recently, we took advantage of bariatric surgery to obtain intact fresh human intestinal specimens to reveal that obese subjects display poor intestinal insulin sensitivity in association with local OxS, inflammation and exaggerated chylomicron formation (15, 16). In the present work, the same experimental approach has been employed to investigate the biochemical manifestations and pathophysiological mechanisms in the liver of obese patients with or without steatosis. We particularly focused on several important questions: (1) Are there significant variations in the liver status of morbidly obese subjects in a given French Canadian cohort? (2) What is the respective hepatic lipid profile of patients with NAFLD versus those without NAFLD? (3) Are patients with higher hepatic steatosis characterized by more severe OxS and inflammation as mechanisms of

action? (4) Is NAFLD characterized by abnormal behaviors of lipogenic and gluconeogenic pathways thereby affecting mitochondrial function? (5) Do central transcription factors play a role in metabolic liver disorders? and (6) Can proteomic analysis highlight a cardiometabolic imprint on NAFLD pathogenesis?

MATERIALS AND METHODS

Subjects and tissue sampling

Thirty-five patients (14 women and 21 men) undergoing biliopancreatic diversion were assigned to 3 groups matched for age, weight and body mass index (BMI), according to hepatic transaminases and steatosis. All the study participants were patients of the Quebec Heart and Lung Institute (IUCPQ) (Quebec City, Canada) and had a BMI $\geq$ 50 kg/m². After the assessment of liver enzymes and steatosis, the patients were subdivided into very minimal (Mi-NAFLD), moderate (Mo-NAFLD) and severe (Se-NAFLD) fatty liver groups, respectively. Exclusion criteria for the study were: i) patients with T2D mellitus, severe peripheral vascular diseases or acute infections; and ii) patients with viral hepatitis, alcoholic hepatitis, drug-induced hepatitis or autoimmune hepatitis. None of these subjects received a therapy with estrogen, hypoglycaemic agents and cholesterol- or triglyceride (TG)-lowering agents. Liver specimens were obtained from the Biobank of the IUCPQ-Université Laval in compliance with Institutional Review Board-approved management modalities. All participants provided written, informed consent. The hepatic samples were immediately transferred to the pathology department or frozen in liquid nitrogen and stored at –80 °C for subsequent analyses. The project was approved by the ethics committees of IUCPQ and Sainte-Justine Hospital Research Center (Montreal, Canada).

Histological evaluation and diagnosis

All liver samples were read by an expert pathologist to define and semi-quantitatively score the NAFLD phenotype, including the degree of steatosis. Tissue slides were prepared and stained with the hematoxylin and eosin staining method. Steatosis was defined by a simple 4 scale grading (from 0 to 3). The pathologist considered macro- and/or medio-vesicular steatosis and assessed the percentage of hepatocytes with steatotic vacuoles. The normal liver (grade 0) contained fat in less than 5% of hepatocytes while grade 1 steatosis referred to less than 33% of steatotic hepatocytes. In grades 2 and 3 steatosis, fat was present in at least 33% and 66% of hepatocytes, respectively (17).

Plasma separation

Samples of fasting venous blood were collected from the patients in tubes containing 1 g EDTA/L, in the early morning before bariatric surgery. Plasma was separated immediately by centrifugation (700 x *g* for 20 min) at 4 °C as described previously (18-20).

Biochemical analyses

Biochemical assessments were carried out as described previously (21). Glucose was assessed using the glucose oxidase method and insulin was quantified with the ultrasensitive insulin assay on the Access® immunoassay system (Beckman Coulter, Brea, USA). The HOMA-IR index was calculated using the following formula: fasting insulin (μUI/mL) x fasting glucose (mmol/L)/22.5. Total cholesterol (TC), free cholesterol (FC), high-density lipoprotein cholesterol (HDL-C) and TG levels were estimated in the plasma by colorimetric enzymatic kits as described previously (18, 19). Low-density lipoprotein cholesterol (LDL-C) was calculated by the Friedewald formula (22). Phospholipid classes were separated on TLC plates, and bands corresponding to phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and sphingomyelin (SP) were scraped off the plates, mixed with scintillation fluid and counted for the amount of radioactivity incorporated.

Fatty acid composition

Fatty acids (FAs) in plasma and liver were assayed by an improved method described previously (18-20). Briefly, each sample to be analyzed was subjected to direct trans-esterification and then injected into a gas chromatograph (GC) using a standard Agilent 7890 GC. The FAs were identified by comparison with the expected retention times of known standards and then analyzed.

Oxidative stress and Inflammatory markers

Prostaglandin E2 (PGE2) was measured by enzyme-linked immunosorbent assay (Arbor Assay, Michigan, USA), while inducible nitric oxide synthase (iNOS) and tumor necrosis factor-alpha (TNFα) were tested by Elisa kits (LifeSpan Biosciences, Inc.). H_2O_2 was measured by using the Hydrogen Peroxide Assay Kit (Colorimetric/Fluorometric, Abcam, USA).

Liver protein expression

Liver samples were homogenized with a polytron in the lysis buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.47 mM KH2PO4 (pH 7.4), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 2 mg/ml pepstatine). Homogenates were agitated at 4°C for 1h in presence of 1% Triton and then centrifuged at 13,000 rpm for 10 min. The Bradford assay (Bio-Rad Laboratories, Mississauga, Canada) was used to determine protein concentration. Homogenates were prepared for Western blotting as described previously (23, 24). Same protein amounts (30 μg) were loaded on the SDS-PAGE and the β-actin protein was used as an internal control. The following human antibodies were employed: β-actin (1:1000), phospho-Acetyl-CoA

Carboxylase (ACC) Ser79 (1/1000), fatty acid synthase (FAS, 1/1000), ACC (1/1000), carnitine palmitoyltransferase-1 (CPT1, 1000), Peroxisome proliferator-activated receptors (PPAR, 1/1000) from Cell Signaling Technology, USA; sterol regulatory element binding transcription factor-1-c (SREBP1-c, 1/500), Fatty acid-binding protein 4 (FABP4, 1/750), farnesoid X receptor (FXR, 1/100), glucose-6-phosphatase (G6P, 1/500), liver X receptor (LXR, 1/500) from Abcam, USA; cluster of differentiation 36 (CD36, 1/1000) and phosphoenolpyruvate carboxykinase (PEPCK, 1/1000) from Santa Cruz Inc. USA; FA transport protein (FATP5, 1/500) from Novus, USA; glucose transporter (GLUT2, 1/500) and acyl-CoA dehydrogenase long chain (ACADL, 1/1000) from Thermofisher Scientific, USA.

Sample preparation for proteomics

Protein concentration from liver samples was determined by the Bradford method using the Biorad protein assay dye reagent (USA), and samples were sent to the Institute for Research in Immunology and Cancer proteomic platform (Université de Montréal, Canada) for proteomic analysis.

Mass spectrometry analysis and protein quantitation

Mass spectrometry analysis was carried out on 4 µg of purified liver from chosen miNAFLD and seNAFLD groups. Sample reduction was performed by adding 50 µl of 5mM TCEP in 100 mM ammonium bicarbonate to the dry sample. Alkylation was performed by adding 50 µl of chloroacetamide 20 mM with ammonium bicarbonate 100 mM. 1 µg of trypsin was added and the digestion was performed for 8 hours at 37°C. Samples were loaded and separated on a home-made C_{18} analytical column (15cm * 150µm i.d.) with a 116-min gradient from 0–30% acetonitrile (0.2% FA) and a 600 Nl/minute flow rate on an Easy nLC (Dionex) connected to a Q-Exactive HF (Thermo Fisher Scientific). Each full MS spectrum acquired with a 60,000-resolution was followed by up to 20 MS/MS spectra, where the 20 most abundant multiply charged ions were selected for MS/MS sequencing. Peptides were identified using Peaks 8.0 (Bioinformatics Solution Inc.) and peptide sequences were blasted against the Human Uniprot database. Tolerance was set at 10 ppm for precursor and 0.01 Da for-fragment ions for protein identification and 7 ppm and 2 min for peptide alignment and profiling. For the post-translational modification of proteins, occurrence of carbamidomethylation, oxidation and deamidation was considered. Volcano plots were made with Perseus 1.5.0.30.

Statistical Analysis

All values are expressed as mean ± SEM. Data were analyzed using a one-way repeated measures ANOVA and the differences between the means were assessed post-hoc using Tukey's test. Statistical significance was defined as $P < 0.05$.

RESULTS

A total of 35 morbidly obese patients were included in the study: 14 females (40%) and 21 males (60%) (**Table 1**). No significant differences were noted in their weight, age, height, BMI, waist and hip measurements. Three groups were clearly apparent on the basis of transaminases and the magnitude of steatosis observed in their liver histology: Mi-NAFL, Mo-NAFLD and Se-NAFLD (**Table 1**). The Se-NAFLD group was characterized by higher levels of alanine transaminase (ALT, 55%), aspartate aminotransferase (AST, 50%), gamma-glutamyl transpeptidase (GGT, 43%) and steatosis (43%) compared to Mo-NAFLD. From its part, the Mo-NAFLD exhibited an elevation of ALT (49.3%), AST (29.0%), GGT (47.1%) and steatosis score (47.9%) compared to Mi-NAFLD.

As excess lipid accumulation is an early signature of NAFLD, we evaluated the TG, TC and phospholipid (PL) classes in the liver of the obese patients. An evident accretion of TG was noted to follow NAFLD severity (**Table 2**). The hepatic TG content in Se-NAFLD exceeded that of Mo-NAFLD by 33.5% whereas TG values in the liver of Mo-NAFLD went beyond those of Mi-NAFLD by 33.3%. But at the same time, the concentrations of PL followed an opposite pattern, showing an obvious decline especially in patients with severe NAFLD. Unlike TG and PL trends, no significant variation was observed in TC in the three groups.

Since changes of certain classes of PLs influence energy metabolism and are linked to general disease progression (25), the characterization of PL distribution in the liver was carried out (**Table 2**). The Se-NAFLD group displayed a significant decrease in PC and an increase in SP. Furthermore, the ratio of PC/PE was calculated and found markedly diminished. Overall, our findings underline the association of hepatic TG enrichment and PL impoverishment with steatosis progression.

To determine whether the groups with liver steatosis had some components of the MetS, we evaluated glycaemia, insulinemia, HOMA-IR index, dyslipidemia and hypertension. Based on the values of plasma glucose and glycated hemoglobin in fasting conditions, there was no indication for diabetes in NAFLD individuals (**Table 3**). However, an increase was evidenced in fasting insulin levels and HOMA-IR index in Se-NAFLD patients, as well as higher TG levels and lower HDL-C concentrations, as compared with Mo-NAFLD subjects. LDL-C/HDL-C ratio was significantly different between the Mi-NAFLD and Se-NAFLD, suggesting an increased risk of

cardiovascular disease in the presence of fatty liver pathogenesis. On the other hand, no significant differences were observed in plasma TC, FC and cholesterol ester between the groups (**Table 3**). The presence of OxS in the liver of NAFLD patients was significantly marked by increased H_2O_2, a biomarker of lipid peroxidation in both Mo-NAFLD and Se-NAFLD groups compared to Mi-NAFLD subjects (**Table 4**). Similarly, knowing that IR in NAFLD is associated to inflammation, we determined the status of inflammatory markers in the liver of our cohort. A rise in the concentrations of PGE_2, iNOS and TNFα was found in the liver tissues of insulin-resistant NAFLD subjects, notably Mo-NAFLD and Se-NAFLD (**Table 4**).

Fatty acid composition

As hepatic FA profile may be related to fatty liver pathogenesis, hepatic FA composition was analyzed (**Table 5**). The FA profile in the liver of Se-NAFLD patients was characterized by a lower percentage of polyunsaturated fatty acids (PUFA). More specifically, the low proportions of eicosapentaenoic, docosapentaenoic, docosahexanoic, homogamma linolenic and arachidonic FAs contributed to the decline of n-3 and n-6 PUFA families. Furthermore, the raised n-6/n-3 ratio indicates a relatively greater lowering of n-3 PUFA. Additional particularities of the FA profile are the low percentages of various FAs belonging to monounsaturated fatty acids (MUFA) as a function of steatosis severity. Interestingly, although we expected an increase in saturated FA (SFA), the opposing trends in palmitic and stearic FAs offset our anticipation.

Considering the FA data provided in absolute concentrations, we noted an increase in SFA, MUFA, PUFA and n-7 FA along with invariable n-3 PUFA families according to the gradation of NAFLD severity. As expected from the alterations of these FA profiles in Se-NAFLD, there was an increase in the ratios of 16:1n-7/18:2n-6 and 20:3n-9/20:4n-6, total n-7/18:2n-6 as well as in 16:00/18:2n-6 and Stearoyl-CoA desaturase 1 (SCD1/Δ9), two relevant indices of liver lipogenesis (**Table 6**), which collectively indicate an exaggerated FA accumulation and an essential fatty acid deficiency (26). In concert, the ratios PUFA/SFA and EFA/NON-EFA exhibit a trend of decrease in proportion to NAFLD severity. The changes in desaturation may be due to low Δ5 and high Δ9 desaturases. Moreover, Mo-NAFLD and Se-NAFLD groups presented a low omega-3 index, suggesting a higher risk for cardiometabolic disorders.

To approach the pathological mechanisms involved in NAFLD-associated IR, we analyzed the expression of key proteins involved in hepatic FA uptake and transport, lipogenesis,

gluconeogenesis and mitochondrial FA β-oxidation in the two extreme groups: Mi-NAFLD and Se-NAFLD.

Hepatic fatty acid uptake and metabolism

Whereas no significant changes were noted in CD36 and FATP5 mediating the transmembrane uptake and transport of long chain FA, a marked raised expression level characterized FABP4, a lipid chaperon facilitating the transport of lipids to intracellular compartments, in Se-NAFLD compared to Mi-NAFLD subjects (**Figure 1A**). As transcription factors regulate the expression and activity of lipid transporters, we focused on their protein expression and detected an induction in SREBP-1c and PPARγ, a down-regulation of LXRα, and no variations in FXR in the liver specimens of Se-NAFLD relative to Mi-NAFLD subjects (**Figure 1B**).

Hepatic lipogenesis

We first appraised the protein expression of ACC, a key protein providing the malonyl CoA substrate for fatty acyl chain elongation and FA biosynthesis with the involvement of FAS. The total protein expression of ACC was not altered in liver specimens of Se-NAFLD subjects. However, using a phosphoserine antibody, a significantly reduced Ser79 phosphorylation of ACC was evidenced, which is indicative of its activation in Se-NAFLD subjects compared to Mo-NAFLD (**Figure 2**). Accordingly, the calculated ratio of phosphorylated ACC to total ACC was significantly reduced in liver samples of Se-NAFLD subjects, confirming ACC activation. Moreover, FAS protein expression was significantly upregulated, substantiating the stimulated lipogenesis process contributing to lipid accumulation in Se-NAFLD compared to Mi-NAFLD, and fully consistent with SREBP1c upregulation (**Figure 2**).

Mitochondrial FA β-oxidation

Evaluation of the main regulatory proteins of the β-oxidation pathway, one of the most important functions of the mitochondria, was carried out. As illustrated in Figure 3, the expression of CPT-1 and ACADL were significantly reduced in Se-NAFLD subjects. The same trend characterized PPARα, known to stimulate FA β-oxidation (**Figure 3**).

Hepatic gluconeogenesis

As increased hepatic gluconeogenesis may represent a cause for NAFLD development, we assessed this hypothesis via the protein expression analysis of G6P, PEPCK and GLUT2 in liver specimens. The protein mass of GLUT2 was significantly higher in Se-NAFLD than in Mi-NAFLD, whereas PEPCK and G6P remained unchanged (**Figure 4**).

Liver proteome analysis

From our morbidly obese NAFLD cohort, we selected five individuals from Mi-NAFLD and Se-NAFLD groups to undertake a preliminary pilot liver proteome evaluation. A total of 1737 proteins were identified using trypsin digestion followed by LC-MS/MS analysis and subsequent quantification after normalization with the median. The major liver proteins identified in both groups originated from the cytoplasm (35%), followed by the nucleus (26%), mitochondria (19%) and endoplasmic reticulum (10%), as well as other cell localizations (ribosome 4%, cytoskeleton 3%, lysosomes 2% and Golgi 1%) (**Figure 5**). However, we noted slight differences in the protein distribution in accordance with homologous intracellular site between the Mi- and Se-NAFLD subjects (**Figure 5**). Moreover, the majority of the identified proteins in the whole group refer to binding (33%), catalytic activity (47%), structural molecule activity (9%) and transport (5%) (**Figure 6**), as well as other processes [translation regulator activity (2%), receptor activity (1%), signal transducer activity (1%) and antioxidant activity (2%)] (**Figure 6**). The comparison between the two groups, illustrated by the Go Term, also displayed a slight difference in the enrichment of proteins in each compartment (**Figure 7**).

To approach protein quantification for each group and to enhance our knowledge on the impact of the degree of steatosis on the localization and function of the identified proteins, **Figure 8** illustrates the volcano plot that represents the change in magnitude of labelled proteins, and discriminanting Se-NAFLD from the Mi-NAFLD group (**Table 7**). Furthermore, to define the interactions between proteins, we separately analyzed the up- and down-regulated proteins in Se-NAFLD compared to Mi-NAFLD subjects. Interestingly, we detected protein-protein networks, either for downregulated (**Figure 9A**) and upregulated (**Figure 9B**) proteins in Se-NAFLD, which suggest regulatory pathways in severe steatosis.

DISCUSSION

The scientific community is facing a serious challenge from the rapidly increasing prevalence of NAFLD, especially considering that related prevention and treatment are not effective. As well, the molecular mechanisms of this pathogenesis seem complex and poorly understood. Furthermore, most of the studies focusing on the causes of disease development and progression were performed in animal models, which makes it difficult to easily extrapolate to humans. Therefore, efforts need to be multiplied to delineate NAFLD mechanisms in humans, which will allow the discovery of new targets for drugs/functional foods, improve patient management and prevent disease complications. It is in this context that our study was conducted, particularly focusing on human liver given the golden opportunity to obtain biological specimens from obese patients undergoing bariatric surgery.

Obesity represents a significant risk factor for metabolic complications, including NAFLD and MetS. However, there is a category of obese subjects who are better protected from these co-morbidities and their clinical condition has been termed metabolically healthy obesity (MHO) (27, 28). Among the important considerations taken in the design of the present study were the strategic inclusion of the MHO and metabolically unhealthy obese (MUO) phenotypes, and the utilisation of human liver specimens during bariatric surgery of morbidly obese individuals. This important approach has recently allowed us to determine that MUO patients developed intestinal IR, probably due to local OxS and inflammation, which led to chylomicron overproduction (15).

The present work is the first to show that NAFLD coexists only in patients who are obese and exhibit features of the MetS. In fact, the insulin-resistant patients with steatosis displayed obesity, IR and dyslipidemia. Second, their liver tissue revealed a high TG content, which confirms the histologic findings and biochemically raised liver transaminases. However, Se-NAFLD subjects were also characterized by a fall in PL, due essentially to decreased PC, along with increased SP and a low PC/PE ratio. Third, their hepatic FA composition was disturbed, more harshly so in Se-NAFLD patients. Fourth, their liver tissue was endowed with OxS and inflammation. Fifth, their liver samples showed lipogenesis upregulation and β-oxidation down-regulation, as evidenced by p-ACC/FAS and CPT-1/ACADL specific biomarkers, respectively. Sixth, in line with these findings, alterations were noted in powerful transcription factors.

Liver is a target organ in MetS, resulting in NAFLD, to the extent that the latter is considered as a component of MetS (29). With the exception of hypertension, all the other components of the MetS (obesity, IR and dyslipidemia) were present in Se-NAFLD group. In particular, IR, which represents the pivotal mechanism in primary NAFLD, is significantly elevated as reflected by the exaggerated HOMA. It appears reasonable to expect that IR triggers SREBP-1c, a robust transcription factor capable of upregulating genes involved in the *de novo* lipogenesis pathway, such as FAS and ACC, thereby mediating hepatic synthesis of FA and TG (30). However, only FAS protein expression was stimulated whereas ACC protein, the rate-limiting enzyme in *de novo* fatty acid synthesis, did not respond to SREBP-1c. Rather, we find that activation of ACC activity was a result of phosphorylation paucity, probably a consequence of p-AMPK deficiency, as AMPK had been shown to phosphorylate and inactivate ACC (31). This provides a potential way to explain how lipogenesis is governed by two mechanisms, dependent on the SREBP-1c transcription factor and AMPK, respectively. These data are compatible with the high activity of SCD1, a key enzyme for the synthesis of MUFA, e.g. palmitoleic acid and oleic acid. In fact, the elevated SCD1 activity, reflected by FA composition in the livers of Se-NAFLD subjects, could markedly enhance the hepatic rate of TG synthesis through direct regulation of SREBP-1c and PPARγ. Interestingly, our study shows unchanged FATP5 and CD36 protein expression in the livers of Se-NAFLD subjects, whereas mice depleted in FATP5 or CD36 exhibited decreases FA uptake and hepatic steatosis (32, 33).

The liver is endowed with a huge capacity for mitochondrial FA β-oxidation. As hepatic steatosis in NAFLD can result not only from lipogenesis, but also from decreased FA β-oxidation, we assessed two important biomarkers: CPT-1, the mitochondrial gateway for FA entry into the matrix and the main controller of the hepatic mitochondrial β-oxidation flux (34), and ACADL, the enzyme that catalyzes the first step in the mitochondrial β-oxidation of long fatty acyl-CoA esters (35). The two degradative enzymes were down-regulated in the liver Se-NAFLD patients. Since PPARα is expressed primarily in tissues rich in mitochondria, such as the liver, and is an important factor regulating the FA β-oxidation (36), we determined its protein expression. Western blot analysis showed a drop of PPARα mass. It is possible that the decline in PPARα affects the link to functional PPARγ response element located on the CPT1 promoter (37) since it has also reported that PPAR may directly regulates CPT1 (36). Other potential mechanisms that may affect the

degradation of FAs in the liver of Se-NAFLD patients are hypersinsulinemia/IR (38) and the low regulation of ACC by phosphorylation. The latter leads to ACC activation and consequently to higher production of malonyl-CoA that inhibits CPT-1, supporting the notion of mitochondrial β-oxidation.

In physiological conditions, once the liver is saturated with glycogen, any additional glucose taken up by hepatocytes is moved to pathways leading to synthesis of FA and exported from the liver as lipoproteins. In this normal state, insulin lowers GLUT2, a facilitative glucose transporter in the liver, and decreases hepatic gluconeogenesis by suppressing expression of the key gluconeogenic enzymes PEPCK and G6P via the activation of PI-3-kinase (39). As a metabolic profile combining hepatic lipid accumulation and activation of hepatic gluconeogenesis characterises insulin resistance (40), we assessed various key enzymes typical of these pathways. Our results show that GLUT2 protein level was upregulated, but in combination with unchanged PEPCK and G6P expression. Additional studies are needed to unravel the foundations of these observations.

Analysis of PL composition disclosed a drop of PC and PC/PE ratio in the liver of Se-NAFLD patients, which may contribute to the initiation and progression of hepatic steatosis. As PC was reported to perform a regulatory function in very low-density lipoprotein assembly in experimental models (41, 42), its impoverishment may hamper the physiological regulation of hepatic lipid export and result in hepato-steatosis. In this context, the mTOR complex 1 was found critical to TG output in mice by upregulating phosphocholine cytidylyltransferase α, the rate-limiting enzyme involved in the synthesis of PC (43). Additional efforts are needed to determine whether mTOR complex 1 may be included among the etiological mechanisms of NAFLD, especially in humans in which the hepatic PL profile has been little explored (44). Similarly, further studies are required to assess the PE *N*-methyltransferase pathway responsible for the production of PC from PE (45), particularly as the ratio of PC/PE influences membrane integrity and NASH in mice (46). Alterations in FA composition were documented in the present work. Nevertheless, our findings cannot be attributed to the direct influence of dietary macronutrients and total calorie intake, given conflicting reports in available literature (47). This aspect remains to be fully elucidated in view of the suggestions that obese patients with NAFLD consume diets high in total fat/SFAs or high in carbohydrates/sugars (48, 49). At this point, we can only state that a decline in n-3 and n-6

families was associated with fat accumulation in the liver of Se-NAFLD patients in our studies and a direct demonstration in humans has yet to be undertaken.

Proteomic profiling and validation

Among the various proteins that showed differences, we undertook a validation process. For example, we selected FABP4 which is an active protein in trafficking of unesterified FA within adipocytes and macrophages (50). Additional functions have been attributed to FABP4 such as lipid metabolism, delivery of lipids to nuclear receptors mediating nuclear transcriptional programs and inflammation (51). A few investigators reported the presence of FABP4 in the liver and particularly in Kupffer cells and suggested its association with NAFLD pathogenesis (52). Our experiments clearly indicated the marked overexpression of FABP4 in the liver of Se-NAFLD patients, in agreement with confirmed previous observations (53). Although speculative, our data may suggest a link between raised FABP4 content and lipid accumulation and inflammation as well as protein-protein interaction in NAFLD given the following available information: (i) FABP4 ablation led to alterations in lipid composition and metabolism, along with resistance to develop chronic inflammation, hyperinsulinemia and lipid metabolism aberrations (54, 55); (ii) the induction of FABP4 in macrophages during differentiation resulted in inflammatory responses (56, 57); and (iii) FABP4 levels are increased in decompensated cirrhosis and correlate with hard clinical outcomes (58). Our results are consistent with a role for FABP4 in NAFLD, but further studies are necessary to investigate uncerlying mechanisms and define whether FABP4 may serve as a surrogate for detection and progression of NAFLD. Finally, additional work is needed to validate various up-and down-regulated proteins.

Table 1: Baseline anthropometric and liver characteristics of NAFLD subjects

Variables	Mi-NAFLD (n=10)	Mo-NAFLD (n=10)	Se-NAFLD (n=15)
Gender	3F/7M	4F/6M	7F/8M
Age (y)	42.70 ± 4.31	42.22 ± 3.20	41.40 ± 2.84
Weight (kg)	153.18 ± 7.47	157.49 ± 10.77	163.85 ± 4.22
Height (cm)	159.02 ± 6.19	172.96 ± 10.57	159.09 ± 4.34
BMI (Kg/m2)	53.16 ± 2.12	54.12 ± 2.83	56.15 ± 2.07
Waist (cm)	143.20 ± 3.64	167.50 ± 12.20	142.90 ± 3.65
Hip (cm)	159.40 ± 5.30	145.63 ± 9.26	150.35 ± 5.39
Waist/Hip	0.90 ± 0.02	1.16 ± 0.07	0.96 ± 0.03
ALT (U/L)	15.60 ± 1.72	30.75 ± 6.07*	47.80 ± 8.60**$^{\#}$
AST (U/L)	15.80 ± 0.86	22.25 ± 2.78*	39.10 ± 5.22*$^{\#}$
ALT/AST	0.99 ± 0.11	1.36 ± 0.20	1.20 ± 0.09
GGT (U/L)	17.80 ± 4.85	33.67 ± 4.91*	48.30 ± 5.91**$^{\#}$
Steatosis (%)	8.10 ± 1.96	15.56 ± 2.42*	63.67 ± 6.79***$^{\#\#\#}$
Steatosis grade	0-1	1-2	≥3

ALT: alanine transaminase; AST: aspartate aminotransferase; BMI: body mass index; GGT: gamma-glutamyl transpeptidase; Mi-NAFLD: minimal non-alcoholic fatty liver disease; Mo-NAFLD: moderate non-alcoholic fatty liver disease; Se-NAFLD: severe non-alcoholic fatty liver disease

All values are expressed as mean ± SEM. Data were analyzed by using Anova Tukey's multiple comparisons. $^{*}P<0.05$, $^{**}P<0.001$, $^{***}P<0.0001$ vs Mi-NAFLD; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.0001$ vs. Mo-NAFLD group.

Table 2: Lipids and phospholipid classes in the liver of NAFLD subjects

Variables	Mi-NAFLD (n=10)	Mo-NAFLD (n=10)	Se-NAFLD (n=15)
Lipids			
TG (mg/g liver)	27.43 ± 5.62	41.15 ± 4.70*	61.89 ± 4.90**#
TC (mg/g liver)	4.45 ± 0.30	4.52 ± 0.43	4.44 ± 0.23
PL (mg/g liver)	175.62 ± 21.32	162.74 ± 22.18	127.37 ± 21.09*
Phospholipids			
Total PL classes (mg/g liver)	28.125 ± 0.47	27.50 ± 2.26	21.90 ± 1.59*
PC (mg/g liver)	12.08 ± 0.52 [43%]	11.91 ± 0.77 [44%]	8.93 ± 0.56 [38%]*#
PE (mg/g liver)	9.14 ± 0.72 [32%]	7.88 ± 0.43 [29%]	7.71 ± 0.22 [33%]
SP (mg/g liver)	1.85 ± 0.16 [7%]	2.06 ± 0.11 [8%]	2.54± 0.45 [11%]*
PS (mg/g liver)	2.46 ± 0.29 [9%]	2.34 ± 0.15 [9%]	2.14 ± 0.28 [9%]
PI (mg/g liver)	2.67 ± 0.18 [9%]	2.71 ± 0.16 [10%]	2.15 ± 0.36 [9%]
PC/PE	1.40 ± 0.15 [35%]	1.40 ± 0.07 [34%]	1.16 ± 0.09 [29%]#

Mi-NAFLD: minimal non-alcoholic fatty liver disease; Mo-NAFLD: moderate non-alcoholic fatty liver disease; Se-NAFLD: severe non-alcoholic fatty liver disease; PL: phospholipids; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; SP: sphingomyelin; TC: total cholesterol; TG: triglyceride.

All values are expressed as mean ± SEM. Data were analyzed by using Anova Tukey's multiple comparisons. *P<0.05, **P<0.001 vs. Mi-NAFLD; #P<0.05 vs. Mo-NAFLD group.

Table 3: Metabolic biomarkers and lipid profile of NAFLD Subjects

Variables	Mi-NAFLD (n=10)	Mo-NAFLD (n=10)	Se-NAFLD (n=15)
Metabolic variables			
Fasting blood Glucose (mmol/L)	5.36 ± 0.17	5.90 ± 0.16	6.35 ± 0.23**
Fasting Insulin (uUI/mL)	12.79 ± 2.29	47.84 ± 6.16**	66.13 ± 9.22***$^{\#}$
HOMA-IR	2.95 ± 0.45	12.50 ± 1.43**	18.78 ± 2.78***$^{\#}$
Glycohemoglobin (%)	6.00 ± 0.00	4.00 ± 0.01	5.00 ± 0.01
SBP (mmHg)	127.60 ± 5.61	142.25 ± 5.30	133.50 ± 3.53
DBP (mmHg)	81.00 ± 3.96	90.50 ± 2.36	80.80 ± 3.35
Lipid profile			
TG (mmol/L)	1.21 ± 0.19	1.60 ± 0.15*	2.21 ± 0.19**$^{\#}$
TC (mmol/L	5.00 ± 0.26	4.88 ± 0.25	5.06 ± 0.39
FC (mmol/L)	1.34 ± 0.10	1.31 ± 0.22	1.28 ± 0.16
CE (mmol/L)	3.27 ± 0.30	2.59 ± 0.24	3.03 ± 0.53
HDL-C (mmol/L)	1.27 ± 0.07	1.14 ± 0.06	1.12 ± 0.06$^{\#}$
LDL-C (mmol/L)	2.83 ± 0.22	2.87 ± 0.25	3.28 ± 0.26
TC/HDL-C	3.99 ± 0.24	4.27 ± 0.10	4.55 ± 0.34
LDL-C/HDL-C	2.31 ± 0.25	2.56 ± 0.22	2.96 ± 0.21*

DBP: diastolic blood pressure; CE: cholesteryl ester; HDL-C: high-density lipoprotein-cholesterol; Mi-NAFLD: minimal non-alcoholic fatty liver disease; Mo-NAFLD: moderate non-alcoholic fatty liver disease; Se-NAFLD: severe non-alcoholic fatty liver disease; LDL-C: low-density lipoprotein-cholesterol; SBP: systolic blood pressure; FC: free cholesterol; TC: total cholesterol; TG: triglyceride.

All values are expressed as mean ± SEM. Data were analyzed by using Anova Tukey's multiple comparisons. $^{*}P<0.05$, $^{**}P<0.001$, $^{***}P<0.0001$ vs. Mi-NAFLD; $^{\#}P<0.05$ vs. Mo-NAFLD group.

Table 4: Oxidative stress and inflammatory biomarkers in the liver of NAFLD subjects

Variables	Mi-NAFLD (n=10)	Mo-NAFLD (n=10)	Se-NAFLD (n=15)
Oxidative stress			
H_2O_2 (umol/g prot)	5.38 ± 0.70	14.63 ± 1.74***	17.30 ± 1.31***
Inflammation			
PGE_2 (pg/mg prot)	4.67 ± 0.54	8.90 ± 1.33*	8.60 ± 1.05*
iNOS (U/mg prot)	0.23 ± 0.03	0.49 ± 0.09*	0.54 ± 0.13*
TNFa (pg/mg prot)	346.17 ± 71.19	1103 ± 161.73**	878.33 ± 136.54*

H_2O_2: Hydrogen peroxide; iNOS: Inducible nitric oxide synthase; Mi-NAFLD: minimal non-alcoholic fatty liver disease; Mo-NAFLD: moderate non-alcoholic fatty liver disease; Se-NAFLD: severe non-alcoholic fatty liver disease; PGE_2: prostaglandin E_2; TNFa: Tumor necrosis factor alpha.

All values are expressed as mean ± SEM. Data were analyzed by using Anova Tukey's multiple comparisons. $^{*}P<0.05$, $^{**}P<0.001$, $^{***}P<0.0001$ vs. Mi-NAFLD group.

Table 5: Liver fatty acids composition in NAFLD subjects

Fatty Acids (FA)	Mi-NAFLD (n=10)	Mo-NAFLD (n=10) (%)	Se-NAFLD (n=15)	Mi-NAFLD (n=10)	Mo-NAFLD (n=10) (µM)	Se-NAFLD (n=15)
SFA						
PA (C16:0)	23.88 ± 0.64	26.72 ± 1.28	30.68 ± 1.50**	220.50 ± 17.09	513.71 ± 92.31*	803.65 ± 134.68**
SA (C18:0)	9.65 ± 0.69	7.41 ± 0.50*	6.32 ± 0.43**#	91.16 ± 5.99	135.39 ± 11.59*	153.86 ± 14.65**
MUFA						
POA (C16 :1n-7)	2.34 ± 0.39	3.05 ± 0.56	3.57 ± 0.57	20.67 ± 5.21	59.97 ± 15.28*	100.38 ± 19.18**
OA (C18:1n9)	26.46 ± 3.16	33.20 ± 0.63	34.05 ± 1.38	212.83 ± 11.97	633.74 ± 95.32*	878.47 ± 116.49**
EIA (C20:1n9)	0.27 ± 0.08	0.25 ± 0.03	0.22 ± 0.03	1.73 ± 0.13	4.72 ± 0.73*	5.49 ± 0.80**
ETA (C20:3n9)	0.10 ± 0.01	0.08 ± 0.01	0.05 ± 0.003**#	0.84 ± 0.05	1.55 ± 0.19*	1.11 ± 0.15
ERA (C22:1n9)	1.35 ± 0.17	0.83 ± 0.07	0.74 ± 0.16*#	13.91 ± 0.07	17.40 ± 2.91	18.02 ± 5.09
NA (C24:1n9)	0.19 ± 0.02	0.09 ± 0.02**	0.09 ± 0.01**	1.86 ± 0.09	2.10 ± 0.99	1.90 ± 0.11
PUFA						
n-3 series						
ALA (C18:3n3)	0.45 ± 0.05	0.53 ± 0.04	0.62 ± 0.04*	4.07 ± 0.41	10.16 ± 1.65	16.25 ± 2.37**
EPA (C20:5n3)	0.24 ± 0.03	0.16 ± 0.02*	0.10 ± 0.02**#	2.34 ± 0.19	2.87 ± 0.15	2.24 ± 0.47
DPA (C22:5n3)	0.35 ± 0.06	0.22 ± 0.03	0.12 ± 0.03**	3.70 ± 0.51	3.99 ± 0.68	2.54 ± 0.68
DHA (C22:6n3)	1.31 ± 0.12	0.73 ± 0.07**	0.42 ± 0.06***#	13.38 ± 1.19	14.47 ± 3.54	10.03 ± 1.89
n-6 series						
LA (C18 :2n-6)	14.86 ± 0.61	12.69 ± 0.56	11.63 ± 0.84*	140.31 ± 10.67	237.80 ± 24.88	293.85 ± 33.22**
GLA (C18:3n6)	0.21 ± 0.02	0.25 ± 0.02	0.19 ± 0.01	2.07 ± 0.20	4.65 ± 0.81	4.66 ± 0.68
HGLA (C20:3n6)	0.90 ± 0.15	0.52 ± 0.05*	0.37 ±0.05**#	9.16 ± 1.31	9.80 ± 0.81	8.79 ± 1.20
AA (C20:4n6)	6.55 ± 1.17	3.76 ± 0.62*	1.79 ± 0.23**#	68.78 ± 6.88	67.93 ± 7.06	41.38 ± 5.53*#
DIA (C22:4n6)	0.03 ± 0.01	0.03 ± 0.01	0.01 ± 0.00	0.16 ± 0.03	0.57 ± 0.37	0.15 ± 0.03
Total n-3 FA	2.45 ± 0.18	1.79 ± 0.12*	1.29 ± 0.13***#	24.52 ± 1.83	34.87 ± 4.53	31.74 ± 4.50
Total n-6 FA	22.69 ± 1.93	17.60 ± 1.16	14.08 ± 1.08**	221.64 ± 17.98	326.60 ± 27.50	351.37 ± 38.05*
Total n-7 FA	5.06 ± 0.50	5.75 ± 0.55	6.44 ± 0.44	44.01 ± 6.91	110.77 ± 20.22	170.43 ± 27.41**

Total n-9 FA	28.38 ± 3.09	34.48 ± 0.69	35.14 ± 1.38	231.1 ± 11.93	660.17 ± 99.54*	904.99 ± 115.45**
Total SFA	38.73 ± 1.52	38.60 ± 0.85	41.61 ± 1.78	370.31 ± 25.71	737.60 ± 112.64*	1075.89 ± 165.36**#
Total MUFA	35.09 ± 3.39	41.31 ± 0.47	42.55 ± 1.33	288.63 ± 18.58	791.74 ± 119.14*	1099.86 ± 141.75**##
Total PUFA	25.24 ± 2.10	19.37 ± 1.20*	15.42 ± 1.18**#	246.99 ± 19.77	360.67 ± 31.88	384.21 ± 41.96*#
Total FA	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	914.67 ± 58.25	1900.23 ± 260.21	2570.25 ± 325.73*#

AA :Arachidonic acid; ALA: Alpha linolenic acid; DHA: Docosahexanoic acid; DPA: Docosapentaenoic acid; DTA: Docosatetraenoic acid; EIA: Eicosenoic acid; EPA: eicosapentaenoic acid; ERA: Erucic acid; ETA: Eicosatrienoic acid; FA: Fatty acid; GLA: Gamma Linoleic acid; Hb: Hemoglobin; HGLA: Homogamma linolenic acid; Mi-NAFLD: minimal non-alcoholic fatty liver disease; Mo-NAFLD: moderate non-alcoholic fatty liver disease; Se-NAFLD: severe non-alcoholic fatty liver disease; LA: Linoleic acid; MA: Myristic acid; MUFA: Monounsaturated fatty acid; NA: Nervonic acid; OA: Oleic acid; PA: Palmitic acid; POA: Palmitoleic acid; PUFA: Polyunsaturated fatty acid; SA: Stearic acid; SFA: Saturated fatty acid. All values are expressed as mean ± SEM. Data were analyzed by using an Anova Tukey's multiple comparison. Statistical significance was defined as *P<0.05; **P<0.001; ***P<0.0001 vs. Mi-NAFLD; $^{\#}P$<0.05 $^{\#\#}$P<0.01 vs. Mo-NAFLD group.

Table 6: Liver fatty acids ratios in NAFLD subjects

	Mi-NAFLD (n=10)	Mo-NAFLD (n=10)	Se-NAFLD (n=15)
16:1n-7/18:2n-6	0.15 ± 0.03	0.25 ± 0.06	0.33 ± 0.03*
18:2n-6/20:4n-6	2.07 ± 0.13	3.63 ± 0.46	7.41 ± 1.02***##
20:3n-9/20:4n-6	0.01 ± 0.00	0.02 ± 0.00*	0.03 ± 0.00**
Total n7/18:2n-6	0.32 ± 0.05	0.46 ± 0.06	0.56 ± 0.04*
PUFA/SFA	0.67 ± 0.03	0.52 ± 0.05	0.37 ± 0.04**
EFA (LA+ALA)/NON-EFA	0.19 ± 0.01	0.15 ± 0.01*	0.13 ± 0.01**
EPA/DHA	0.18 ± 0.01	0.20 ± 0.03	0.22 ± 0.01
DHA/AA	0.20 ± 0.01	0.24 ± 0.04	0.24 ± 0.02
ALA/EPA	1.78 ± 0.24	3.53 ± 0.49	8.33 ± 1.82**##
ALA/LA	0.03 ± 0.00	0.04 ± 0.00	0.05 ± 0.00**
n-6/n-3	9.10 ± 0.16	9.64 ± 0.62	11.51 ± 0.92*
Δ5 (20:4n-6/20 :3n-6)	7.67 ± 0.50	7.01 ± 0.75	4.72 ± 0.08**#
Δ6 (20:3n-6/18:2n-6)	0.03 ± 0.01	0.04 ± 0.00	0.04 ± 0.00
Δ7 (16:1n-7/16:0)	0.09 ± 0.02	0.11 ± 0.01	0.12 ± 0.11
Δ9 (18:1n-9/18:0)	2.34 ± 0.12	4.62 ± 0.33*	5.68 ± 0.63***##
Omega-3 Index : %(EPA+DHA)	1.55 ± 0.14	0.89 ± 0.07***	0.50 ± 0.08***#
Lipogenesis index (16:00/18:2n-6)	1.57 ± 0.07	2.11 ± 0.20***	2.72 ± 0.23***#

AA: Arachidonic acid; ALA: Alpha linolenic acid; DHA: Docosahexanoic acid; EFA: Essential fatty acid; EPA: eicosapentaenoic acid; Mi-NAFLD: minimal non-alcoholic fatty liver disease; Mo-NAFLD: moderate non-alcoholic fatty liver disease; Se-NAFLD: severe non-alcoholic fatty liver disease; LA: Linoleic acid; PUFA: Polyunsaturated fatty acid; SFA: Saturated fatty acid. All values are expressed as mean ± SEM. Data were analyzed by using an Anova Tukey's multiple comparison. Statistical significance was defined as $^{**}P<0.001$; $^{***}P<0.0001$ vs. Mi-NAFLD; $^{\#}P<0.05$ vs. Mo-NAFLD.

Table 7: Summary of statistically different proteins and their related functions expressed in NAFLD subjects.

Function	#	Protein name	Accession number
Catalytic activity			
	57	Kynurenine/alpha-aminoadipate aminotransferase mitochondrial	Q8N5Z0|AADAT_HUMAN
	55	Acyl-coenzyme A synthetase mitochondrial	Q6NUN0|ACSM5_HUMAN
	61	Acetyl-coenzyme A synthetase cytoplasmic	Q9NR19|ACSA_HUMAN
	53	Cytosolic 10-formyltetrahydrofolate dehydrogenase	O75891|AL1L1_HUMAN
	76	L-xylulose reductase	Q7Z4W1|DCXR_HUMAN
	78	Aspartyl aminopeptidase	Q9ULA0|DNPEP_HUMAN
	69	Glyoxylate reductase/hydroxypyruvate reductase	Q9UBQ7|GRHPR_HUMAN
	74	Glycogenin-2	O15488|GLYG2_HUMAN
	70	3-hydroxyanthranilate 3 4-dioxygenase	P46952|3HAO_HUMAN
	63	Beta-lactamase-like protein 2	Q53H82|LACB2_HUMAN
	62	Methylmalonyl-CoA epimerase mitochondrial	Q96PE7|MCEE_HUMAN
	79	Aldehyde oxidase	Q06278|AOXA_HUMAN
	66	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 4	Q9Y237|PIN4_HUMAN
	77	Peptidyl-prolyl cis-trans isomerase C	P45877|PPIC_HUMAN
	73	Glycogen phosphorylase brain form	P11216|PYGB_HUMAN
	71	Beta-ureidopropionase	Q9UBR1|BUP1_HUMAN
GTPase activity			
	45	GTPase HRas	P01112|RASH_HUMAN
	8	Ras-related protein Rab-1B	Q9H0U4|RAB1B_HUMAN
	41	Ras-related protein Rab-35	tr|F5H157|F5H157_HUMAN
	13	Ras-related protein Rab-8B (Fragment)	tr|H0YNE9|H0YNE9_HUMAN

	16	Signal recognition particle receptor subunit alpha	P08240|SRPRA_HUMAN
	49	Tubulin beta-8 chain	Q3ZCM7|TBB8_HUMAN
Small molecule binding			
	34	Actin aortic smooth muscle	P62736|ACTA_HUMAN
	50	Actin-related protein 2	P61160|ARP2_HUMAN
	31	Serum albumin	tr|A0A0C4DGB6|A0A0C4DGB6_HUMAN
	1	Cleavage stimulation factor subunit 2	tr|E7EWR4|E7EWR4_HUMAN
	7	Fatty acid-binding protein (adipocity)	P15090|FABP4_HUMAN
	51	Fatty acid-binding protein (epidermal)	Q01469|FABP5_HUMAN
	4	Heterogeneous nuclear ribonucleoprotein H	tr|E9PCY7|E9PCY7_HUMAN
	45	GTPase HRas	P01112|RASH_HUMAN
	2	Heat shock 70 kDa protein 6	P17066|HSP76_HUMAN
	18	Myosin light chain kinase smooth muscle	Q15746|MYLK_HUMAN
	8	Ras-related protein Rab-1B	Q9H0U4|RAB1B_HUMAN
	41	Ras-related protein Rab-35	tr|F5H157|F5H157_HUMAN
	13	Ras-related protein Rab-8B (Fragment)	tr|H0YNE9|H0YNE9_HUMAN
	16	Signal recognition particle receptor subunit alpha	P08240|SRPRA_HUMAN
	49	Tubulin beta-8 chain	Q3ZCM7|TBB8_HUMAN
	12	Valine--tRNA ligase	P26640|SYVC_HUMAN
Anion binding			
	34	Actin aortic smooth muscle	P62736|ACTA_HUMAN
	50	Actin-related protein 2	P61160|ARP2_HUMAN
	31	Serum albumin	tr|A0A0C4DGB6|A0A0C4DGB6_HUMAN
	7	Fatty acid-binding protein (adipocity)	P15090|FABP4_HUMAN
	51	Fatty acid-binding protein (epidermal)	Q01469|FABP5_HUMAN
	11	Glutathione S-transferase	P09488|GSTM1_HUMAN
	45	GTPase HRas	P01112|RASH_HUMAN
	2	Heat shock 70 kDa protein 6	P17066|HSP76_HUMAN

	5	Lactotransferrin (Fragment)	tr|E7EQB2|E7EQB2_HUMAN
	18	Myosin light chain kinase smooth muscle	Q15746|MYLK_HUMAN
	8	Ras-related protein Rab-1B	Q9H0U4|RAB1B_HUMAN
	41	Ras-related protein Rab-35	tr|F5H157|F5H157_HUMAN
	13	Ras-related protein Rab-8B (Fragment)	tr|H0YNE9|H0YNE9_HUMAN
	16	Signal recognition particle receptor subunit alpha	P08240|SRPRA_HUMAN
	49	Tubulin beta-8 chain	Q3ZCM7|TBB8_HUMAN
	12	Valine--tRNA ligase	P26640|SYVC_HUMAN
Fatty acid binding			
	31	Serum albumin	tr|A0A0C4DGB6|A0A0C4DGB6_HUMAN
	7	Fatty acid-binding protein (adipocity)	P15090|FABP4_HUMAN
	51	Fatty acid-binding protein (epidermal)	Q01469|FABP5_HUMAN
Binding			
	34	Actin aortic smooth muscle	P62736|ACTA_HUMAN
	50	Actin-related protein 2	P61160|ARP2_HUMAN
	31	Serum albumin	tr|A0A0C4DGB6|A0A0C4DGB6_HUMAN
	29	Zinc-alpha-2-glycoprotein	P25311|ZA2G_HUMAN
	23	Coronin-1C	Q9ULV4|COR1C_HUMAN
	7	Fatty acid-binding protein (adipocity)	P15090|FABP4_HUMAN
	51	Fatty acid-binding protein (epidermal)	Q01469|FABP5_HUMAN
	9	Fibulin-1	P23142|FBLN1_HUMAN
	43	Filaggrin	P20930|FILA_HUMAN
	14	Fragile X mental retardation protein 1	tr|A8MQB8|A8MQB8_HUMAN
	11	Glutathione S-transferase	P09488|GSTM1_HUMAN
	20	Hemoglobin subunit alpha	P69905|HBA_HUMAN
	21	HLA class I histocompatibility antigen alpha chain G	tr|Q8MH48|Q8MH48_HUMAN
	48	High mobility group protein HMG-I/HMG-Y	P17096|HMGA1_HUMAN
	45	GTPase HRas	P01112|RASH_HUMAN
	2	Heat shock 70 kDa protein 6	P17066|HSP76_HUMAN

42	Inhibin beta E chain	P58166|INHBE_HUMAN
5	Lactotransferrin (Fragment)	tr|E7EQB2|E7EQB2_HUMAN
6	Mannose-binding protein	P11226|MBL2_HUMAN
18	Myosin light chain kinase smooth muscle	Q15746|MYLK_HUMAN
19	Optineurin	Q96CV9|OPTN_HUMAN
47	Perilipin-1	O60240|PLIN1_HUMAN
10	Trypsin-1	tr|E7EQ64|E7EQ64_HUMAN
8	Ras-related protein Rab-1B	Q9H0U4|RAB1B_HUMAN
41	Ras-related protein Rab-35	tr|F5H157|F5H157_HUMAN
13	Ras-related protein Rab-8B (Fragment)	tr|H0YNE9|H0YNE9_HUMAN
32	Protein transport protein Sec24B	tr|G5EA31|G5EA31_HUMAN
24	Nuclear autoantigen Sp-100	P23497|SP100_HUMAN
16	Signal recognition particle receptor subunit alpha	P08240|SRPRA_HUMAN
26	Stress-induced-phosphoprotein 1	P31948|STIP1_HUMAN
49	Tubulin beta-8 chain	Q3ZCM7|TBB8_HUMAN
12	Valine--tRNA ligase	P26640|SYVC_HUMAN

in the table represents the protein number on the representative volcano plot in Figure 8.

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REFERENCES

1. Wild SH, Walker JJ, Morling JR, McAllister DA, Colhoun HM, Farran B, et al. Cardiovascular Disease, Cancer, and Mortality Among People With Type 2 Diabetes and Alcoholic or Nonalcoholic Fatty Liver Disease Hospital Admission. Diabetes care. 2018;41(2):341-7.
2. Kim BJ, Kim HS, Kang JG, Kim BS, Kang JH. Association of epicardial fat volume and nonalcoholic fatty liver disease with metabolic syndrome: From the CAESAR study. J Clin Lipidol. 2016;10(6):1423-30 e1.
3. Rosso C, Mezzabotta L, Gaggini M, Salomone F, Gambino R, Marengo A, et al. Peripheral insulin resistance predicts liver damage in nondiabetic subjects with nonalcoholic fatty liver disease. Hepatology. 2016;63(1):107-16.
4. Carr RM, Oranu A, Khungar V. Nonalcoholic Fatty Liver Disease: Pathophysiology and Management. Gastroenterol Clin North Am. 2016;45(4):639-52.
5. Kuppan G, Anjana RM, Deepa M, Paramasivam P, Chandrakumar S, Kaliyaperumal V, et al. Inflammatory markers in relation to nonalcoholic fatty liver disease in urban South Indians. Diabetes Technol Ther. 2012;14(2):152-8.
6. Cusi K. Role of obesity and lipotoxicity in the development of nonalcoholic steatohepatitis: pathophysiology and clinical implications. Gastroenterology. 2012;142(4):711-25 e6.
7. Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, et al. The diagnosis and management of non-alcoholic fatty liver disease: Practice guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. The American journal of gastroenterology. 2012;107(6):811-26.
8. Park SK, Seo MH, Shin HC, Ryoo JH. Clinical availability of nonalcoholic fatty liver disease as an early predictor of type 2 diabetes mellitus in Korean men: 5-year prospective cohort study. Hepatology. 2013;57(4):1378-83.
9. Rowe IA. Too much medicine: overdiagnosis and overtreatment of non-alcoholic fatty liver disease. Lancet Gastroenterol Hepatol. 2018;3(1):66-72.

10. Lindenmeyer CC, McCullough AJ. The Natural History of Nonalcoholic Fatty Liver Disease-An Evolving View. Clin Liver Dis. 2018;22(1):11-21.
11. Dongiovanni P, Anstee QM, Valenti L. Genetic predisposition in NAFLD and NASH: impact on severity of liver disease and response to treatment. Current pharmaceutical design. 2013;19(29):5219-38.
12. Petta S, Di Marco V, Pipitone RM, Grimaudo S, Buscemi C, Craxi A, et al. Prevalence and Severity of NAFLD by Transient Elastography: Genetic and Metabolic Risk Factors in a General Population. Liver international : official journal of the International Association for the Study of the Liver. 2018.
13. Kim SS, Cho HJ, Kim HJ, Kang DR, Berry JR, Kim JH, et al. Nonalcoholic fatty liver disease as a sentinel marker for the development of diabetes mellitus in non-obese subjects. Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver. 2018;50(4):370-7.
14. Rinella ME, Lominadze Z, Loomba R, Charlton M, Neuschwander-Tetri BA, Caldwell SH, et al. Practice patterns in NAFLD and NASH: real life differs from published guidelines. Therap Adv Gastroenterol. 2016;9(1):4-12.
15. Veilleux A, Grenier E, Marceau P, Carpentier AC, Richard D, Levy E. Intestinal lipid handling: evidence and implication of insulin signaling abnormalities in human obese subjects. Arteriosclerosis, thrombosis, and vascular biology. 2014;34(3):644-53.
16. Veilleux A, Mayeur S, Berube JC, Beaulieu JF, Tremblay E, Hould FS, et al. Altered intestinal functions and increased local inflammation in insulin-resistant obese subjects: a gene-expression profile analysis. BMC gastroenterology. 2015;15:119.
17. Bedossa P. Pathology of non-alcoholic fatty liver disease. Liver international : official journal of the International Association for the Study of the Liver. 2017;37 Suppl 1:85-9.
18. Spahis S, Alvarez F, Dubois J, Ahmed N, Peretti N, Levy E. Plasma fatty acid composition in French-Canadian children with non-alcoholic fatty liver disease: Effect of n-3 PUFA supplementation. Prostaglandins, leukotrienes, and essential fatty acids. 2015;99:25-34.

19. Spahis S, Vanasse M, Belanger SA, Ghadirian P, Grenier E, Levy E. Lipid profile, fatty acid composition and pro- and anti-oxidant status in pediatric patients with attention-deficit/hyperactivity disorder. Prostaglandins, leukotrienes, and essential fatty acids. 2008;79(1-2):47-53.
20. Belanger SA, Vanasse M, Spahis S, Sylvestre MP, Lippe S, L'Heureux F, et al. Omega-3 fatty acid treatment of children with attention-deficit hyperactivity disorder: A randomized, double-blind, placebo-controlled study. Paediatr Child Health. 2009;14(2):89-98.
21. Lambert M, Paradis G, O'Loughlin J, Delvin EE, Hanley JA, Levy E. Insulin resistance syndrome in a representative sample of children and adolescents from Quebec, Canada. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity. 2004;28(7):833-41.
22. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem. 1972;18(6):499-502.
23. Levy E, Spahis S, Garofalo C, Marcil V, Montoudis A, Sinnet D, et al. Sar1b transgenic male mice are more susceptible to high-fat diet-induced obesity, insulin insensitivity and intestinal chylomicron overproduction. The Journal of nutritional biochemistry. 2014;25(5):540-8.
24. Levy E, Spahis S, Ziv E, Marette A, Elchebly M, Lambert M, et al. Overproduction of intestinal lipoprotein containing apolipoprotein B-48 in Psammomys obesus: impact of dietary n-3 fatty acids. Diabetologia. 2006;49(8):1937-45.
25. van der Veen JN, Kennelly JP, Wan S, Vance JE, Vance DE, Jacobs RL. The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease. Biochimica et biophysica acta. 2017;1859(9 Pt B):1558-72.
26. Lepage G, Levy E, Ronco N, Smith L, Galeano N, Roy CC. Direct transesterification of plasma fatty acids for the diagnosis of essential fatty acid deficiency in cystic fibrosis. Journal of lipid research. 1989;30(10):1483-90.
27. Stefan N, Haring HU, Hu FB, Schulze MB. Metabolically healthy obesity: epidemiology, mechanisms, and clinical implications. Lancet Diabetes Endocrinol. 2013;1(2):152-62.

28. Primeau V, Coderre L, Karelis AD, Brochu M, Lavoie ME, Messier V, et al. Characterizing the profile of obese patients who are metabolically healthy. International journal of obesity. 2011;35(7):971-81.
29. Wree A, Broderick L, Canbay A, Hoffman HM, Feldstein AE. From NAFLD to NASH to cirrhosis-new insights into disease mechanisms. Nature reviews Gastroenterology & hepatology. 2013;10(11):627-36.
30. Ameer F, Scandiuzzi L, Hasnain S, Kalbacher H, Zaidi N. De novo lipogenesis in health and disease. Metabolism: clinical and experimental. 2014;63(7):895-902.
31. Harmel E, Grenier E, Bendjoudi Ouadda A, El Chebly M, Ziv E, Beaulieu JF, et al. AMPK in the small intestine in normal and pathophysiological conditions. Endocrinology. 2014;155(3):873-88.
32. Hajri T, Han XX, Bonen A, Abumrad NA. Defective fatty acid uptake modulates insulin responsiveness and metabolic responses to diet in CD36-null mice. The Journal of clinical investigation. 2002;109(10):1381-9.
33. Doege H, Baillie RA, Ortegon AM, Tsang B, Wu Q, Punreddy S, et al. Targeted deletion of FATP5 reveals multiple functions in liver metabolism: alterations in hepatic lipid homeostasis. Gastroenterology. 2006;130(4):1245-58.
34. Eaton S. Control of mitochondrial beta-oxidation flux. Progress in lipid research. 2002;41(3):197-239.
35. Nakamura MT, Yudell BE, Loor JJ. Regulation of energy metabolism by long-chain fatty acids. Progress in lipid research. 2014;53:124-44.
36. Chen Y, Wang Y, Huang Y, Zeng H, Hu B, Guan L, et al. PPARalpha regulates tumor cell proliferation and senescence via a novel target gene carnitine palmitoyltransferase 1C. Carcinogenesis. 2017;38(4):474-83.
37. Louet JF, Chatelain F, Decaux JF, Park EA, Kohl C, Pineau T, et al. Long-chain fatty acids regulate liver carnitine palmitoyltransferase I gene (L-CPT I) expression through a peroxisome-proliferator-activated receptor alpha (PPARalpha)-independent pathway. The Biochemical journal. 2001;354(Pt 1):189-97.
38. Hamel FG, Bennett RG, Upward JL, Duckworth WC. Insulin inhibits peroxisomal fatty acid oxidation in isolated rat hepatocytes. Endocrinology. 2001;142(6):2702-6.

39. Barthel A, Schmoll D. Novel concepts in insulin regulation of hepatic gluconeogenesis. American journal of physiology Endocrinology and metabolism. 2003;285(4):E685-92.
40. Regnell SE, Lernmark A. Hepatic steatosis in type 1 diabetes. Rev Diabet Stud. 2011;8(4):454-67.
41. Hashidate-Yoshida T, Harayama T, Hishikawa D, Morimoto R, Hamano F, Tokuoka SM, et al. Fatty acid remodeling by LPCAT3 enriches arachidonate in phospholipid membranes and regulates triglyceride transport. Elife. 2015;4.
42. Yao ZM, Vance DE. The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. The Journal of biological chemistry. 1988;263(6):2998-3004.
43. Quinn WJ, 3rd, Wan M, Shewale SV, Gelfer R, Rader DJ, Birnbaum MJ, et al. mTORC1 stimulates phosphatidylcholine synthesis to promote triglyceride secretion. The Journal of clinical investigation. 2017;127(11):4207-15.
44. Arendt BM, Ma DW, Simons B, Noureldin SA, Therapondos G, Guindi M, et al. Nonalcoholic fatty liver disease is associated with lower hepatic and erythrocyte ratios of phosphatidylcholine to phosphatidylethanolamine. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme. 2013;38(3):334-40.
45. Cui Z, Houweling M. Phosphatidylcholine and cell death. Biochimica et biophysica acta. 2002;1585(2-3):87-96.
46. Li Z, Agellon LB, Allen TM, Umeda M, Jewell L, Mason A, et al. The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and steatohepatitis. Cell metabolism. 2006;3(5):321-31.
47. Parry SA, Hodson L. Influence of dietary macronutrients on liver fat accumulation and metabolism. J Investig Med. 2017;65(8):1102-15.
48. Yki-Jarvinen H. Nutritional Modulation of Non-Alcoholic Fatty Liver Disease and Insulin Resistance. Nutrients. 2015;7(11):9127-38.
49. Green CJ, Hodson L. The influence of dietary fat on liver fat accumulation. Nutrients. 2014;6(11):5018-33.

50. Kralisch S, Fasshauer M. Adipocyte fatty acid binding protein: a novel adipokine involved in the pathogenesis of metabolic and vascular disease? Diabetologia. 2013;56(1):10-21.
51. Storch J, Thumser AE. Tissue-specific functions in the fatty acid-binding protein family. The Journal of biological chemistry. 2010;285(43):32679-83.
52. Hu B, Guo Y, Garbacz WG, Jiang M, Xu M, Huang H, et al. Fatty acid binding protein-4 (FABP4) is a hypoxia inducible gene that sensitizes mice to liver ischemia/reperfusion injury. Journal of hepatology. 2015;63(4):855-62.
53. Thompson KJ, Austin RG, Nazari SS, Gersin KS, Iannitti DA, McKillop IH. Altered fatty acid-binding protein 4 (FABP4) expression and function in human and animal models of hepatocellular carcinoma. Liver international : official journal of the International Association for the Study of the Liver. 2017.
54. Hotamisligil GS, Bernlohr DA. Metabolic functions of FABPs--mechanisms and therapeutic implications. Nature reviews Endocrinology. 2015;11(10):592-605.
55. Uysal KT, Scheja L, Wiesbrock SM, Bonner-Weir S, Hotamisligil GS. Improved glucose and lipid metabolism in genetically obese mice lacking aP2. Endocrinology. 2000;141(9):3388-96.
56. Hui X, Li H, Zhou Z, Lam KS, Xiao Y, Wu D, et al. Adipocyte fatty acid-binding protein modulates inflammatory responses in macrophages through a positive feedback loop involving c-Jun NH2-terminal kinases and activator protein-1. The Journal of biological chemistry. 2010;285(14):10273-80.
57. Makowski L, Brittingham KC, Reynolds JM, Suttles J, Hotamisligil GS. The fatty acid-binding protein, aP2, coordinates macrophage cholesterol trafficking and inflammatory activity. Macrophage expression of aP2 impacts peroxisome proliferator-activated receptor gamma and IkappaB kinase activities. The Journal of biological chemistry. 2005;280(13):12888-95.
58. Graupera I, Coll M, Pose E, Elia C, Piano S, Sola E, et al. Adipocyte Fatty-Acid Binding Protein is Overexpressed in Cirrhosis and Correlates with Clinical Outcomes. Sci Rep. 2017;7(1):1829.

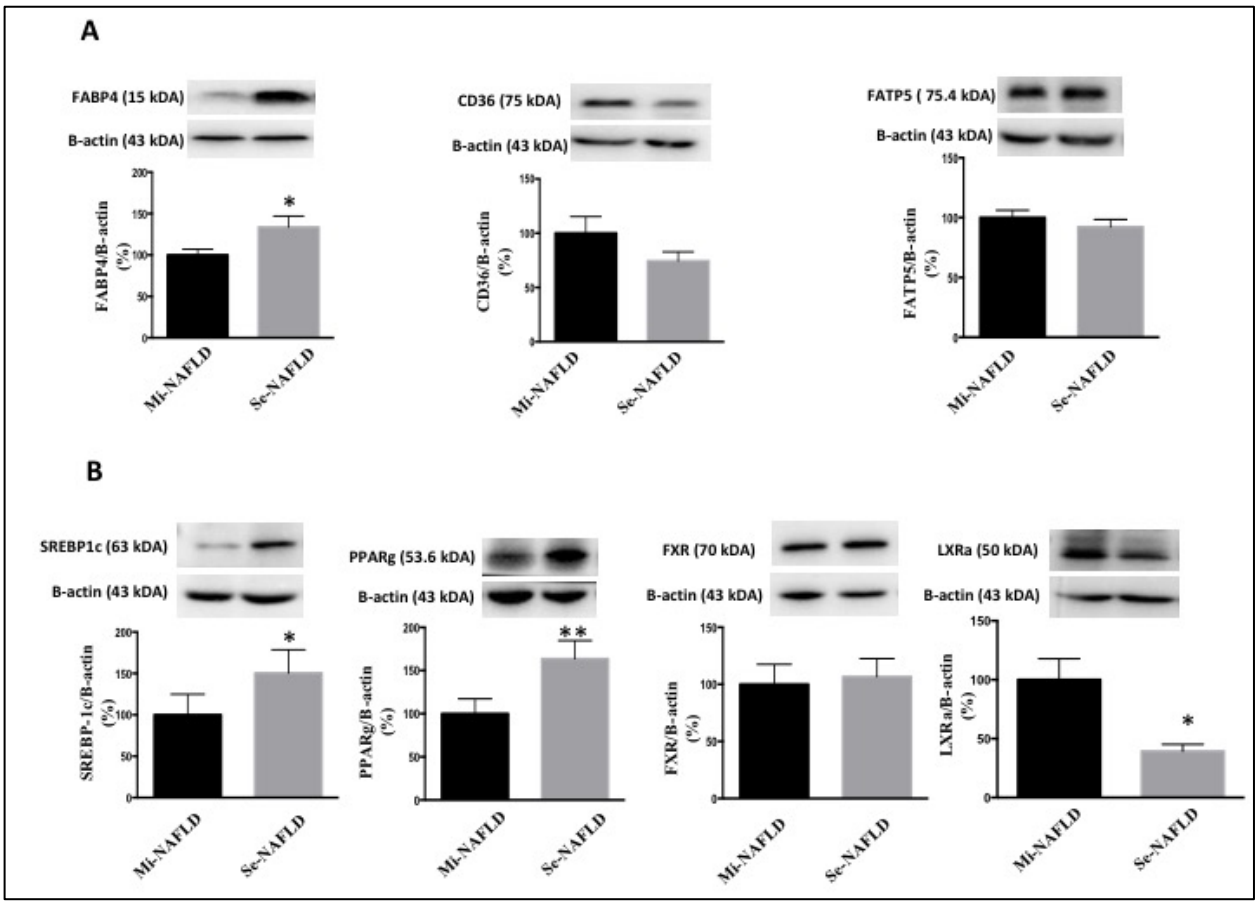


Figure 1. Hepatic fatty acid uptake and metabolism in NAFLD subjects.

Protein extracts from liver total cell lysates were resolved by SDS/PAGE and immunoblotted for FABP4, CD36, FATP5, SERBP1-C, PPPARγ, FXR and LXRα for protein mass. The protein content was normalized to β-actin as a housekeeping gene. Results represent the means ± SEM for n=5 for each group. *P<0.05 compared to Mi-NAFLD group.

Mi-NAFLD: minimal non-alcoholic fatty liver disease; Se-NAFLD: severe non-alcoholic fatty liver disease.

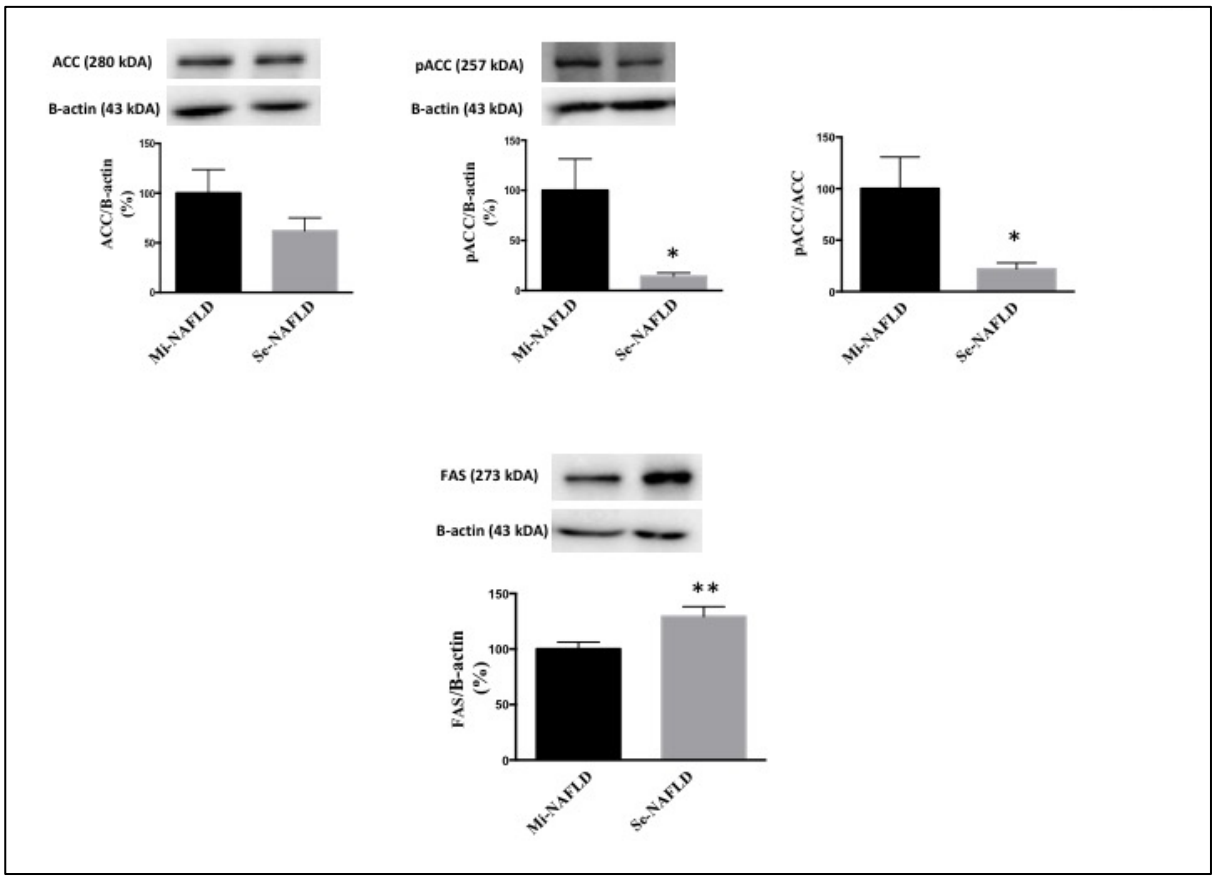


Figure 2. **Hepatic lipogenesis expression in NAFLD subjects.**

Protein extracts from liver total cell lysates were resolved by SDS/PAGE and immunoblotted for ACC, phospho-ACC-Ser79 and FAS for protein expression. The ratio of total ACC protein content and phospho-ACC was then calculated. Results represent the means ± SEM for n=5 for each group. *P<0.05 compared to Mi-NAFLD group.

Mi-NAFLD: minimal non-alcoholic fatty liver disease; Se-NAFLD: severe non-alcoholic fatty liver disease.

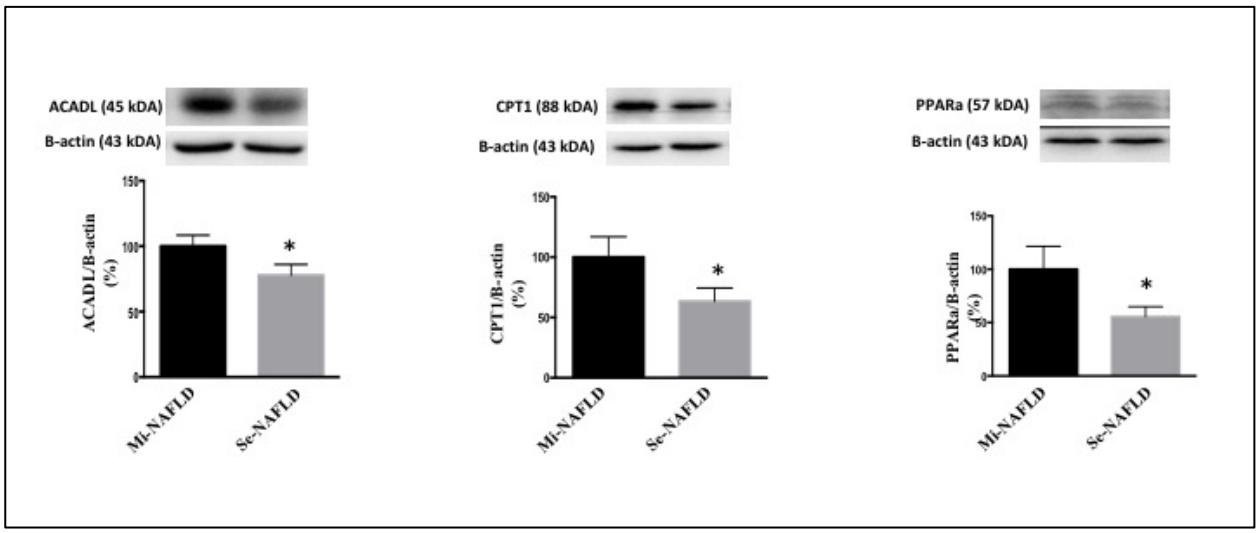


Figure 3. **Mitochondrial fatty acid β-oxidation estimation in NAFLD.**

The expressions of the key enzymes (CPT1 and ACADL) as well as PPARα transcription factor was analyzed by Western blot as described in "Materials and Methods." Results represent the means ± SEM for n=5 for each group. *P<0.05 compared to Mi-NAFLD group.

Mi-NAFLD: minimal non-alcoholic fatty liver disease; Se-NAFLD: severe non-alcoholic fatty liver disease.

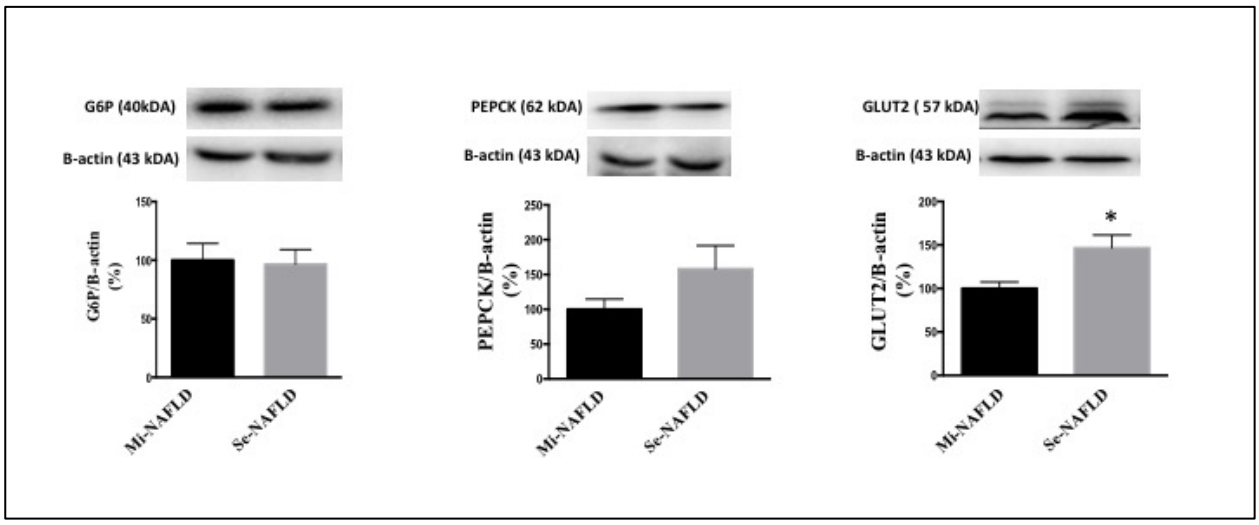


Figure 4. **Hepatic gluconeogenesis in NAFLD patients.**

Protein mass of G6P, PEPCK and GLUT2 was analysed by Western blot as described in "Materials and Methods." Results represent the means ± SEM for n=5 for each group. *P<0.05 compared to Mi-NAFLD group.

Mi-NAFLD: minimal non-alcoholic fatty liver disease; Se-NAFLD: severe non-alcoholic fatty liver disease.

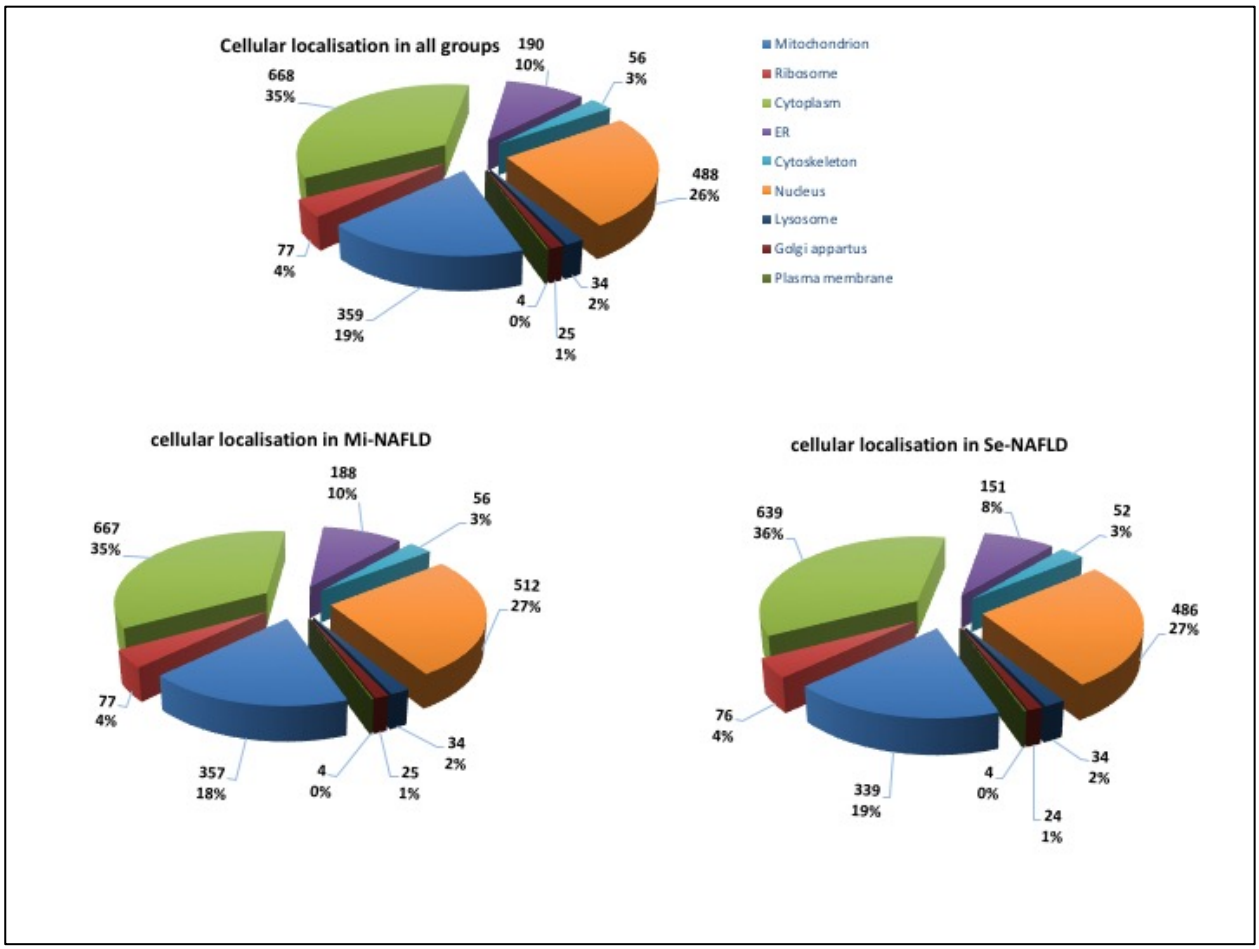


Figure 5. Annotation of Gene Ontology (GO) category cellular component of the different proteins found in the liver.

Localization of proteins identified in hepatic tissues was defined from the GO terms built from the UniProt Knowledgebase (n=1737 proteins). All the localizations that applied for a single protein were considered. Results refer to: localization, number and % of proteins detected in each intracellular site. Localizations are colour coded and described in the affiliated legend.

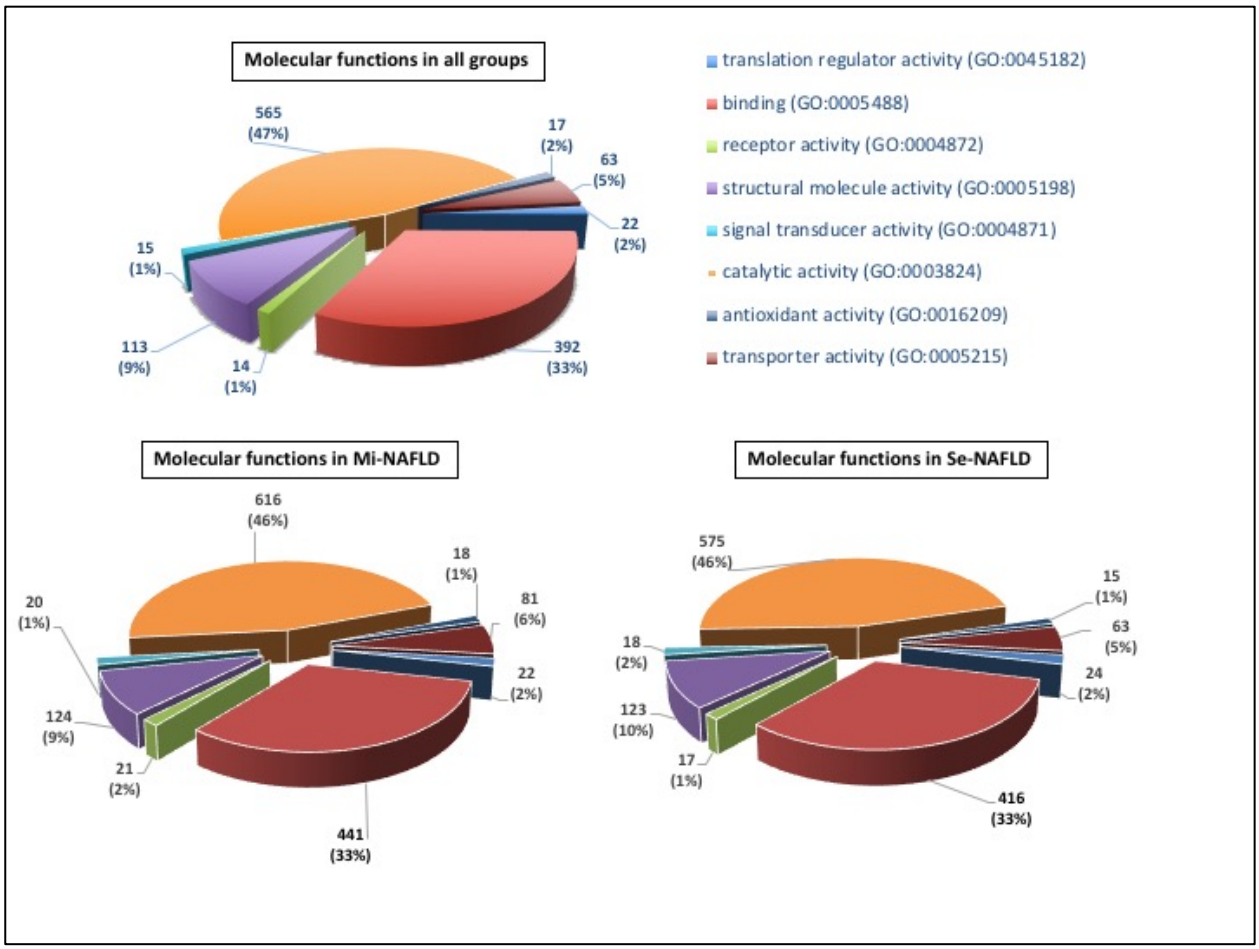


Figure 6. Related functions of the different proteins in the liver.

Classification of proteins identified in the liver in each group was defined from the Gene Ontology terms from the UniProt Knowledgebase (n=1737 proteins). All the functions that applied for a single protein were considered. Results are expressed as: function; number of proteins involved in this function; and % of proteins involved in this function. Functions are colour coded and described in the affiliated legend.

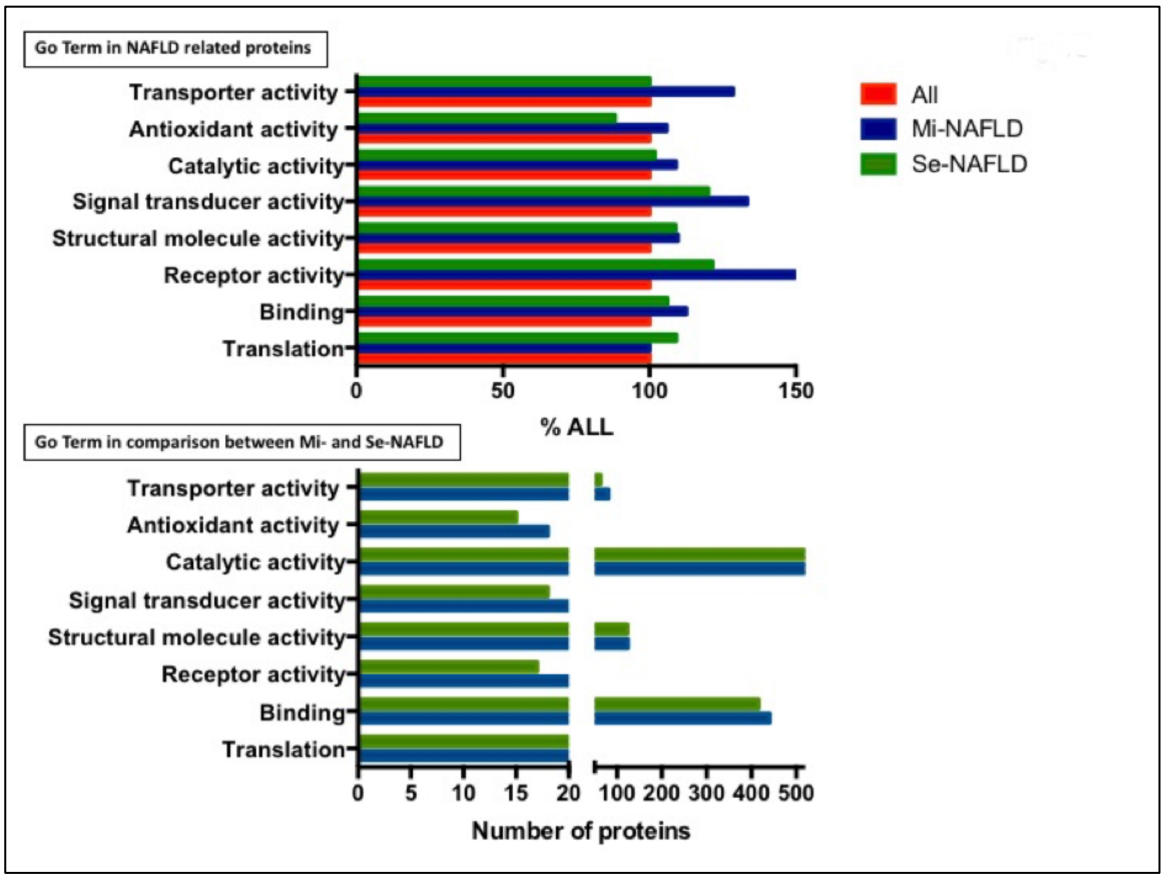
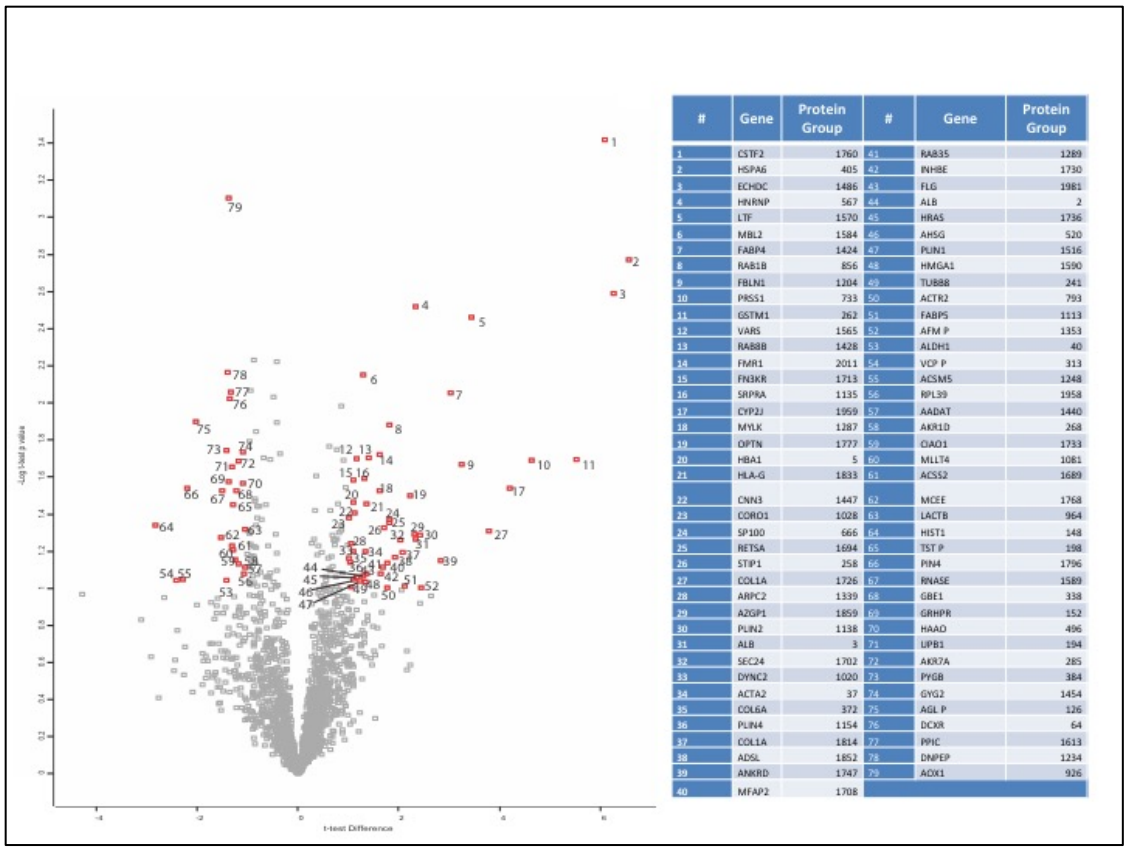


Figure 7. Functional annotation of the different proteins in the liver.

The proteins derived from the Gene Ontology terms built from the UniProt Knowledgebase (n=1737 proteins) were calculated according to their enrichment: (A) total differentially expressed proteins in the whole NAFLD group and subgroups (moderate and severe NAFLD subjects) and (B) between minimal and severe NAFLD groups.



#	Gene	Protein Group	#	Gene	Protein Group
1	CSTF2	1760	41	RAB35	1289
2	HSPA6	405	42	INHBE	1730
3	ECHDC	1486	43	FLG	1981
4	HNRNP	567	44	ALB	2
5	LTF	1570	45	HRAS	1736
6	MBL2	1584	46	AHSG	520
7	FABP4	1424	47	PLIN1	1516
8	RAB1B	856	48	HMGA1	1590
9	FBLN1	1204	49	TUBB8	241
10	PRSS1	733	50	ACTR2	793
11	GSTM1	262	51	FABP5	1113
12	VARS	1565	52	AFM P	1353
13	RAB8B	1428	53	ALDH1	40
14	FMR1	2011	54	VCP P	313
15	FN3KR	1713	55	ACSM5	1248
16	SRPRA	1135	56	RPL39	1958
17	CYP2J	1959	57	AADAT	1440
18	MYLK	1287	58	AKR1D	268
19	OPTN	1777	59	OAO1	1733
20	HBA1	5	60	MLLT4	1081
21	HLA-G	1833	61	ACSS2	1689
22	CNN3	1447	62	MCEE	1768
23	CORO1	1028	63	LACTB	964
24	SP100	666	64	HIST1	148
25	RETSA	1694	65	TST P	198
26	STIP1	258	66	PIN4	1796
27	COL1A	1726	67	RNASE	1589
28	ARPC2	1339	68	GBE1	338
29	AZGP1	1859	69	GRHPR	152
30	PLIN2	1138	70	HAAO	496
31	ALB	3	71	UPB1	194
32	SEC24	1702	72	AKR7A	285
33	DYNC2	1020	73	PYGB	384
34	ACTA2	37	74	GYG2	1454
35	COL6A	372	75	AGL P	126
36	PLIN4	1154	76	DCXR	64
37	COL1A	1814	77	PPIC	1613
38	ADSL	1852	78	DNPEP	1234
39	ANKRD	1747	79	AOX1	926
40	MFAP2	1708			

Figure 8. **Volcano plot representation of statistically different proteins expressed in NAFLD subjects.**

X-axis represents the variation in expression [t-test (fold change)] while y-axis represents the statistical significance [- log *t-test* (p-value)]. Proteins with coordinates (x,y) where x is <-1 or > 1 and y is > 1 are statistically halved or doubled in a group of samples. Statistically reproducible proteins have been numbered. Proteins on the right of x=0 (labelled 1-52) are up regulated whereas those on the left of x=0 (labelled 53-79) are down regulated as described in **Table 7**.

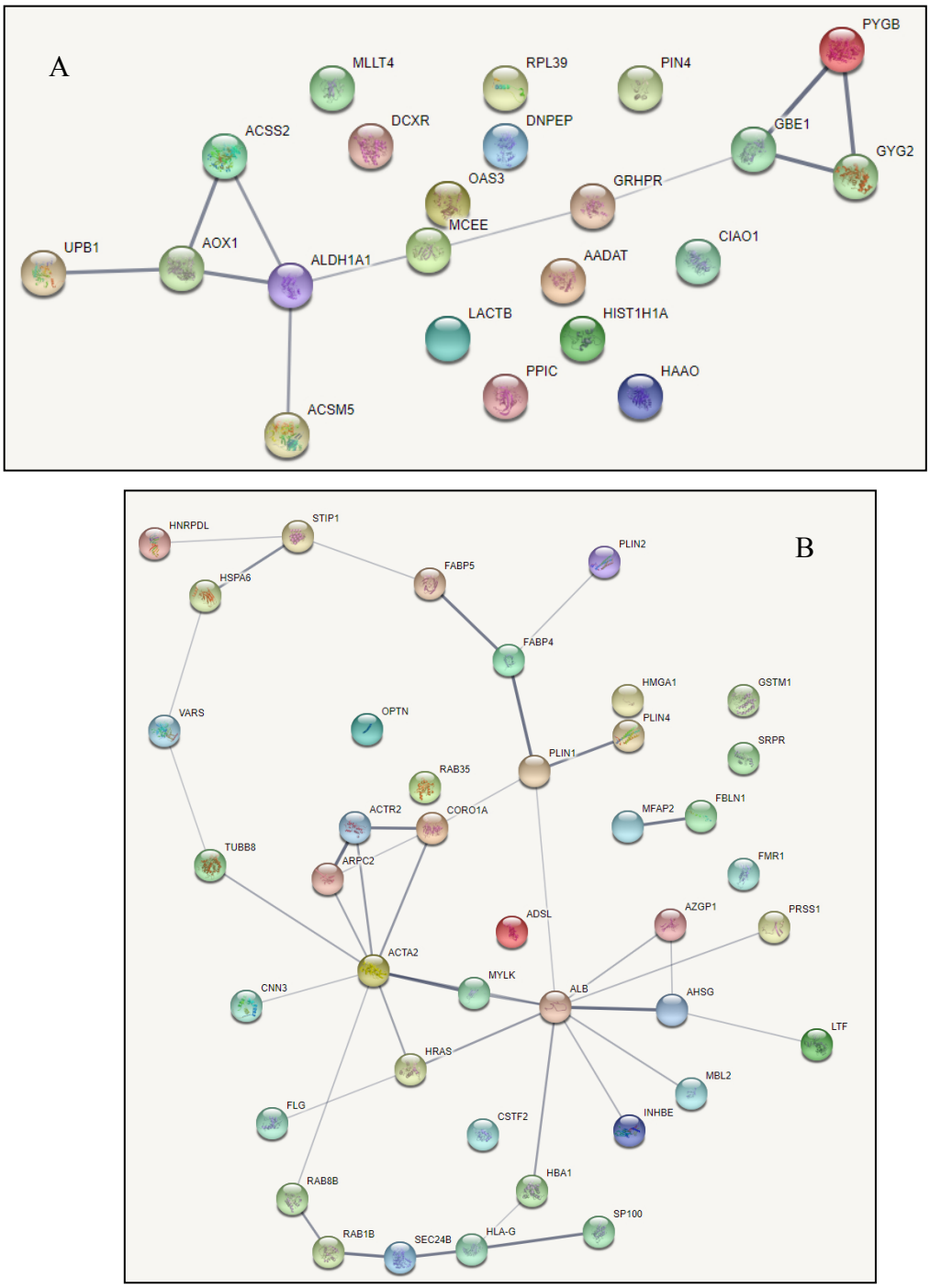


Figure 9. Relevance of protein-protein interactions in severe NAFLD subjects.

A representative network is illustrated for (A) significant downregulated and (B) upregulated protein-interactions constructed using the bioinformatic tool STRING v.10.00. Proteins are expressed according to the gene name.

Article 4

«Plasma fatty acid composition in French-Canadian children with non-alcoholic fatty liver disease: Effect of n-3 PUFA supplementation»

Spahis S, Alvarez F, Dubois J, Ahmed N, Peretti N, Levy E. *Prostaglandins Leukot Essent Fatty Acids*. 2015 Aug;99:25-34. doi:10.1016/j.plefa.2015.04.010. Epub 2015 May 8.

CONTRIBUTION DES AUTEURS

Spahis S a été impliquée dans la conception et la planification de l'étude ; la mise au point du protocole de recherche clinique auprès de Santé Canada et du Comité d'éthique à la recherche ainsi que la création des ''Case Report Form'' pour chaque patient. Elle s'est occupée du recrutement et le suivi des patients; l'acquisition des données biochimiques, leur analyse et leur interprétation. Elle a contribué à la rédaction, soumission et révision du manuscrit sous la supervision du Directeur de recherche, Dr Levy E.

Alvarez F et Ahmed N ont contribué au recrutement et au suivi médical des patients.

Dubois J a contribué au suivi médical des patients.

Peretti N a participé à la conception de l'étude.

Levy E a contribué au concept et à la supervision de l'étude, aux ressources financières et matérielles pour la réalisation de l'étude. Dr Levy E a participé à la rédaction, l'analyse critique des résultats et la révision du manuscrit.

Plasma fatty acid composition in French-Canadian children with non-alcoholic fatty liver disease: Effect of n-3 PUFA supplementation

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Running Title: Fatty acid profile and efficacy of omega 3 in pediatric NAFLD

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) represents one of the most common causes of liver disease worldwide. As the NAFLD pathogenesis is associated with diet and lifestyle, the aims of the present work are to assess FA composition in NAFLD young French-Canadian, to determine whether treatment with n-3 FA improves the plasma FA profile, and to define the time on the effectiveness of n-3 FA supplementation. Baseline characteristics of the NAFLD subjects show increased, anthropometric and biochemical parameters. Their plasma FA composition is characterized by a percent increase in total n-6 FA and a high proportion of saturated and total monounsaturated FA, as well as a decrease in Δ5 and increase in Δ6 desaturases. In conclusion, our results document for the first time the composition of plasma FAs in NAFLD young French Canadian and the efficacy of 3-month supplementation to improve the proportion of n-3 FA in their plasma.

Keywords: Fatty acids, PUFA, n-3 fatty acids, supplementation, obesity, NAFLD

Abbreviations: AA, Arachidonic acid; CVD, Cardiovascular disease; DHA, Docosahexaenoic acid; EPA, Eicosapentaenoic acid; FA, Fatty acids; HDL, High-density lipoprotein; LA, Linoleic acid; NAFLD, Non-alcoholic fatty liver disease; PUFA, Polyunsaturated fatty acid; SFA, Saturated fatty acid; TG, Triglycerides

1. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is increasingly regarded as the most common liver condition in the developed world (1). It is defined as fat accumulation in the liver, greater than 5% by weight in the absence of excessive alcohol consumption, and affects up 30% in countries patients with abnormal liver functions worldwide (1). NAFLD is closely related to obesity and to characteristic components of the metabolic syndrome (2). It begins with simple steatosis in the hepatocytes, which can then evolve and progress to non-alcoholic steatohepatitis with variable degrees of fibrosis, cirrhosis and even hepatocellular carcinoma (1, 3). Approximately 10-25% NAFLD patients develop NASH (3), while the subtype NASH becomes a major health issue throughout the world.

The prevalence of NAFLD in the general pediatric population is estimated to be nearly 13%, while among obese and overweight children and, particularly, adolescents it raises up to 70% (4, 5). The gender and ethnic background represent two critical risk factors for the development of NAFLD that, in fact, is more common in boys than in girls with a male to female ratio of 2:1 (6). The excessive accumulation of fatty acids (FA) and triglycerides (TG) in the liver is mainly due to several aberrant events, including the reduction of FA oxidation, the influx increase, and efflux reduction of FA and, hence, increased *de novo* lipogenesis (7). Lipid accretion and subsequent lipotoxicity trigger intracellular signaling pathways, which result in pro-inflammatory cytokines that are responsible for immune cellular recruitment (8, 9). Disease progression is likely to depend on complex interplay between genetic predisposition (10, 11) and environmental factors (12-15). Although the prognosis of children with NAFLD is still unknown, high concentrations of serum saturated free FAs (SFA) are important in the pathogenesis severity since they create hepatotoxic impulse (16, 17).

As fatty liver does not have a proven pharmacological treatment in childhood (4, 5), various groups have used probiotics, metformin, ursodeoxycholic acid, L-carnitine and antioxidants, which showed conflicting and heterogeneous data in terms of improvement of different parameters (radiology, biochemistry, histology) (5, 18-20). Surprisingly, there are only limited studies on FA acid composition pertaining to youth NAFLD and on valuable impact of polyunsaturated fatty acids (PUFA) in childhood although diets rich in n-3 PUFAs may confer anti-inflammatory and cardiovascular benefits, particularly when

they replace SFAs and n-6 PUFA (21). However, patients with NAFLD display low intake of n-3 PUFA (22, 23), indicating that diets may represent a confining factor for circulating content of n-3 PUFA. On the other hand, ethnicity groups may have divergent fish consumption habits as recently reported (24).

The aims of our studies are to determine the plasma FA composition and to assess changes in the latter in response to n-3 PUFA supplementation in French-Canadian youth since (i) none of the available pharmacological agents could be recommended for treatment of children with NAFLD (25, 26); (ii) n-3 PUFA are quite safe diet supplements that showed efficacy in the prevention and therapy of cardiovascular diseases, dyslipidemia and metabolic syndrome (26-28); (iii) loss of n-3 PUFA dietary intake was found in pediatric NAFLD (24) and (iv) no attention has been given to French-Canadian population, which is primarily and historically located in the province of Quebec, has the highest prevalence worldwide of lipoprotein lipase deficiency, includes a large pool of individuals at risk for atherosclerosis and other lipid-related diseases (29, 30), and exhibits a founder effect among the 8,000 ancestors of present-day French-Canadians, who have had relatively little cross-breeding with individuals from other national origin groups (31).

2. MATERIALS AND METHODS

2.1 Subjects

The present randomized clinical trial, registered as NCT02201160 on www.clinicaltrials.gov, was performed on 30 NAFLD children followed as outpatients at the Gastroenterology/Hepatology and Nutrition clinic of MUHC Sainte-Justine and the Gastroenterology division of the Montreal Children's Hospital in Montreal. The children were between 8 and 18 years of age, with obesity and a diagnosis of NAFLD based on the results of a clinical evaluation, liver echography and magnetic resonance imaging-proton density fat fraction.

Importantly, in the registration of our study on the website of clinical trials, we mentioned that one of the specific outcomes will be to assess the impact of n-3 FAs on plasma transaminases in order to dissect their beneficial effects on liver abnormalities and NAFLD management. This study that was ongoing at the time has been completed and we are in the active process of data compilation and result analysis, which will allow us to bring the findings to the scientific community's attention. In this article, we mainly focused on the FA profile assessment to caracterize for the first time the young French Canadian subjects with NAFLD.

2.2 Inclusion and exclusion criteria

The children were eligible for the study if they were French Canadian, boys (according to the literature review on NAFLD prevalence), with a body weight $\geq 95^{th}$ percentile (based on the CDC 2000 Chart), aged <18 years, have a diffusely hyperechogenic liver at ultrasonography (consistent with NAFLD diagnosis), and have normal or elevated transaminases (> 2N). Moreover, the exclusion criteria include subjects having pin or cochlear implants that may affect the magnetic resonance imaging examination, subjects who consumed natural medicine products possibly leading to increased risk of haemorrhage, and those who were found to consume fish, flaxseed oil and foods enriched with n-3 PUFA, probiotics, vitamin E or drugs known to induce fatty liver during the study.

2.3 Study design

The present study was a 6-month, double-blind, one-way, crossover randomized study. The study samples were randomly assigned to two groups (**Figure**). The treatment consisting of n-3 PUFA supplement (NutriSanté Inc./Ponroy, Canada) was administered in two

phases, each of 3-month duration. In the first phase, an NAFLD group received an active n-3 PUFA supplement and another received equivalent quantities of sunflower oil as a placebo. During the second phase (after the first 3-month period), all NAFLD subjects received an active n-3 PUFA supplement. The study was approved by the Clinical Research Ethics Committee of MUHC Sainte-Justine (Montreal, Quebec). Informed consent was obtained from all subjects or parents before starting experimental procedures, and the study followed the Helsinki guidelines. A total of 30 NAFLD subjects were selected for the study but only twenty one French Canadian NAFLD boys (12 in n-3 PUFA and 9 in placebo group) succeeded in completing 6 months and were retained for the data analysis.

2.4 Fatty acid supplements

The dose supplementation considered for this study is 2.0 g of fish oil (NutriSanté Inc./Ponroy) per day, providing a total of 1.2 g of n-3 PUFA. This dose was chosen according to official recommendations (32) and based on our previous studies (33) and paediatric trials (34, 35). For comparison, in North American diets people consume about 500-1000 mg/day of n-3 PUFA [34] while the Inuit consumption is about 10 g/day, which gives them a plasma profile with high levels of n-3 PUFA (docosahexaenoic acid: DHA, eicosapentaenoic acid: EPA) and low n-6 FA (Arachidonic acid: AA) (36). Overall, 21 NAFLD subjects completed the randomized double-blind study with placebo (sunflower oil) or n-3 PUFA at a rate of 4 capsules per day. More specifically, each capsule of the n-3 PUFA supplement contained 500 mg of fish oil (each capsule providing 300 mg of EPA+DHA with 3.75 U vitamin E to prevent peroxidation). The placebo capsule contained 500 mg of sunflower oil with 3.75 U vitamin E. Noteworthy, the capsules were approved by Santé Canada.

Compliance to the study treatment was evaluated by pill count at every visit, review of medication records, and direct interview of patients by the physicians (FA and NA). At each visit, subjects and parents were specifically asked about n-3 usual food frequency consumption, adverse events, and the physician checked for any association between the treatment and adverse events.

2.5 Biochemical Analysis

Blood samples were collected in tubes containing 1 g EDTA/L after subjects had fasted for 12h overnight. Plasma was separated immediately by centrifugation (700*g* for 20 min) at 4

°C as described previously [37]. Blood tests measurements, including lipid and fatty acid analysis were performed (33, 37).

2.6 Fatty acid Analysis

FAs in whole plasma were assayed by an improved method described previously (33, 37). Briefly, each sample to be analyzed was subjected to direct trans-esterification and then injected into a gas chromatograph by using the Varian 8400 GC AutoSampler system (Varian Inc, Canada). The FAs were identified by comparison with the expected retention times of known standards and then analyzed with Galaxy Chromatography Data System software (Varian Inc).

2.7 Statistical Analysis

All values are expressed as mean ± SEM. Data were analyzed by using Student's two-tailed *t* test or a one-way ANOVA analysis variance and the differences between the means were assessed post-hoc using Tukey's test. Statistical significance was defined as $P < 0.05$.

3. RESULTS

3.1 Anthropometric and metabolic characteristics

Baseline anthropometric and liver characteristics of the young subjects are listed in **Table 1**. Overall, no differences were noted in the mean age distribution of NAFLD young patients compared to their healthy controls paired for age and sex. However, NAFLD subjects exhibited a significant (P <0.001) elevation in body weight and body mass index. In addition, a marked increase was evidenced in the hepatic alanine transaminase while aspartate transaminase and gamma-glutamyl transpeptidase remained in the normal range. Although there were no differences in glucose values between NAFLD and healthy groups, an increase was evidenced in fasting insulin levels and HOMA-IR index, suggesting an insulin resistance (IR) state in the NAFLD patients (**Table 2**). The latter were also characterized by inflammation given the high significant C-reactive protein value. According to the data of **Table 2,** NAFLD subjects presented higher TG levels, lower high-density lipoprotein (HDL)-cholesterol and apolipoprotein (apo) A-1 concentrations as compared with healthy controls. In view of their low HDL-cholesterol, NAFLD patients displayed higher total cholesterol/HDL-cholesterol and Apo B/Apo A-I ratios, which suggests an increased risk of cardiovascular disease (CVD). On the other hand, total and low-density lipoprotein cholesterol concentrations as well as systolic and diastolic blood pressure were not significantly different between the two groups. The elevated concentration of C-reactive protein suggests an inflammatory state in NAFLD subjects.

3.2 Plasma fatty acid composition in NAFLD compared to healthy control subjects

The first objective of the present study was to examine baseline FA composition of 21 NAFLD young patients in comparison with 33 healthy controls because no data is available for the pediatric French Canadian subjects. A small but significant increment was noted in palmitic and stearic acids, which enhanced the total proportion of SFA in NAFLD subjects (**Table 3**). A similar pattern was observed in total monounsaturated FA (probably due to oleic acid) of NAFLD subjects (**Table 3**). Chromatographic analysis revealed divergent profiles of PUFAs: while an increase was evidenced in total n-3 FA with the contribution of EPA (20:5n3), DPA (22:5n3) and DHA (22:6n3) acids, a decline characterized total n-6 FA owing mainly to linoleic acid (LA, 18:2n-6), but with an increase of AA (20:4n-6). **Table 4** clearly shows low ratios of elongase activity (18:2n-6/20:4n-6, 24:0/22:0 and

24:0/20:0) and high ratios of Δ6 (20:3n-6/18:2n-6) and Δ7 (16:1n-7/16:0) desaturase activities in NAFLD subjects. Δ5 (20:4n-6/20:3n-6) and Δ9 (18:1n-9/18:0) desaturase activities were lower in the NAFLD group that also exhibited low PUFA/saturates, essential FA (EFA)/non EFA and n-6/n-3 ratios. The calculation of the stearoyl-CoA desaturase (SCD) indices, a biomarkers of FA synthesis and liver lipogenesis, showed a significant increase in SCD1, SCD16 and lower SCD18, suggesting an association with increased obesity and metabolic syndrome.

3.3 Efficacy of n-3 PUFA supplementation in NAFLD subjects compared to placebo

To assess the effect of n-3 PUFA on NAFLD, two groups from our cohort were double blind supplemented either with n-3 PUFA or sunflower (as a placebo) for 3 months. The total NAFLD patients (n = 21) were randomized to either n-3 PUFA (n = 12) or placebo (n = 9) for the first three months. In a second step, the n-3 PUFA NAFLD subjects continued with the same dose of n-3 PUFA supplementation while the placebo group was placed on n-3 PUFA for another 3-month period. **Tables 5A and 5B** show a significant raise of EPA and DHA after 3 months in the group administered n-3 PUFA compared to the placebo group. This amelioration was confirmed by augmented total n-3 FA, DHA/AA ratio and Δ5 along with a lower n-6/n-3 ratio and SCD1 index (**Table 6**).

As the second objective was to assess whether a longer period of n-3 PUFA supplementation has a more beneficial effects in NAFLD individuals, we recorded the data after 6 months. **Tables 5A and 5B** document the same FA profile with only a slight increase in EPA, DHA, total n-3 and DHA/AA ratio along with a decrease of n-6/n-3 ratio, SCD1 and SCD16 indices in the two groups of NAFLD subjects (**Table 6**).

3.4 Plasma fatty acid composition in NAFLD after n-3 PUFA supplementation

The final objective of the current investigation was to determine whether n-3 PUFA supplementation improves FA composition of NAFLD patients. Clearly, **Table 7** illustrates that n-3 PUFA intake significantly raised the proportion of EPA and DHA at the expense of n-6 FAs, including AA and docosatetraenoic (22:4n-6) acids. Consequently, there was a raise in total n-3 FA, DHA/AA ratio and Δ5 as well as a decrease of monounsaturated FA, n-6/n-3, SCD1 and SCD16 indices (**Table 8**).

4. DISCUSSION

Available literature indicates that n-3 FA may be instrumental in the treatment of NAFLD (38-42), the most common liver disease in Western countries (43). As n-3 PUFA may be effective in reducing triglyceride content, IR and inflammation, all processes related to NAFLD (44, 45), they appear as a potential, natural therapeutic approach for liver steatosis (24). However, variable and inconsistent results are apparent from the reported trials centring on cardio-metabolic diseases (46). In addition, only limited studies are available in childhood (38, 39). Finally, little focus has been placed on the French-Canadians, a population with a known founder effect (47), characterized by specific mutations, and in which fewer gene variants are expected to contribute to the determination of complex traits, such as blood pressure, dyslipidemia and metabolic syndrome (48-50). Therefore, the aims of the current study were to establish, for the first time, the distribution of plasma FA in French-Canadian children with NAFLD, to test the clinical tolerability and impact of safety n-3 PUFA supplements on plasma FA composition, and to explore whether longer periods in the six-month window are more beneficial. Our results point out the high levels of all FA classes, which likely contributed to the significant elevation of total FA in French-Canadians children and adolescents with NAFLD. Against our expectations, the baseline of palmitoleic (16:1n-7) and oleic (18:1n-9) acids was elevated suggesting specific alterations in hepatic lipid composition characterizing the spectrum of NAFLD (51) and it was accompanied by a significant raising of the n-3 FA family. However, various ratios reflecting the activities of elongase and desaturase enzymes suggest irregular metabolic interrelationships among FAs in our young NAFLD patients. These observations were confirmed by the lowered SCD18 and elevated SCD1 and SCD16 indices, where some studies reported their association with obesity and metabolic syndrome (52, 53). Interestingly, supplementation of n-3 PUFA led to circulating long-chain n-3 FA, including EPA, DPA DHA and DHA/AA ratio, thereby resulting in reduced n-6/n-3 ratio. These captivating data were obtained with 3-month supplementation with little amelioration of an additional 3-month period.

Previous experimental interventions showed augmented insulin sensitivity, lowering of intra-hepatic TG content and improvement of steatohepatitis in animals following the administration of n-3 PUFA (54, 55). Furthermore, only limited human studies reported a

positive effect on fatty liver: the first with 42 NAFLD patients aged 56 to 62 years (56), the second with 23 NASH patients aged 27 to 74 years and with biopsy-proven NASH (57), and the third with 60 children with biopsy-proven NAFLD (41). Noteworthy, no FA profile was provided in these three trials and no control healthy group was available, except the placebo group. For the first time, our study examined the baseline FA profiles in French-Canadian children and reported the influence of n-3 long-chain FA supplementation on FA pattern. We could not detect depletion of plasma n-3 FA either in absolute concentrations (μM) or in percent (%) distribution. However, various alterations were noticed: (i) the n-6 family was characterized by low % of LA, an essential FA and high % of AA acid whose major metabolic pathways are relevant for inflammation and metabolic syndrome manifestations, including liver steatosis (58, 59); (ii) the raised palmitic (16:00), stearique (18:00) and dihomo-γ-linolenic (20:3n-6) acids reflecting a possible correlation with the inflammatory markers (60); (iii) there was an increased ratios of 16:1n-7/18:2n-6 and total n-7/18:2n-6, indicative of EFA deficiency (61)**;** (iv) the elongation and desaturation of LA was stimulated as suggested by the lower Δ5 (20:4n-6/20:3n-6) and 18:2n-6/20:4n-6 as well as a raise of Δ6 (20:3n-6/18:2n-6) ratios, thereby contributing the increased risk of CVD and NAFLD (62, 63); (v) in contrast, evidence was obtained as to the low concentration of PUFA and high proportions of long-chain FAs such as EPA, DPA, DHA and DHA/AA ratio that improve the prognosis of CVD and NAFLD (62-66); and (vi) On the other hand, there was a less conversion of EPA to DHA as advocated by the ALA/EPA ratio, suggesting a lipid peroxidation (37). Overall, our data are not completely in favour or against the cardio-metabolic risks given the opposite trends of n-3 and n-6 in French-Canadian children with NAFLD.

Many FA can be elongated and desaturated by specific enzymes. In particular, FA desaturases introduce a double bond in a specific position of long-chain FA, which enhances the degree of FA unsaturation, thereby impacting on the physical properties of membrane phospholipids. Furthermore, metabolites of PUFA may be used as signaling molecules in many systems. Δ9 desaturase catalyzes the biogenesis of monounsaturates whereas Δ6 and Δ5 desaturases contribute to the synthesis of highly unsaturated FA that compose phospholipids and maintain membrane fluidity. There are indications from the present study that the activity of Δ6 is stimulated and that of Δ5 is down-regulated (as

estimated using FA product/ precursor ratios), which were found to correlate to the metabolic syndrome (62, 63, 67, 68). Noteworthy, the role of desaturases in the development of excess liver fat is not fully understood, but the 16:1n–7 to 16:0 ratio used to estimate SCD16 or SD1 (16:1n-7/18:1n-9) activity has been proposed as an indirect biomarker of *de novo* lipogenesis (52), obesity and IR (53), a pathways known to be increasingly recognized as an important contributors to hepatic TG concentrations in NAFLD (69, 70). In our NAFLD patients, the SCD1 and SCD16 indices concomitantly with circulating TGs and IR were found elevated, which suggests not only a raised *de novo* lipogenesis, but also various the development of metabolic disorders as reported by other groups (71-73).

Recently, it has been postulated that potential alterations in the metabolic pathway of PUFA synthesis could constitute a fundamental trigger in the initiation and propagation of metabolic abnormalities, such as IR (62, 63, 74). In our study, treatment of NAFLD children during 3 months with n-3 PUFA had a significant effect on the absolute values and proportions of plasma n-3 FA as was recently reported by Nobili et al (38, 41). There was a concomitantly significant increment of EPA and DHA, as well as a decrease in LA, which essentially reflected the dietary FA composition. On the other hand, a consistent AA decline characterized n-6 FA family. Taken together, the observed findings may be significant with regards to the role of n-3 PUFA in NAFLD-related disorders. Indeed, although limited, the clinical studies performed so far have reported reduced hepatic lipid content by directly testing liver biopsies (57) or via ultrasonography (56, 75) in response to n-3 long-chain FA administration. Similarly, treatment with n-3 long-chain FA resulted in improved liver function markers along with beneficial effects on circulating TG and TNF-α (56, 57, 75, 76). It is important to mention that n-3 long-chain FAs were fully safe in our young NAFLD patients. According to our careful monitoring, no adverse reactions or especially complaints about bleeding were reported. N-3 treatment seemed to be safe following duration of both 3 and 6 months. Nevertheless, no amplified beneficial effects were observed with the long-term period. Our data are therefore in line with previous studies that reported the safety of n-3 PUFA supplements (26, 27, 75). This important feature, on the one hand, and the paucity of effective treatment with pharmacological drugs in both children and adults with NAFLD, on the other hand (77) suggests that n-3 PUFA

may represent an interesting and useful option. In this context, two clinical trials employing vitamin E and pioglitazone in NAFLD have recently showed conflicting results (18, 78).

5. CONCLUSIONS

Our results document for the first time the composition of plasma FAs in French Canadian children with NAFLD. They also reveal the efficacy of supplementation to improve the proportion of n-3 PUFA in their plasma. Additional studies are necessary to highlight the efficacy to improve the total symptom score in these patients.

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SS was involved in the study concept and design, acquisition, analysis and interpretation of data, revision of the manuscript, technical support, statistical analysis and study supervision.

EL contributed to the study concept and design, data analysis, drafting, writing and critical revision of the manuscript, for important intellectual content, obtained funding, material support and study supervision.

SS, FA and NA contributed to recruiting patients and data analysis.

JD and NP contributed to study concept and design

All authors read and approved the final manuscript.

REFERENCES

(1) B.W. Smith, L.A. Adams, Non-alcoholic fatty liver disease, Crit Rev Clin Lab Sci, 48 (2011) 97-113.

(2) E. Hui, A. Xu, Y.H. Bo, K.S. Lam, Obesity as the common soil of non-alcoholic fatty liver disease and diabetes: Role of adipokines, J Diabetes Investig, 4 (2013) 413-425.

(3) E. Vanni, E. Bugianesi, A. Kotronen, M.S. De, H. Yki-Jarvinen, G. Svegliati-Baroni, From the metabolic syndrome to NAFLD or vice versa?, Dig Liver Dis, 42 (2010) 320-330.

(4) J.B. Schwimmer, R. Deutsch, T. Kahen, J.E. Lavine, C. Stanley, C. Behling, Prevalence of fatty liver in children and adolescents, Pediatrics, 118 (2006) 1388-1393.

(5) S. Berardis, E. Sokal, Pediatric non-alcoholic fatty liver disease: an increasing public health issue, Eur J Pediatr, 173 (2014) 131-139.

(6) J.D. Browning et al., Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity, Hepatology, 40 (2004) 1387-1395.

(7) S. Parekh, F.A. Anania, Abnormal lipid and glucose metabolism in obesity: implications for nonalcoholic fatty liver disease, Gastroenterology, 132 (2007) 2191-2207.

(8) F. Marra, A. Gastaldelli, B.G. Svegliati, G. Tell, C. Tiribelli, Molecular basis and mechanisms of progression of non-alcoholic steatohepatitis, Trends Mol Med, 14 (2008) 72-81.

(9) V. Bieghs, C. Trautwein, The innate immune response during liver inflammation and metabolic disease, Trends Immunol, 34 (2013) 446-452.

(10) Y.C. Lin, P.F. Chang, M.H. Chang, Y.H. Ni, Genetic variants in GCKR and PNPLA3 confer susceptibility to nonalcoholic fatty liver disease in obese individuals, Am J Clin Nutr, 99 (2014) 869-874.

(11) H. Karaman et al., Investigation of genome instability in patients with non-alcoholic steatohepatitis, World J Gastroenterol, 19 (2013) 5295-5301.

(12) C.F. Del et al., Meta-omic platforms to assist in the understanding of NAFLD gut microbiota alterations: tools and applications, Int J Mol Sci, 15 (2014) 684-711.

(13) R. Kelishadi, P. Poursafa, Obesity and air pollution: global risk factors for pediatric non-alcoholic fatty liver disease, Hepat Mon, 11 (2011) 794-802.

(14) A. Alisi, S. Cianfarani, M. Manco, C. Agostoni, V. Nobili, Non-alcoholic fatty liver disease and metabolic syndrome in adolescents: pathogenetic role of genetic background and intrauterine environment, Ann Med, 44 (2012) 29-40.

(15) A. Hamabe et al., Impact of cigarette smoking on onset of nonalcoholic fatty liver disease over a 10-year period, J Gastroenterol, 46 (2011) 769-778.

(16) V. Nobili, Non-alcoholic fatty liver disease in children and adolescents, Clin Biochem, (2014).

(17) K. Nanda, Non-alcoholic steatohepatitis in children, Pediatr Transplant, 8 (2004) 613-618.

(18) J.E. Lavine et al., Effect of vitamin E or metformin for treatment of nonalcoholic fatty liver disease in children and adolescents: the TONIC randomized controlled trial, JAMA, 305 (2011) 1659-1668.

(19) L.N. Bell et al., Relationship between adipose tissue insulin resistance and liver histology in nonalcoholic steatohepatitis: a pioglitazone versus vitamin E versus placebo for the treatment of nondiabetic patients with nonalcoholic steatohepatitis trial follow-up study, Hepatology, 56 (2012) 1311-1318.

(20) M. Malaguarnera et al., L-carnitine supplementation to diet: a new tool in treatment of nonalcoholic steatohepatitis--a randomized and controlled clinical trial, Am J Gastroenterol, 105 (2010) 1338-1345.

(21) E.M. McCarthy, M.E. Rinella, The role of diet and nutrient composition in nonalcoholic Fatty liver disease, J Acad Nutr Diet, 112 (2012) 401-409.

(22) B. Koletzko et al., Dietary intake of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in children - a workshop report, Br J Nutr, 103 (2010) 923-928.

(23) P.M. Kris-Etherton, J.A. Grieger, T.D. Etherton, Dietary reference intakes for DHA and EPA, Prostaglandins Leukot Essent Fatty Acids, 81 (2009) 99-104.

(24) D.E. St-Jules et al., Estimation of fish and omega-3 fatty acid intake in pediatric nonalcoholic fatty liver disease, J Pediatr Gastroenterol Nutr, 57 (2013) 627-633.

(25) P. Vajro, G. Paolella, M. Poeta, C. Pizza, M. Sangermano, G. Massa, Pediatric non alcoholic fatty liver disease: more on novel treatment targets, BMC Pediatr, 13 (2013) 109.

(26) P. Vajro et al., Therapeutic options in pediatric non alcoholic fatty liver disease: current status and future directions, Ital J Pediatr, 38 (2012) 55.

(27) C. Wang et al., n-3 Fatty acids from fish or fish-oil supplements, but not alpha-linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review, Am J Clin Nutr, 84 (2006) 5-17.

(28) J.H. Lee, J.H. O'Keefe, C.J. Lavie, R. Marchioli, W.S. Harris, Omega-3 fatty acids for cardioprotection, Mayo Clin Proc, 83 (2008) 324-332.

(29) Y. Ma et al., A mutation in the human lipoprotein lipase gene as the most common cause of familial chylomicronemia in French Canadians, N Engl J Med, 324 (1991) 1761-1766.

(30) E. Leitersdorf, E.J. Tobin, J. Davignon, H.H. Hobbs, Common low-density lipoprotein receptor mutations in the French Canadian population, J Clin Invest, 85 (1990) 1014-1023.

(31) B.M. De et al., Founder effect in familial hyperchylomicronemia among French Canadians of Quebec, Hum Hered, 41 (1991) 168-173.

(32) M.E. Surette, The science behind dietary omega-3 fatty acids, CMAJ, 178 (2008) 177-180.

(33) S.A. Belanger et al., Omega-3 fatty acid treatment of children with attention-deficit hyperactivity disorder: A randomized, double-blind, placebo-controlled study, Paediatr Child Health, 14 (2009) 89-98.

(34) N. Chahal, C. Manlhiot, H. Wong, B.W. McCrindle, Effectiveness of Omega-3 Polysaturated Fatty Acids (Fish Oil) Supplementation for Treating Hypertriglyceridemia in Children and Adolescents, Clin Pediatr (Phila), 53 (2014) 645-651.

(35) G.P. Amminger et al., Omega-3 fatty acid supplementation in adolescents with borderline personality disorder and ultra-high risk criteria for psychosis: a post hoc subgroup analysis of a double-blind, randomized controlled trial, Can J Psychiatry, 58 (2013) 402-408.

(36) N. Kromann, A. Green, Epidemiological studies in the Upernavik district, Greenland. Incidence of some chronic diseases 1950-1974, Acta Med Scand, 208 (1980) 401-406.

(37) S. Spahis, M. Vanasse, S.A. Belanger, P. Ghadirian, E. Grenier, E. Levy, Lipid profile, fatty acid composition and pro- and anti-oxidant status in pediatric patients with attention-deficit/hyperactivity disorder, Prostaglandins Leukot Essent Fatty Acids, 79 (2008) 47-53.

(38) V. Nobili et al., Docosahexaenoic acid for the treatment of fatty liver: randomised controlled trial in children, Nutr Metab Cardiovasc Dis, 23 (2013) 1066-1070.

(39) W. Janczyk et al., Omega-3 fatty acids for treatment of non-alcoholic fatty liver disease: design and rationale of randomized controlled trial, BMC Pediatr, 13 (2013) 85.

(40) E. Scorletti et al., Design and rationale of the WELCOME trial: a randomised, placebo controlled study to test the efficacy of purified long chain omega-3 fatty treatment in non-alcoholic fatty liver disease, Contemp Clin Trials, 37 (2014) 301-311.

(41) V. Nobili et al., Docosahexaenoic acid supplementation decreases liver fat content in children with non-alcoholic fatty liver disease: double-blind randomised controlled clinical trial, Arch Dis Child, 96 (2011) 350-353.

(42) F. Sofi et al., Effects of a 1-year dietary intervention with n-3 polyunsaturated fatty acid-enriched olive oil on non-alcoholic fatty liver disease patients: a preliminary study, Int J Food Sci Nutr, 61 (2010) 792-802.

(43) J.P. Ong, Z.M. Younossi, Epidemiology and natural history of NAFLD and NASH, Clin Liver Dis, 11 (2007) 1-16, vii.

(44) F. Bril et al., Relationship between disease severity, hyperinsulinemia, and impaired insulin clearance in patients with nonalcoholic steatohepatitis, Hepatology, 59 (2014) 2178-2187.

(45) C.D. Byrne, G. Targher, Ectopic fat, insulin resistance, and nonalcoholic Fatty liver disease: implications for cardiovascular disease, Arterioscler Thromb Vasc Biol, 34 (2014) 1155-1161.

(46) D.J. Lou et al., Serum phospholipid omega-3 polyunsaturated fatty acids and insulin resistance in type 2 diabetes mellitus and non-alcoholic fatty liver disease, J Diabetes Complications, (2014).

(47) C. Betard, A.M. Kessling, M. Roy, A. Chamberland, S. Lussier-Cacan, J. Davignon, Molecular genetic evidence for a founder effect in familial hypercholesterolemia among French Canadians, Hum Genet, 88 (1992) 529-536.

(48) B.M. De, Hereditary disorders in Saguenay-Lac-St-Jean (Quebec, Canada), Hum Hered, 41 (1991) 141-146.

(49) P. Hamet et al., Quantitative founder-effect analysis of French Canadian families identifies specific loci contributing to metabolic phenotypes of hypertension, Am J Hum Genet, 76 (2005) 815-832.

(50) H.H. Hobbs, M.S. Brown, D.W. Russell, J. Davignon, J.L. Goldstein, Deletion in the gene for the low-density-lipoprotein receptor in a majority of French Canadians with familial hypercholesterolemia, N Engl J Med, 317 (1987) 734-737.

(51) P. Puri et al., The plasma lipidomic signature of nonalcoholic steatohepatitis, Hepatology, 50 (2009) 1827-1838.

(52) J.J. Lee et al., Palmitoleic acid is elevated in fatty liver disease and reflects hepatic lipogenesis, Am J Clin Nutr, 101 (2015) 34-43.

(53) I. Rudkowska et al., Cardiometabolic risk factors are influenced by Stearoyl-CoA Desaturase (SCD) -1 gene polymorphisms and n-3 polyunsaturated fatty acid supplementation, Mol Nutr Food Res, 58 (2014) 1079-1086.

(54) M. Rossmeisl et al., Omega-3 phospholipids from fish suppress hepatic steatosis by integrated inhibition of biosynthetic pathways in dietary obese mice, Biochim Biophys Acta, 1841 (2014) 267-278.

(55) C.M. Depner, K.A. Philbrick, D.B. Jump, Docosahexaenoic acid attenuates hepatic inflammation, oxidative stress, and fibrosis without decreasing hepatosteatosis in a Ldlr(-/-) mouse model of western diet-induced nonalcoholic steatohepatitis, J Nutr, 143 (2013) 315-323.

(56) M. Capanni et al., Prolonged n-3 polyunsaturated fatty acid supplementation ameliorates hepatic steatosis in patients with non-alcoholic fatty liver disease: a pilot study, Aliment Pharmacol Ther, 23 (2006) 1143-1151.

(57) N. Tanaka, K. Sano, A. Horiuchi, E. Tanaka, K. Kiyosawa, T. Aoyama, Highly purified eicosapentaenoic acid treatment improves nonalcoholic steatohepatitis, J Clin Gastroenterol, 42 (2008) 413-418.

(58) J.P. Allard et al., Nutritional assessment and hepatic fatty acid composition in non-alcoholic fatty liver disease (NAFLD): a cross-sectional study, J Hepatol, 48 (2008) 300-307.

(59) J. Monteiro, M. Leslie, M.H. Moghadasian, B.M. Arendt, J.P. Allard, D.W. Ma, The role of n - 6 and n - 3 polyunsaturated fatty acids in the manifestation of the metabolic syndrome in cardiovascular disease and non-alcoholic fatty liver disease, Food Funct, 5 (2014) 426-435.

(60) M. Perreault et al., Plasma levels of 14:0, 16:0, 16:1n-7, and 20:3n-6 are positively associated, but 18:0 and 18:2n-6 are inversely associated with markers of inflammation in young healthy adults, Lipids, 49 (2014) 255-263.

(61) G. Lepage, E. Levy, N. Ronco, L. Smith, N. Galeano, C.C. Roy, Direct transesterification of plasma fatty acids for the diagnosis of essential fatty acid deficiency in cystic fibrosis, J Lipid Res, 30 (1989) 1483-1490.

(62) J. Mayneris-Perxachs et al., Plasma fatty acid composition, estimated desaturase activities, and their relation with the metabolic syndrome in a population at high risk of cardiovascular disease, Clin Nutr, 33 (2014) 90-97.

(63) L.M. Steffen et al., Serum phospholipid and cholesteryl ester fatty acids and estimated desaturase activities are related to overweight and cardiovascular risk factors in adolescents, Int J Obes (Lond), 32 (2008) 1297-1304.

(64) A. Yamamoto, T. Yamamura, A. Kawaguchi, K. Kameda, Y. Matsuzawa, Triglyceride and glucose intolerance as a risk factor for coronary heart disease, Cardiology, 78 (1991) 185-193.

(65) K. Mayer et al., Short-time infusion of fish oil-based lipid emulsions, approved for parenteral nutrition, reduces monocyte proinflammatory cytokine generation and adhesive interaction with endothelium in humans, J Immunol, 171 (2003) 4837-4843.

(66) L. Tavazzi et al., Effect of n-3 polyunsaturated fatty acids in patients with chronic heart failure (the GISSI-HF trial): a randomised, double-blind, placebo-controlled trial, Lancet, 372 (2008) 1223-1230.

(67) M.M. Sethom et al., Plasma fatty acids profile and estimated elongase and desaturases activities in Tunisian patients with the metabolic syndrome, Prostaglandins Leukot Essent Fatty Acids, 85 (2011) 137-141.

(68) A. Kawashima et al., Plasma fatty acid composition, estimated desaturase activities, and intakes of energy and nutrient in Japanese men with abdominal obesity or metabolic syndrome, J Nutr Sci Vitaminol (Tokyo), 55 (2009) 400-406.

(69) K.L. Donnelly, C.I. Smith, S.J. Schwarzenberg, J. Jessurun, M.D. Boldt, E.J. Parks, Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease, J Clin Invest, 115 (2005) 1343-1351.

(70) F. Diraison, P. Moulin, M. Beylot, Contribution of hepatic de novo lipogenesis and reesterification of plasma non esterified fatty acids to plasma triglyceride synthesis during non-alcoholic fatty liver disease, Diabetes Metab, 29 (2003) 478-485.

(71) R. Gutierrez-Juarez et al., Critical role of stearoyl-CoA desaturase-1 (SCD1) in the onset of diet-induced hepatic insulin resistance, J Clin Invest, 116 (2006) 1686-1695.

(72) A. Dobrzyn, J.M. Ntambi, The role of stearoyl-CoA desaturase in body weight regulation, Trends Cardiovasc Med, 14 (2004) 77-81.

(73) Z.Z. Li, M. Berk, T.M. McIntyre, A.E. Feldstein, Hepatic lipid partitioning and liver damage in nonalcoholic fatty liver disease: role of stearoyl-CoA desaturase, J Biol Chem, 284 (2009) 5637-5644.

(74) J. Araya, R. Rodrigo, P. Pettinelli, A.V. Araya, J. Poniachik, L.A. Videla, Decreased liver fatty acid delta-6 and delta-5 desaturase activity in obese patients, Obesity (Silver Spring), 18 (2010) 1460-1463.

(75) F.S. Zhu, S. Liu, X.M. Chen, Z.G. Huang, D.W. Zhang, Effects of n-3 polyunsaturated fatty acids from seal oils on nonalcoholic fatty liver disease associated with hyperlipidemia, World J Gastroenterol, 14 (2008) 6395-6400.

(76) L. Spadaro et al., Effects of n-3 polyunsaturated fatty acids in subjects with nonalcoholic fatty liver disease, Dig Liver Dis, 40 (2008) 194-199.

(77) P. Socha, A. Horvath, P. Vajro, P. Dziechciarz, A. Dhawan, H. Szajewska, Pharmacological interventions for nonalcoholic fatty liver disease in adults and in children: a systematic review, J Pediatr Gastroenterol Nutr, 48 (2009) 587-596.

(78) A.J. Sanyal et al., Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis, N Engl J Med, 362 (2010) 1675-1685.

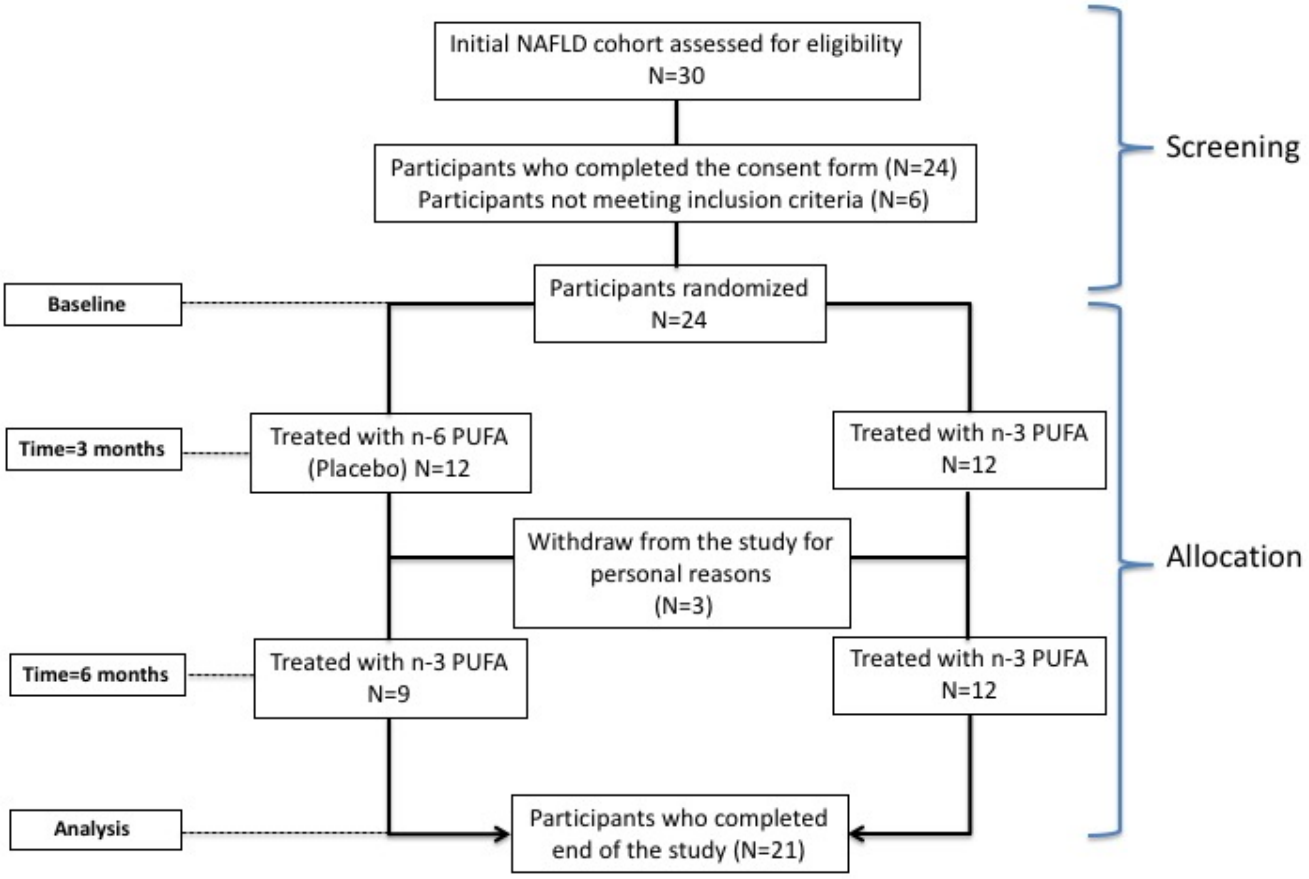
Initial NAFLD cohort assessed for eligibility
N=30
Participants who completed the consent form (N=24)
Participants not meeting inclusion criteria (N=6)
Participants randomized
N=24
Screening
Baseline
Time=3 months
Treated with n-6 PUFA
(Placebo) N=12
Treated with n-3 PUFA
N=12
Withdraw from the study for
personal reasons
(N=3)
Allocation
Time=6 months
Treated with n-3 PUFA
N=9
Treated with n-3 PUFA
N=12
Analysis
Participants who completed
end of the study (N=21)

Table 1: Baseline anthropometric and liver characteristics of NAFLD patients compared to healthy controls

Variables	Controls (N=33)	NAFLD (N=21)	*P values*
Age (y)	14.22 ± 0.64	14.09 ± 0.70	NS
Body Weight (kg)	54.25 ± 3.50	85.91 ± 5.55	<0.001
BMI (Kg/m2)	20.25 ± 0.70	31.88 ± 1.09	<0.001
ALT (U/L)	NR: (5-25)	38.95 ± 4.50	-
AST (U/L)	NR: (11-43)	29.4 ± 1.78	-
GGT (U/L)	NR: (3-43)	25.73 ± 4.16	-

BMI, Body mass index; ALT, alanine transaminase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase;

NS: Not significant; NR: Normal range.

All values are expressed as mean ± SD or normal range of values. Data were analyzed by using a Student's *t* test. Statistical significance was defined as $P<0.05$.

Table 2: Baseline biochemical characteristics of NAFLD patients compared to healthy controls

Variables	Controls (N=33)	NAFLD (N=21)	*P values*
Metabolic biomarkers			
Fasting blood Glucose (mmol/L)	5.25 ± 0.05	5.27 ± 0.06	NS
Insulin (pmol/L)	36.43 ± 3.43	147 ± 17.14	<0.001
HOMA-IR	1.24 ± 0.12	6.88 ± 1.07	<0.001
SBP (mmHg)	115.06 ± 2.77	114.90 ± 3.18	NS
DBP (mmHg)	57.32 ± 1.19	58.05 ± 1.40	NS
Lipids			
Triglycerides (mmol/L)	0.84 ± 0.07	1.44 ±0.20	<0.01
Total cholesterol (mmol/L)	3.96 ± 0.11	4.00 ± 0.23	NS
HDL-C (mmol/L)	1.22 ± 0.01	0.95 ± 0.05	<0.001
LDL-C (mmol/L)	2.35 ± 0.10	2.40 ± 0.18	NS
Total cholesterol/HDL-C	3.25 ± 0.09	4.39 ± 0.29	<0.001
Apo AI (g/L)	1.65 ± 0.04	1.14 ± 0.02	<0.001
Apo B (g/L)	0.66 ± 0.03	0.73 ± 0.05	<0.001
Apo B/Apo AI	0.48 ± 0.03	0.63 ± 0.04	<0.001
Inflammatory biomarker			
CRP (mg/L)	0.44 ± 0.08	3.43 ± 1.13	<0.001

Apo, Apolipoprotein; DBP, Diastolic blood pressure; CRP, C reactive protein; HDL, High-density lipoprotein; LDL, Low-density lipoprotein; NS, Not significant; SBP, Systolic blood pressure

All values are expressed as mean ± SD. Data were analyzed by using a Student's *t* test. Statistical significance was defined as $P<0.05$ vs. Controls.

Table 3 : Plasma fatty acid profile in paediatric NAFLD patients compared to healthy controls

Fatty Acids (FA)	CTR (N=33)	NAFLD (N=21)	*P values*	CTR (N=33)	NAFLD (N=21)	*P values*
	Composition (%)			Distribution (µM)		
SFA						
MA (C14:0)	1.29 ± 0.08	1.35 ± 0.08	NS	166.65 ± 15.90	209.63 ± 27.58	NS
PA (C16:0)	25.46 ± 0.25	25.29 ± 0.45	NS	3178.58 ± 129.78	3795.15 ± 353.16	<0.05
SA (C18:0)	6.27 ± 0.09	7.08 ± 0.14	<0.001	775.62 ± 24.84	1028.59 ± 64.64	<0.001
MUFA						
POA (C16 :1n-7)	2.00 ± 0.11	2.49 ± 0.15	<0.05	251.62 ± 19.81	375.65 ± 43.00	<0.001
OA (C18:1n9)	18.27 ± 0.37	19.82 ± 0.54	<0.01	2299.95 ± 124.76	2967.26 ± 294.50	<0.05
EIA (C20:1n9)	18.27 ± 0.37	0.13 ± 0.00	<0.001	20.30 ± 2.11	19.52 ± 1.79	<NS
ETA (C20:3n9)	0.08 ± 0.00	0.10 ± 0.00	<0.01	10.16 ± 0.82	15.66 ± 1.73	<0.01
ERA (C22:1n9)	0.25 ± 0.01	0.12 ± 0.01	<0.001	31.75 ± 2.10	17.29 ± 1.61	<0.001
NA (C24:1n9)	0.58 ± 0.02	0.76 ± 0.03	<0.001	71.07 ± 2.43	109.75 ± 7.64	<0.001
PUFA						
n-3 series						
ALA (C18:3n3)	0.78 ± 0.06	0.75 ± 0.05	NS	100.23 ± 10.73	115.21 ± 13.86	NS
EPA (C20:5n3)	0.28 ± 0.01	0.62 ± 0.06	<0.001	35.29 ± 2.26	91.91 ± 11.02	<0.001
DPA (C22:5n3)	0.27 ± 0.01	0.37 ± 0.02	<0.001	33.68 ± 1.57	58.76 ± 10.21	<0.01
DHA (C22:6n3)	0.82 ± 0.05	1.29 ± 0.10	<0.001	99.47 ± 5.54	194.49 ± 22.68	<0.001
n-6 series						
LA (C18 :2n-6)	32.03 ± 0.65	27.44 ± 0.76	<0.001	3953.27 ± 125.48	4002.72 ± 266.30	NS
GLA (C18:3n6)	0.40 ± 0.02	0.51 ± 0.03	<0.01	51.31 ± 4.16	78.41 ± 9.76	<0.01
HGLA (C20:3n6)	1.17 ± 0.11	1.67 ± 0.07	<0.01	146.93 ± 16.93	248.94 ± 26.47	<0.01
AA (C20:4n6)	5.16 ± 0.18	5.83 ± 0.19	<0.05	631.75 ± 22.68	858.70 ± 66.35	<0.001
DIA (C22:2n6)	0.00 ± 0.00	0.02 ± 0.00	<0.001	0.43 ± 0.09	3.20 ± 0.63	<0.001

DTA (C22:4n6)	0.10 ± 0.00	0.13 ± 0.00	<0.01	13.01 ± 0.83	21.66 ± 3.41	<0.01
Total n-3 FA	2.20 ± 0.07	3.09 ± 0.19	<0.001	273.69 ± 12.57	466.37 ± 52.80	<0.001
Total n-6 FA	39.05 ± 0.58	35.79 ± 0.82	<0.01	4819.98 ± 138.06	5237.76 ± 360.29	NS
Total n-7 FA	3.45 ± 0.11	3.89 ± 0.17	<0.05	433.06 ± 23.20	587.27 ± 61.48	<0.01
Total n-9 FA	19.37 ± 0.37	20.94 ± 0.52	<0.05	2433.24 ± 127.03	3129.50 ± 303.17	<0.05
Total SFA	34.98 ± 0.30	35.65 ± 0.49	NS	4361.62 ± 170.56	5311.82 ± 457.41	<0.01
Total MUFA	23.18 ± 0.43	25.07 ± 0.57	<0.001	2909.37 ± 147.75	3749.41 ± 358.20	NS
Total PUFA	41.34 ± 0.58	38.98 ± 0.84	<0.001	5103.84 ± 146.77	5719.80 ± 406.12	NS
TOTAL FA				12434.06 ± 435.06	14822.45 ± 1191.69	<0.001

AA, Arachidonic acid; ALA, Alpha linolenic acid; CTR, Controls; DHA, Docosahexanoic acid; DIA, Docosadienoic acid; DPA, Docosapentaenoic acid ; DTA, Docosatetraenoic acid; EIA, Eicosenoic acid; EPA, Eicosapentaenoic acid; ERA, Erucic acid; ETA, Eicosatrienoic acid; FA, Fatty acid; GLA, Gamma linoleic acid; HGLA, Homogamma linolenic acid; LA, Linoleic acid; MA, Myristic acid; MUFA, Monounsaturated fatty acid; NA, Nervonic acid; OA, Oleic acid; PA, Palmitic acid; POA, Palmitoleic acid; PUFA, Polyunsaturated fatty acid; SA, Stearic acid; SFA, Saturated fatty acid

All values are expressed as mean ± SD. Data were analyzed by using a Student's *t* test. Statistical significance was defined as $P<0.05$ vs. Controls.

Table 4: Plasma Fatty acid ratios in NAFLD patients compared to healthy controls

Ratios	CTR (N=33)	NAFLD (N=21)	*P values*
16:1n-7/18:2n-6	0.06 ± 0.00	0.09 ± 0.00	<0.001
18:2n-6/20:4n-6	6.41 ± 0.28	4.80 ± 0.20	<0.001
20:3n-9/20:4n-6	0.01 ± 0.00	0.01 ± 0.00	NS
Total n-7/18:2n-6	0.10 ± 0.00	0.14 ± 0.01	<0.001
24:0/22:0	1.05 ± 0.22	0.54 ± 0.01	NS
24:0/20:0	2.64 ± 0.54	1.52 ± 0.13	<0.05
PUFA/SFA	1.26 ± 0.02	1.10 ± 0.03	<0.001
EFA (LA+ALA)/NON-EFA	0.49 ±0.01	0.39 ±0.01	<0.001
EPA/DHA	0.36 ± 0.02	0.50 ± 0.04	<0.01
DHA/AA	0.16 ± 0.00	0.22 ± 0.01	<0.01
ALA/EPA	3.13 ± 0.37	1.44 ± 0.17	<0.001
ALA/LA	0.02 ± 0.00	0.02 ± 0.00	NS
n-6/n-3	18.16 ± 0.66	12.44 ± 0.74	<0.001
Δ5 (20:4n-6/20 :3n-6)	4.99 ± 0.29	3.60 ± 0.18	<0.001
Δ6 (20:3n-6/18:2n-6)	0.03 ± 0.00	0.06 ± 0.00	<0.001
SCD1 index (16:1n-7/18:1n-9)	0.09 ± 0.00	0.13 ± 0.00	<0.001
SCD16 index (16:1n-7/16:0)	0.06 ± 0.00	0.09 ± 0.00	<0.001
SCD18 index (18:1n-9/18:0)	2.94 ± 0.08	2.83 ± 0.10	<0.05

AA, Arachidonic acid; ALA, Alpha linolenic acid; CTR, Controls; DHA, Docosahexanoic acid; EFA, Essential fatty acid; EPA, Eicosapentaenoic acid; FA, Fatty acid; LA, Linoleic acid; PUFA, Polyunsaturated fatty acid; SFA, Saturated fatty acid

All values are expressed as mean ± SD. Data were analyzed by using a Student's *t* test. Statistical significance was defined as $P<0.05$ vs. Controls.

Table 5A: Comparative plasma fatty acid composition in NAFLD patients following 3- and 6-month treatments with n-3 PUFA or n-6 placebo

	Baseline		3 month- treatment		6 month-treatment	
	n-6 Placebo (N=9)	n-3 PUFA (N=12)	n-6 Placebo (N=9)	n-3 PUFA (N=12)	n-3 PUFA (N=9)	n-3 PUFA (N=12)
Fatty Acids (FA)			(%)			
SFA						
MA (C14:0)	1.34 ± 0.14	1.37 ± 0.10	1.25 ± 0.10	1.34 ± 0.13	1.34 ± 0.17	1.21 ± 0.13$^{\#}$
PA (C16:0)	25.23 ± 0.83	25.35 ± 0.52	25.05 ± 0.66	25.44 ± 0.74	25.55 ± 1.06	24.93 ± 0.83
SA (C18:0)	7.02 ± 0.25	7.12 ± 0.17	6.81 ± 0.31	7.13 ± 0.23	6.73 ± 0.36	7.19 ± 0.21
MUFA						
OA (C18:1n9)	20.43 ± 0.61	19.37 ± 0.83	21.61 ± 0.67	18.62 ± 0.82*	20.70 ± 0.79	18.68 ± 0.54
EIA (C20:1n9)	0.14 ± 0.01	0.12 ± 0.01	0.15 ± 0.01	0.11 ± 0.01***	0.13 ± 0.01	0.12 ± 0.01
ETA (C20:3n9)	0.10 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.08 ± 0.01$^{\#}$	0.09 ± 0.01	0.08 ± 0.01$^{\#}$
ERA (C22:1n9)	0.11 ± 0.02	0.13 ± 0.01	0.10 ± 0.00	0.10 ± 0.01$^{\#}$	0.10 ± 0.01	0.10 ± 0.00$^{\#}$
NA (C24:1n9)	0.67 ± 0.03	0.83 ± 0.05	0.70 ± 0.04	0.84 ± 0.05*	0.74 ± 0.05	0.88 ± 0.07
PUFA						
n-3 series						
ALA (C18:3n3)	0.88 ± 0.09	0.66 ± 0.05	0.83 ± 0.06	0.72 ± 0.05	0.75 ± 0.04	0.76 ± 0.08
EPA (C20:5n3)	0.56 ± 0.08	0.68 ± 0.11	0.58 ± 0.08	1.30 ± 0.16***$^{\#\#\#}$	0.72 ± 0.11*	1.41 ± 0.10***$^{\#\#\#}$
DPA (C22:5n3)	0.37 ± 0.04	0.38 ± 0.04	0.34 ± 0.03	0.41 ± 0.03	0.35 ± 0.02	0.40 ± 0.03
DHA (C22:6n3)	1.27 ± 0.16	1.31 ± 0.15	1.27 ± 0.11	2.09 ± 0.19***$^{\#\#\#}$	1.66 ± 0.15**	2.12 ± 0.16***$^{\#\#\#}$
n-6 series						
LA (C18 :2n-6)	27.63 ± 1.22	27.30 ± 1.03	27.28 ± 1.27	27.94 ± 1.42	27.44 ± 1.68	28.44 ± 1.43

GLA (C18:3n6)	0.49 ± 0.06	0.54 ± 0.03	0.51 ± 0.07	0.47 ± 0.03	0.46 ± 0.09	0.47 ± 0.05
HGLA (C20:3n6)	1.58 ± 0.11	1.74 ± 0.09	1.61 ± 0.09	1.47 ± 0.07$^{\#}$	1.30 ± 0.08	1.41 ± 0.05$^{\#}$
AA (C20:4n6)	5.51 ± 0.32	6.08 ± 0.22	5.27 ± 0.37	5.48 ± 0.17$^{\#\#\#}$	5.09 ± 0.30	5.46 ± 0.26$^{\#\#\#}$
DIA (C22:2n6)	0.02 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
DTA (C22:4n6)	0.13 ± 0.02	0.14 ± 0.01	0.13 ± 0.01	0.10 ± 0.01$^{\#\#\#}$	0.10 ± 0.01	0.09 ± 0.01$^{\#\#\#}$
Total n-3 FA	3.12 ± 0.29	3.07 ± 0.28	3.06 ± 0.16	4.55 ± 0.39***$^{\#\#\#}$	3.53 ± 0.26**	4.72 ± 0.28$^{\#\#\#}$
Total n-6 FA	35.55 ± 1.33	35.97 ± 1.10	34.99 ± 1.30	35.63 ± 1.43	34.58 ± 1.73	36.02 ± 1.52
Total n-7 FA	3.97 ± 0.31	3.84 ± 0.22	3.98 ± 0.27	3.57 ± 0.29**	4.12 ± 0.33	3.35 ± 0.23**$^{\#\#\#}$
Total n-9 FA	21.45 ± 0.62	20.56 ± 0.81	22.64 ± 0.65	19.76 ± 0.81	21.76 ± 0.77	19.86 ± 0.52
Total SFA	35.33 ± 0.87	35.91 ± 0.59	34.78 ± 0.67	35.90 ± 0.92	35.36 ± 1.20	35.44 ± 0.96
Total MUFA	25.63 ± 0.76	24.65 ± 0.84	26.84 ± 0.83	23.58 ± 1.03	26.12 ± 1.00	23.45 ± 0.65
Total PUFA	38.77 ± 1.34	39.15 ± 1.13	38.15 ± 1.24	40.26 ± 1.57	38.20 ± 1.91	40.83 ± 1.50

AA, Arachidonic acid; ALA, Alpha linolenic acid; DHA, Docosahexanoic acid; DIA, Docosadienoic acid; DPA, Docosapentaenoic acid ; DTA, Docosatetraenoic acid; EIA, Eicosenoic acid; EPA, Eicosapentaenoic acid; ERA, Erucic acid; ETA, Eicosatrienoic acid; FA, Fatty acid; GLA, Gamma linoleic acid; HGLA, Homogamma linolenic acid; LA, Linoleic acid; MA, Myristic acid; MUFA, Monounsaturated fatty acid; NA, Nervonic acid; OA, Oleic acid; PA, Palmitic acid; POA, Palmitoleic acid; PUFA, Polyunsaturated fatty acid; SA, Stearic acid; SFA, Saturated fatty acid

All values are expressed as mean ± SD. Data were analyzed by using a one-way analysis variance and the differences between the means were assessed post-hoc using Tukey's test. *P<0.05; **P<0.01; ***P<0.001 vs. n-6 placebo group (3 month-treatment); $^{\#}$P<0.05; $^{\#\#\#}$P<0.001 vs. n-3 PUFA group (Baseline).

Table 5B: Comparative plasma fatty acid distribution in NAFLD patients following 3- and 6-month treatments with n-3 PUFA or n-6 placebo

	Baseline		3 month-treatment		6 month-treatment	
	n-6 Placebo (N=9)	n-3 PUFA (N=12)	n-6 Placebo (N=9)	n-3 PUFA (N=12)	n-3 PUFA (N=9)	n-3 PUFA (N=12)
Fatty Acids (FA)				(µM)		
SFA						
MA (C14:0)	208.27 ± 36.60	210.65 ± 41.12	191.20 ± 23.93	199.62 ± 32.18	189.26 ± 31.91	177.34 ± 32.75
PA (C16:0)	3820.26 ± 424.68	3776.32 ± 546.27	3761.48 ± 336.05	3673.42 ± 370.35	3541.96 ± 326.39	3529.11 ± 364.70
SA (C18:0)	1033.25 ± 56.91	1025.11 ± 107.36	1005.24 ± 63.94	1010.78 ± 79.11	919.49 ± 65.39	995.44 ± 65.72
MUFA						
OA (C18:1n9)	3037.76 ± 228.05	2914.38 ± 497.01	3261.08 ± 328.08	2693.14 ± 294.39**	2868.45 ± 257.33	2639.91 ± 249.09**
EIA (C20:1n9)	21.55 ± 1.83	18.01 ± 2.82	21.76 ± 1.93	16.03 ± 1.56*	18.19 ± 1.35	16.17 ± 1.41*
ETA (C20:3n9)	14.65 ± 1.87	16.43 ± 2.75	14.44 ± 2.09	12.02 ± 1.59	12.71 ± 2.59	11.49 ± 1.41
ERA (C22:1n9)	15.97 ± 2.36	18.30 ± 2.25	14.08 ± 0.98	13.94 ± 0.74	13.22 ± 0.70	13.71 ± 0.66
NA (C24:1n9)	98.88 ± 7.55	117.91 ± 11.87	103.52 ± 8.46	117.16 ± 7.79	101.04 ± 9.01	120.64 ± 10.25
PUFA						
n-3 series						
ALA (C18:3n3)	132.85 ± 16.80	101.98 ± 20.50	125.19 ± 14.37	104.25 ± 13.82	104.25 ± 9.45	107.87 ± 16.41
EPA (C20:5n3)	83.17 ± 12.77	98.48 ± 17.00	83.26 ± 10.14	185.76 ± 34.02***###	96.97 ± 14.17	194.01 ± 17.32***###
DPA (C22:5n3)	54.67 ± 5.84	61.85 ± 17.65	51.15 ± 6.34	61.30 ± 11.06	48.95 ± 4.99	56.21 ± 6.97
DHA (C22:6n3)	193.47 ±31.72	195.27 ± 33.00	190.23 ± 21.89	302.47 ± 45.13***###	222.97 ± 21.74	296.47 ± 29.36***###
n-6 series						
LA (C18 :2n-6)	4055.29 ± 233.48	3963.29 ± 442.26	4028.28 ± 291.56	3943.77 ± 314.44	3731.64 ± 297.56	3950.74 ± 310.27

GLA (C18:3n6)	72.34 ± 10.02	82.98 ± 15.62	76.12 ± 11.03	68.86 ± 9.02	66.91 ± 14.75	65.91 ± 8.30
HGLA (C20:3n6)	234.51 ± 23.22	259.77 ± 43.72	239.96 ± 19.78	210.44 ± 20.83	179.19 ± 16.54	195.29 ± 14.40
AA (C20:4n6)	816.12 ± 66.09	890.64 ± 106.82	786.03 ± 75.68	782.10 ± 64.70$^{\#\#}$	694.59 ± 53.69*	751.84 ± 49.73$^{\#\#}$
DIA (C22:2n6)	3.47 ± 1.21	3.00 ± 0.68	1.69 ± 0.20	2.00 ± 0.16	2.16 ± 0.54	2.62 ± 0.59
DTA (C22:4n6)	20.07 ± 2.74	22.87 ± 5.71	18.98 ± 2.37	15.08 ± 2.47	14.53 ± 1.48	11.65 ± 1.27
Total n-3 FA	470.50 ± 60.08	463.29 ± 83.07	455.80 ± 39.06	658.86 ± 100.36***$^{\#\#\#}$	479.19 ± 42.08	658.89 ± 62.42***$^{\#\#\#}$
Total n-6 FA	5227.27 ± 295.61	5245.63 ± 604.44	5177.28 ± 356.17	5042.57 ± 389.40	4711.66 ± 341.06	4997.69 ± 354.04
Total n-7 FA	606.43 ± 84.88	572.91 ± 89.76	602.19 ± 73.65	519.14 ± 66.86*	575.10 ± 70.97	474.35 ± 63.31*$^{\#\#}$
Total n-9 FA	3188.80 ± 236.85	3085.04 ± 511.32	3414.89 ± 335.91	2852.30 ± 301.89	3013.61 ± 262.89	2801.92 ± 256.27
Total SFA	5319.43 ± 525.59	5306.12 ± 717.66	5205.70 ± 415.34	5162.06 ± 489.87	4887.97 ± 413.07	4994.64 ± 469.50
Total MUFA	3827.48 ± 319.22	3690.86 ± 593.26	4049.31 ± 403.86	3405.80 ± 363.39	3621.01 ± 324.00	3310.07 ± 315.80
Total PUFA	5712.42 ± 337.63	5725.35 ± 680.15	5647.52 ± 378.43	5713.46 ± 470.96	5203.56 ± 377.44	5668.06 ± 390.45
TOTAL FA	14899.70 ± 1119.71	14764.52 ± 1958.06	14936.95 ± 1118.92	14317.94 ± 1228.43	13756.58 ± 912.13	14012.23 ± 1068.38

AA, Arachidonic acid; ALA, Alpha linolenic acid; DHA, Docosahexanoic acid; DIA, Docosadienoic acid; DPA, Docosapentaenoic acid ; DTA, Docosatetraenoic acid; EIA, Eicosenoic acid; EPA, Eicosapentaenoic acid; ERA, Erucic acid; ETA, Eicosatrienoic acid; FA, Fatty acid; GLA, Gamma linoleic acid; HGLA, Homogamma linolenic acid; LA, Linoleic acid; MA, Myristic acid; MUFA, Monounsaturated fatty acid; NA, Nervonic acid; OA, Oleic acid; PA, Palmitic acid; POA, Palmitoleic acid; PUFA, Polyunsaturated fatty acid; SA, Stearic acid; SFA, Saturated fatty acid

All values are expressed as mean ± SD. Data were analyzed by using a one-way analysis variance and the differences between the means were assessed post-hoc using Tukey's test. *P<0.05; **P<0.01; ***P<0.001 vs. n-6 placebo group (3 month-treatment); $^{\#\#}$P<0.01; $^{\#\#\#}$P<0.001 vs. n-3 PUFA group (Baseline).

Table 6: Comparative fatty acid ratios in NAFLD patients following 3- and 6-month supplementations with n-3 PUFA or n-6 placebo

	Baseline		3 month-treatment		6 month-treatment	
Ratios	**n-6 Placebo (N=9)**	**n-3 PUFA (N=12)**	**n-6 Placebo (N=9)**	**n-3 PUFA (N=12)**	**n-3 PUFA (N=9)**	**n-3 PUFA (N=12)**
16:1n-7/18:2n-6	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.10 ± 0.02	0.08 ± 0.01
18:2n-6/20:4n-6	5.14 ± 0.35	4.54 ± 0.22	5.43 ± 0.54	5.13 ± 0.27	5.50 ± 0.41	5.30 ± 0.30
20:3n-9/20:4n-6	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
Total n-7/18:2n-6	0.15 ± 0.02	0.15 ± 0.01	0.15 ± 0.02	0.14 ± 0.02	0.16 ± 0.02	0.13 ± 0.02
24:0/22:0	0.50 ± 0.01	0.58 ± 0.03	0.52 ± 0.01	0.57 ± 0.03	0.53 ± 0.02	0.59 ± 0.03
24:0/20:0	1.26 ± 0.11	1.73 ± 0.20	1.31 ± 0.12	1.63 ± 0.21	1.22 ± 0.06	1.81 ± 0.21
PUFA/SFA	1.11 ± 0.06	1.10 ± 0.05	1.10 ± 0.05	1.14 ± 0.07	1.10 ± 0.08	1.17 ± 0.07$^{\#}$
EFA (LA+ALA)/NON-EFA	0.40 ± 0.02	0.39 ± 0.02	0.39 ± 0.02	0.41 ± 0.03	0.40 ± 0.03	0.42 ± 0.03$^{\#}$
EPA/DHA	0.44 ± 0.04	0.56 ± 0.07	0.45 ± 0.05	0.62 ± 0.05**	0.45 ±0.06	0.69 ± 0.06**
DHA/AA	0.23 ± 0.03	0.22 ± 0.02	0.24 ± 0.02	0.39 ± 0.04***$^{\#\#\#}$	0.33 ± 0.03**	0.40 ± 0.03***$^{\#\#\#}$
ALA/EPA	1.90 ± 0.35	1.10 ± 0.09	1.90 ± 0.50	0.64 ± 0.08***$^{\#\#\#}$	1.24 ± 0.18***	0.55 ± 0.04***$^{\#\#}$
ALA/LA	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
n-6/n-3	12.17 ± 1.16	12.65 ± 1.02	11.80 ± 0.97	8.47 ± 0.79***$^{\#\#\#}$	10.01 ± 0.52**	8.03 ± 0.79***$^{\#\#\#}$
Δ5 (20:4n-6/20 :3n-6)	3.60 ± 0.84	3.61 ± 0.24	4.04 ± 0.38	3.89 ± 0.24$^{\#}$	4.04 ± 0.38	3.94 ± 0.24$^{\#}$
Δ6 (20:3n-6/18:2n-6)	0.06 ± 0.01	0.07 ± 0.00	0.06 ± 0.00	0.06 ± 0.00$^{\#}$	0.05 ± 0.01	0.05 ± 0.00$^{\#}$
SCD1 index (16:1n-7/18:1n-9)	0.10 ± 0.04	0.13 ± 0.00	0.12 ± 0.01	0.11 ± 0.00$^{\#}$	0.12 ±0.01	0.11 ± 0.00$^{\#}$
SCD16 index (16:1n-7/16:0)	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01$^{\#}$	0.10 ± 0.01	0.08 ± 0.00$^{\#}$
SCD18 index (18:1n-9/18:0)	2.94 ± 0.14	2.75 ± 0.16	3.25 ± 0.22	2.66 ± 0.18*	3.14 ± 0.19	2.64 ± 0.13*

AA, Arachidonic acid; ALA, Alpha linolenic acid; DHA, Docosahexanoic acid; EFA, Essential fatty acid; EPA, Eicosapentaenoic acid; FA, Fatty acid; LA, Linoleic acid; PUFA, Polyunsaturated fatty acid; SFA, Saturated fatty acid

All values are expressed as mean ± SD. Data were analyzed by using a one-way analysis variance and the differences between the means were assessed post-hoc using Tukey's test. *P<0.05; **P<0.01; ***P<0.001 vs. n-6 placebo group (3 month-treatment); $^{\#}$P<0.05; $^{\#\#}$P<0.01; $^{\#\#\#}$P<0.001 vs. n-3 PUFA group (Baseline).

Table 7: Plasma fatty acid profile in all paediatric NAFLD patients before and after 6-month supplementation with n-3 PUFA

Fatty Acids (FA)	Before treatment (Baseline) (N=21) (%)	After treatment (6 months)	*P values*	Before treatment (Baseline) (N=21) (µM)	After treatment (6 months)	*P values*
SFA						
MA (C14:0)	1.36 ± 0.08	1.26 ± 0.10	NS	209.63 ± 27.59	182.45 ± 22.66	NS
PA (C16:0)	25.29 ± 0.45	25.20 ± 0.64	NS	3795.15 ± 353.17	3534.62 ± 245.08	NS
SA (C18:0)	7.08 ± 0.14	7.00 ± 0.20	NS	1028.60 ± 64.64	962.89 ± 46.48	NS
MUFA						
POA (C16 :1n-7)	2.49 ± 0.155	2.28 ± 0.18	NS	375.65 ± 43.00	323.65 ± 38.27	NS
OA (C18:1n9)	19.82 ± 0.54	19.55 ± 0.50	NS	2967.26 ± 294.51	2737.86 ± 177.46	<0.05
EIA (C20:1n9)	0.13 ± 0.01	0.12 ± 0.00	NS	19.53 ± 1.80	17.04 ± 0.99	<0.05
ETA (C20:3n9)	0.10 ± 0.01	0.08 ± 0.01	NS	15.67 ± 1.74	12.01 ± 1.34	NS
ERA (C22:1n9)	0.12 ± 0.01	0.10 ± 0.00	<0.001	17.30 ± 1.62	13.50 ± 0.47	<0.001
NA (C24:1n9)	0.76 ± 0.04	0.82 ± 0.05	NS	109.76 ± 7.65	112.24 ± 7.19	NS
PUFA						
n-3 series						
ALA (C18:3n3)	0.76 ± 0.05	0.76 ± 0.05	NS	115.21 ± 13.86	106.32 ± 10.00	NS
EPA (C20:5n3)	0.63 ± 0.07	1.11 ± 0.11	<0.05	91.92 ± 11.03	152.42 ± 15.62	<0.05
DPA (C22:5n3)	0.37 ± 0.03	0.38 ± 0.02	NS	58.77 ± 10.22	53.10 ± 4.49	<0.001
DHA (C22:6n3)	1.29 ± 0.11	1.92 ± 0.12	<0.01	194.50 ± 22.69	264.97 ± 20.45	<0.05
n-6 series						
LA (C18 :2n-6)	27.44 ± 0.77	28.01 ± 1.07	NS	4002.72 ± 266.31	3856.84 ± 214.53	NS
GLA (C18:3n6)	0.52 ± 0.03	0.47 ± 0.05	NS	78.42 ± 9.76	66.34 ± 7.68	NS
HGLA (C20:3n6)	1.67 ± 0.07	1.36 ± 0.05	<0.001	248.94 ± 26.48	188.39 ± 10.74	<0.001
AA (C20:4n6)	5.84 ± 0.19	5.30 ± 0.19	<0.05	858.70 ± 66.36	727.31 ± 36.21	<0.01

DIA (C22:2n6)	0.02 ± 0.00	0.02 ± 0.00	NS	3.20 ± 0.63	2.42 ± 0.40	NS
DTA (C22:4n6)	0.14 ± 0.01	0.09 ± 0.01	<0.05	21.67 ± 3.41	12.88 ± 0.99	<0.001
Total n-3 FA	3.09 ± 0.20	4.21 ± 0.23	<0.001	466.37 ± 52.80	581.87 ± 43.85	<0.001
Total n-6 FA	35.79 ± 0.83	35.41 ± 1.12	NS	5237.76 ± 360.29	4875.10 ± 245.63	<0.001
Total n-7 FA	3.90 ± 0.18	3.68 ± 0.21	<0.001	587.27 ± 61.48	517.52 ± 47.40	<0.001
Total n-9 FA	20.94 ± 0.53	20.68 ± 0.48	NS	3129.50 ± 303.17	2892.64 ± 181.75	NS
Total SFA	35.66 ± 0.49	35.41 ± 0.73	NS	5311.82 ± 457.41	4948.92 ± 314.11	<0.01
Total MUFA	25.07 ± 0.58	24.59 ± 0.63	NS	3749.41 ± 358.20	3443.32 ± 224.77	<0.05
Total PUFA	38.99 ± 0.84	39.70 ± 1.19	<0.05	5719.80 ± 406.12	5468.99 ± 273.82	<0.05
TOTAL FA				14822.45 ± 1191.69	13902.660 ± 708.6356	NS

AA, Arachidonic acid; ALA, Alpha linolenic acid; DHA, Docosahexanoic acid; DIA, Docosadienoic acid; DPA, Docosapentaenoic acid; DTA, Docosatetraenoic acid; EIA, Eicosenoic acid; EPA, Eicosapentaenoic acid; ERA, Erucic acid; ETA, Eicosatrienoic acid; FA, Fatty acid; GLA, Gamma linoleic acid; HGLA, Homogamma linolenic acid; LA, Linoleic acid; MA, Myristic acid; MUFA, Monounsaturated fatty acid; NA, Nervonic acid; OA, Oleic acid; PA, Palmitic acid; POA, Palmitoleic acid; PUFA, Polyunsaturated fatty acid; SA, Stearic acid; SFA, Saturated fatty acid

All values are expressed as mean ± SD. Data were analyzed by using a Student's *t* test. Statistical significance was defined as $P<0.05$ vs. Baseline.

Table 8: Plasma fatty acid ratios in NAFLD patients before and after 6-month supplementation with n-3 PUFA

Ratios	Before treatment (Baseline) (N=21)	After treatment (6 months)	*P values*
16:1n-7/18:2n-6	0.09 ± 0.01	0.09 ± 0.01	NS
18:2n-6/20:4n-6	4.80 ± 0.20	5.38 ± 0.24	<0.001
20:3n-9/20:4n-6	0.02 ± 0.00	0.02 ± 0.00	NS
Total n7/18:2n-6	0.15 ± 0.01	0.14 ± 0.01	NS
24:0/22:0	0.54 ± 0.02	0.56 ± 0.02	NS
24:0/20:0	1.53 ± 0.13	1.56 ± 0.14	NS
PUFA/Saturated FA	1.10 ± 0.04	1.14 ± 0.05	<0.05
EFA (LA+ALA)/NON-EFA	0.40 ± 0.01	0.41 ± 0.02	NS
EPA/DHA	0.51 ± 0.05	0.58 ± 0.05	<0.001
DHA/AA	0.22 ± 0.02	0.37 ± 0.02	<0.001
ALA/EPA	1.44 ± 0.18	0.84 ± 0.11	<0.05
ALA/LA	0.03 ± 0.00	0.03 ± 0.00	NS
n-6/n-3	12.45 ± 0.75	8.88 ± 0.54	<0.001
Δ5 (20:4n-6/20 :3n-6)	3.61 ± 0.18	3.98 ± 0.21	<0.05
Δ6 20:3n-6/18:2n-6	0.06 ± 0.00	0.05 ± 0.00	NS
SCD1 index (16:1n-7/18:1n-9)	0.13 ± 0.00	0.11 ± 0.00	<0.05
SCD16 index (16:1n-7/16:0)	0.10 ± 0.00	0.09 ± 0.00	<0.05
SCD18 index (18:1n-9/18:0)	2.83 ± 0.11	2.80 ± 0.12	NS

AA, Arachidonic acid; ALA, Alpha linolenic acid; DHA, Docosahexanoic acid; EFA, Essential fatty acid; EPA, eicosapentaenoic acid; FA, Fatty acid; LA, Linoleic acid; PUFA, Polyunsaturated fatty acid; SFA, Saturated fatty acid

All values are expressed as mean ± SD. Data were analyzed by using a Student's *t* test. Statistical significance was defined as $P<0.05$ vs. Baseline.

Article 5

«Non-alcoholic fatty liver disease severity and metabolic complications in obese children: impact of Omega-3 fatty acids»

Spahis S, Alvarez F, Ahmed N, Dubois J, Jalbout R, Paganelli M, Grzywacz K, Delvin E, Peretti N, Emile Levy. *J Nutr Biochem* 2018 Aug;58:28-36. doi: 10.1016/j.jnutbio.2018.03.025

CONTRIBUTION DES AUTEURS

Spahis S a été impliquée dans la conception et la planification de l'étude ; la mise au point du protocole de recherche clinique auprès de Santé Canada et du Comité d'éthique à la recherche ainsi que la création des ''Case Report Form'' pour chaque patient. Elle s'est occupée du recrutement et le suivi des patients; l'acquisition des données biochimiques, leur analyse et leur interprétation. Elle a contribué à la rédaction, soumission et révision du manuscrit sous la supervision du Directeur de recherche, Dr Levy E.

Alvarez F et Ahmed N ont contribué au recrutement et au suivi médical des patients.

Dubois J a contribué au suivi médical des patients.

Jalbout R, Paganelli M, Grzywacz K et Delvin E et **Peretti N** ont participé à la révision du manuscrit.

Levy E a contribué au concept et à la supervision de l'étude, aux ressources financières et matérielles pour la réalisation de l'étude. Dr Levy E a participé à la rédaction, l'analyse critique des résultats et la révision du manuscrit.

NON-ALCOHOLIC FATTY LIVER DISEASE SEVERITY AND METABOLIC COMPLICATIONS IN OBESE CHILDREN: IMPACT OF OMEGA-3 FATTY ACIDS

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Running Head: NAFLD and n-3 PUFA benefits

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ABSTRACT

Although n-3 polyunsaturated fatty acids (PUFA) revealed promising therapeutic results in non-alcoholic fatty liver disease (NAFLD), which is considered as the most prevalent cause of chronic hepatic disease, inconsistencies are calling for further confirmatory trials to demonstrate therapeutic efficacy and safety. The study, registered as NCT02201160 on www.clinicaltrials.gov, was designed to compare two groups of NAFLD with a different severity, and to evaluate the efficacy of n-3 PUFA supplementation. Twenty young male participants of French Canadian origin with NAFLD were enrolled and classified into moderate (mNAFLD) and severe (sNAFLD) fatty liver groups, according to transaminase levels, ultrasonography, NAFLD Activity Score and fatty liver index (FLI). The sNAFLD patients were assigned to consume 2 g of n-3 PUFA for 6 months. sNAFLD patients displayed higher insulinemia, insulin resistance (IR), oxidative stress (OxS), systolic blood pressure and the risk lipid indicators of cardiovascular diseases. Supplementation of n-3 PUFA for 6 months resulted in a significant increase in concentrations of eicosapentaenoic and docosahexaenoic acids in RBC along with an attenuation of hepatic steatosis as reflected by the reduction of the FLI, ALT and ALT/AST ratio. Moreover, the n-3 PUFA improved the lipid profile and carotid intima-media thickness, while reducing metabolic and OxS markers and raising adiponectin. In conclusion, NAFLD severity was essentially related to IR. Treatment with n-3 PUFA has an evidently beneficial effect on liver steatosis and related metabolic abnormalities. Furthermore, the cross association of omega-3 index with cardiometabolic markers may serve as a predictor for cardiovascular risk disorders in NAFLD.

Key words: NAFLD, PUFA, fatty liver index, lipids, insulin resistance, inflammation, oxidative stress, red blood cell fatty acid composition.

Abbreviations: APO, Apolipoprotein; BMI, body mas index; CTR, control; CVD, cardiovascular disease; DHA, Docosahexaenoic acid; DBP, diastolic blood pressure; EPA, Eicosapentaenoic acid; FA, Fatty acids; IL, Interleukin; IMT, Intima-media thickness; IR, Insulin resistance; FLI, Fatty liver index; GC, gas chromatography; GGT, γ-glutamyl transferase; LDL-C, Low-density lipoprotein cholesterol; MDA, Malondialdehyde; MetS, metabolic syndrome; NAFLD, Non-alcoholic fatty liver disease; OxS, oxidative stress; OxLDL, Oxidized LDL; PUFA,

Polyunsaturated fatty acid; RBC, red blood cell; SBP, systolic blood pressure; TG, Triglycerides; VLCFA, Very long chain FA

1.1 INTRODUCTION

In close association with obesity, non-alcoholic fatty liver disease (NAFLD) is becoming the most widespread cause of chronic liver disease in Western countries (1). Children are no exception to this increasingly diagnosed medical condition in addition with the possibility of developing hepatocellular fibrosis and carcinoma, as well as high morbidity and mortality (2). Cardiometabolic health is also affected in the presence of NAFLD given the observed abnormalities in peripheral insulin sensitivity, lipid profile, glucose metabolism and vascular integrity, thereby giving rise to metabolic syndrome (MetS) (3). Elevated carotid intima-media thickness (IMT) has frequently been reported in adult subjects with NAFLD and may stratify cardiovascular risks (4).

Currently, various nutritional and pharmacological treatments are recommended for NAFLD (5-8), but most of them seem ineffective in the long term. Among the most powerful pharmacological agents, insulin-sensitizing pioglitazone exhibits a beneficial impact on liver biochemical and histological parameters, but unfortunately with adverse events (9). Other drugs such as liraglutide, elafibranor, obeticholic acid and statins have been assessed but the number of randomised controlled trials is quite limited, and further clarification is required as to their long-term benefits and safety (9-13). It has to be acknowledged that no medically approved treatment is available for NAFLD (14). Even pediatric trials are not convincing as they are scarce and characterized by a very small sample size and duration.

However, appropriate diet, exercise and lifestyle implementation may ameliorate hepatic steatosis and complications (15). For example, the combination of α-tocopherol and metformin decreased liver enzymes and complications, but the absence of control (CTR) groups restricts the interpretation of the data (16-18). The effects of omega-3 polyunsaturated fatty acids (n-3 PUFA) were also evaluated in young patients with NAFLD in view of their hypotriglyceridemic and anti-inflammatory features. The treatment of children with docosahexaenoic acid (DHA) resulted in the reduction of metabolic disorders and fat in the liver (19-21). However, other trials with DHA could not show convincing improvement in transaminases and body overweight (22). Furthermore, inconsistent findings were also reported in adult subjects concerning transaminase levels, hepatic fat content, circulating triglyceride (TG) and glycaemia concentrations (23-28). Discrepancy was

also evident in two systematic meta-analyses (29, 30). The small doses of administered n-3 PUFA and patient compliance were probably the limiting factors (31).

The purpose of the present work is twofold: (i) to compare two levels of NAFLD severity in obese adolescent patients with respect to liver transaminases, dyslipidemia, metabolic abnormalities, oxidative stress (OxS), inflammation and carotid IMT; and (ii) to evaluate the efficacy and safety response to n-3 PUFAs treatment. This interventional trial was carried out with 2000 mg of fish oil administered daily to the young patients who were continually motivated for adherence and persistence (21).

1.2 MATERIALS AND METHODS

1.2.1 Subjects and Inclusion/ exclusion criteria

The present clinical trial was performed on 20 NAFLD male children followed as outpatients at the Gastroenterology/Hepatology and Nutrition Clinic of Mother University Hospital Child (MUHC) Sainte-Justine and the Gastroenterology division of the Montreal Children's Hospital in Montreal. The children were between 8 and 18 years of age, and with obesity (>95th Percentile) considering the CDC 2000 Chart. The diagnosis of NAFLD was based on the results of a clinical and biochemical evaluation (transaminase levels), ultrasound fat grading and magnetic resonance imaging fat quantification as per our recent publication (21, 32). Moreover, the exclusion criteria include subjects having pins or cochlear implants that may affect the magnetic resonance imaging examination, subjects who consumed natural medicine products possibly leading to increased risk of haemorrhage, and those who were found to consume fish, flaxseed oil and food enriched with n-3 PUFA, probiotics, vitamin E or drugs known to induce fatty liver during the study (21).

1.2.2 Study design

The young NAFLD subjects were classified in two groups: the moderate NAFLD (mNAFLD) group that was characterized by normal transaminases, lower score steatosis (1-2) and mild fatty liver index (FLI) ≤30), whereas severe NAFLD (sNFLD) subjects had higher transaminases (>2 normal), steatosis score (≤5) according to the NAFLD Activity Score (NAS) (33), and FLI (≥60) (34). The present study included a 6-month-treatment with an active n-3 PUFA supplements (NutriSanté Inc./Ponroy, Canada). The study was approved by Health Canada and the Clinical Research Ethics Committee of MUHC Sainte-Justine (Montreal, Quebec). Informed consent was obtained from all subjects or parents before starting experimental procedures, and the study followed the Helsinki guidelines. A total of 20 French Canadian children with NAFLD were selected: 9 mNAFLD and 11 sNAFLD) as they succeeded in completing the 6 month-treatment with n-3 PUFA. Therefore, to meet the objectives of the present study, only the sNAFLD were considered for the data analysis. For biochemical references and fatty acid composition, 20 unrelated age-matched healthy CTRs were recruited at the Orthopedic Clinic of the MUHC Sainte-Justine.

1.2.3 Fatty acid supplements

The dose supplementation considered for this study is 2 g of fish oil per day, providing a total of 1.2 g of n-3 PUFA at a rate of 4 capsules per day (21).

1.2.4 Plasma and red blood cell separation

Blood samples were collected in tubes containing 1 g EDTA/L after subjects had fasted for 12h overnight. Plasma and erythrocytes were separated immediately by centrifugation (700 x *g* for 20 min) at 4 °C as described previously (21, 35, 36).

1.2.5 NAFLD Activity Score and Fatty liver index

The NAFLD severity was assessed by evaluating the steatosis score according to the NAS (37): Absence of steatosis/normal liver echo-texture (grade 0); mild steatosis/slight and diffuse increase in fine parenchymal echoes with normal visualization of diaphragm and portal vein borders (grade 1); moderate steatosis/moderate and diffuse increase in fine echoes with slightly impaired visualization of diaphragm and portal vein borders (grade 2); and severe steatosis/fine echoes with poor or no visualization of diaphragm, portal vein borders and posterior portion of the right lobe (grade 3).

The level of FLI is an algorithm, generally used to detect fatty liver and to predict the NAFLD risk (38). FLI was calculated using the validated algorithm: $[e^{0.953\times\log e(TG)+0.139\times BMI+0.718\times\log e(GGT)+0.053\times waist\ circumference-15.745}]/[1+e^{0.953\times\log e(triglycerides)+0.139\times BMI+0.718\times\log e(GGT)+0.053\times waist\ circumference-15.745}] \times 100$ and included four markers: body mass index (BMI), waist circumference, plasma TGs and γ-glutamyl transferase (GGT) (34).

1.2.6 Biochemical parameters

Biochemical assessments were carried out as described previously (39). Plasma apolipoproteins (Apo) A-I, B and Cs, oxidized LDL (oxLDL), interleukin (IL)-6, and serum adipokines (adiponectin, acyl-ghrelin and leptin) were determined by commercial ELISA kits. Insulin resistance (IR) was assessed by the homeostasis model assessment HOMA-IR index (40) calculated with the formula: [fasting insulin (mU/mL) × fasting glucose (mmol/L)] / 22.5.

1.2.7 Carotid Doppler

Longitudinal images of the common carotid arteries were acquired by combination of 2-D mode and color Doppler examination using a high-resolution linear ultrasound transducer. Common carotid IMT was measured by calculating the mean value of 3 consecutive measurements of the deep wall thickness of the vessel, 10 mm below the carotid bulb (41).

1.2.8 Malondialdehyde

Plasma free malondialdehyde (MDA) concentrations were measured according to our high-performance liquid chromatography method described previously (35).

1.2.9 Fatty acid composition

Fatty acids (FAs) in erythrocytes were assessed by an improved gas chromatography method described previously (21, 35, 36).

The omega-3 index was calculated as the total percentage of (EPA,20:5n-3) and (DHA,22:6n-3) content of erythrocyte cell membranes.

1.2.10 Statistical Analysis

All values are expressed as mean ± SEM. Data were analyzed by a pairwise using Student's two-tailed *t* test (if two groups) or one-way ANOVA analysis variance (three groups) and the differences between the means were assessed post-hoc using Tukey's test. The association with the metabolic markers was evaluated by univariate analysis correlation with a Spearman test for non-normally distributed variable. Statistical significance was defined as $P < 0.05$.

1.3 RESULTS

1.3.1 Anthropometric, biochemical and clinical data

20 young male NAFLD patients with the mean age of 13.77 ± 0,78 yr (ranging 8-18 yr) completed the study. The anthropometric, clinical and laboratory data from each individual were obtained and distributed into subgroups on the basis of fatty liver severity, which was characterized by elevated transaminases, steatosis score and FLI (**Table 1**). These two subgroups formed according to the severity of fatty liver disease did not display any significant difference in age, BMI and IMT. Noteworthy, twenty healthy subjects, paired for age and sex, were included in the study for reference values.

Based on the values of fasting plasma glucose, there was no evidence of diabetes in the two NAFLD groups (**Table 2**). However, sNAFLD patients exhibited more marked hyperinsulinemia compared to mNAFLD individuals (**Table 2**). As expected from these findings, IR was higher in the sNAFLD group in view of the calculated HOMA-IR index. Similarly, sNAFLD patients displayed high systolic blood pressure (SBP) whereas their diastolic blood pressure (DBP) remained unchanged in comparison with the mNAFLD group.

The lipid profile, lipoproteins and Apos are shown in **Table 2**. Interestingly, no significant changes between the moderate and severe NAFLD groups were observed in TGs, total cholesterol and LDL-C, as well as in Apos (A-I, B, Cs), with the exception of a significant increase in Apo C2/C3 ratio that characterized the sNAFLD group. However, compared to the CTR group, most of the NAFLD subjects exhibited significantly severe metabolic derangements in their lipid profile as shown by TGs, HDL-C and Apos (AI, B and C3), as well as by the ratios of total cholesterol/HDL-C, LDL-C/HDL-C, B/AI and B/TG (**Table 2**), thereby worsening the risk of cardiovascular diseases (CVD). No significant alterations were noticed in lipid peroxidation/OxS (as reflected by MDA and oxLDL), inflammation (as shown by hCRP and IL-6), and adipose tissue-derived hormones (as indicated by acyl-ghrelin, adiponectin, leptin and adiponectin/leptin ratio) between the moderate and severe NAFLD groups (**Table 3**). However, all these categories of markers were substantially divergent between NAFLD groups and CTR subjects (**Table 3**).

As FA levels are proposed as useful biomarkers for the estimation of dietary FA intake and risk of CVD (42, 43), we analyzed their absolute concentrations and percent distribution in red blood cells (RBC) of CTR and NAFLD subjects (**Tables 4 and 5**). Marked disturbances were apparent in the FA composition profile of NAFLD patients as evidenced by the increase of saturated FA and

decrease of PUFA compared to healthy CTR subjects. On another side, the sNAFLD patients displayed a significantly higher concentration of total FA in the RBC, lower levels of DHA and EPA, and a decreased omega-3 index, which constitutes an elevated risk of CVD (**Tables 4-5**).

1.3.2 Impact of n-3 PUFA supplementation on NAFLD

To study the effect of n-3 PUFA on NAFLD, we chose only the subgroup displaying the greatest fatty liver severity (sNAFLD). The administration of n-3 PUFA for 6 months significantly improved ALT, ALT/AST ratios and FLI (Table 6) as well as IMTL, fasting insulinemia and the HOMA-IR index (Table 7). Moreover, the long n-3 PUFAs were able to alter the lipid profile by reducing TGs, LDL-C and Apo B, as well as the ratios of Apo B/AI, total cholesterol/HDL-C, LDL-C/HDL-C and Apo B/TG without ameliorating HDL-C, Apo A-I and Apo C family members (**Table 7**).

Moreover, treatment of the sNAFLD young subjects with n-3 PUFAs decreased the magnitude of OxS and inflammation as evidenced by the lowering of serum oxLDL, leptin and acyl-ghrelin, while favourably increasing adiponectin (**Table 8**). Finally, with the n-3 PUFA supplementation, statistically significant changes were observed in RBC FA composition of sNAFLD patients (**Table 9**). An evident decline was noted in saturates along with a rise in monounsaturated FA and n-3 PUFA. The n-3 family was enriched in DHA and EPA. As expected from the alterations of this FA composition, there was an elevation of the ratios of 18:2n-6/20:4n-6, PUFA/saturated FA, EPA/DHA, DHA/AA and omega-3 index (Table 8). On the other hand, a marked decrease characterized n-6/n-3. Moreover, the Δ5 (20:4n-6/20:3n-6) and Δ9 (18:1n-9/18:0) ratios were higher while that of Δ6 (20:3n-6/18:2n-6) was lower (**Table 10**).

1.3.3 n-3 PUFA-associated cardiometabolic biomarkers in NAFLD

To better assess the impact of the n-3 PUFA treatment on the cardiometabolic factors associated with NAFLD and to uncover important biomarkers, we first evaluated the possible interactions between omega-3 index and biochemical/clinical parameters. Noteworthy, the omega-3 index has been reported to be associated with a lower risk of CVD and coronary mortality in epidemiological studies (44, 45). Our findings documented that the Δ (changed values between the basal and the end of 6-month treatment) omega-3 index was negatively correlated with IMT, IR and lipids while being positively associated with the very long chain FA (VLCFA) (**Table 11**). On the other hand, the n-6/n-3 ratio was only correlated with cholesteryl ester. In these conditions, not only n-3 PUFAs were able to lower FLI (**Table 6**) and n-6/n-3 ratio (**Table 10**), but they also promoted

their negative correlation with phospholipids and omega-3 index, respectively, suggesting that the omega-3 index may be used as a relevant biomarker in NAFLD status.

1.4 DISCUSSION

As obese subjects are increasingly susceptible to develop NAFLD, we evaluated the steatosis features of unrelated young male French Canadian along with its association with the MetS components and response to n-3 PUFA supplementation. Thus, we are joining the biomedical community endeavor to identify reliable cardiometabolic biomarkers for diagnosis, follow-up and response to treatments, in the hope of attenuating or reversing NAFLD evolution, particularly its progression towards steatohepatitis. The findings of the present study showed that the patients were segregated into two groups according to their steatosis score, which differentiated between grades 1-2 (n=9, mNAFLD) and grades 2-4 (n=11, sNAFLD) in combination with FLI. Compared to healthy CTRs, the NAFLD subjects of the two groups showed abnormalities in BMI, insulin sensitivity, lipid levels, adipokines and inflammatory factors as well as derangements in fatty acid composition, which collectively indicate the presence of the MetS and potential risks for CVD. Although the sNAFLD group, classified as severe given its steatosis magnitude based on higher levels of transaminases, NAS and IR (raised levels of HOMA-IR), it did not display more metabolic disturbances. However, our findings highlight the beneficial impact of n-3 PUFA on its RBC FA composition and metabolic variables, suggesting an alleviation of the disorder.

As per the definition of NAFLD, our patients who underwent ultrasonography, an imaging technique widely used for diagnosis of fatty liver (46), were characterized by an increased deposit of lipids exceeding 5% of total liver weight in the absence of significant alcohol consumption. In addition, the positive correlation between hepatic accumulation and elevated body weight supports the growing evidence that NAFLD is more prevalent among obese individuals (47). This also applies to the important factor of IR evaluated by HOMA-IR in our investigation, therefore confirming the well-established link between IR and the pathological stages of NAFLD (48-50). Our finding is in line with various studies, which documented a higher rate of the MetS in NAFLD patients (47, 48, 50-52). Indeed, a number of cross-sectional investigations reported the association between NAFLD and MetS (53-55). NAFLD may even predict the prevalence of MetS according to prospective reports (56-58). Interestingly, our results could not demonstrate that NAFLD severity was linked to an amplification of the MetS despite the presence of increased HOMA-IR and lower omega-3 index. No marked divergences were noted between moderate and severe NAFLD in terms of dyslipidemia, hypertension, OxS and inflammation, all of which are

components of the MetS. Noteworthy, NAFLD was documented to be more prevalent in patients with type 2 diabetes, but none of our patients showed any signs of diabetes (47).

Visceral obesity appears as a key metabolic contributor to NAFLD (59, 60). However, in our studies, quantitative measures of central adiposity by DEXA did not show significant differences between moderate and more severe NAFLD (data not shown). Furthermore, n-3 PUFA supplementation did not result in alterations in visceral fat. In this context, it is important to mention the conflicting data reported in pertinent literature. Some groups of investigators have described the direct relationship between visceral fat and NAFLD (47, 61) while others failed to show such association (62, 63). Similarly, previous work demonstrated that the gradation in the degree of obesity was not correlated with clinical and biochemical parameters and did not reflect NAFLD severity in children (3). Probably, the limitation of the current study is the absence of very high to extremely severe NAFLD, which could have been more informative about the relationship between the degree of NAFLD and various metabolic complications.

1.4.1 Omega-3 index-associated NAFLD and metabolic derangements

The omega-3 index has been developed by Harris'group to predict cardiometabolic disorders (45, 64). Indeed, lower levels of omega-3 index are associated with CVD. In our cohort, the sNAFLD subjects presented a very low level of omega-3 index, and the dietary intake of n-3 PUFA increased the index value while improving the MetS parameters with a beneficial impact on NAFLD subjects. Furthermore, the omega-3 index displayed a negative association with metabolic variables related to MetS such as HOMA-IR, LDL/HDL, Cholesterol/HDL and IMT, suggesting that the omega-3 index can be an appropriate predictor for MetS in NAFLD (65). On the other hand, FAs with 20 or more carbons [e.g., arachidic acid (20:0), behenic acid (22:0) and lignoceric acid (24:0)] were classified as VLCFAs. Various studies reported a significant reduction in the proportion of VLCFA in subjects with MetS (66) whereas VLCFA intake resulted in beneficial effects on CVD (67, 68). However, the association between dietary VLCFAs and NAFLD remains misunderstood. In our NAFLD cohort study, changes between baseline and the end of n-3 PUFA intake revealed a negative correlation of omega-3 index with VLCFAs suggesting a possible link with NAFLD, whereas the n-6/n-3 ratio was correlated only with cholesteryl ester. This observation requires further investigation in order to establish that omega-3 index can definitely serve as a biomarker for NAFLD.

Our data demonstrate the benefits of n-3 PUFA for cardiometabolic complications and NAFLD. This finding adds to the growing clinical and preclinical literature indicating that n-3 PUFA should be included in any treatment regime for cardiometabolic disorder and NAFLD. Indeed, n-3 PUFA have been valuable for MetS and particularly IR, which is central in NAFLD pathogenesis (69). Previous trials reported the advantageous influence of n-3 PUFA on hypertriglyceridemia (70), adiponectin levels (71) and IR (22), along with an amelioration of insulin sensitivity, hepatic transaminases and liver histology (69, 72-74). Noteworthy, testing a pediatric French Canadian population with liver steatosis is particularly important as the population of Quebec is more prone to develop cardiometabolic disorders, including hyperlipidemia, hypercholesterolemia, and other hard conditions.

The present investigation did not assess physical activity or food intake by self-reporting or questionnaire since the main hypothesis was to evaluate the specific efficacy of n-3 PUFA on metabolic parameters in NAFLD without any interference from environmental factors. Such an approach is the most optimal for testing the effectiveness of n-3 PUFA. However, future studies will allow to examine the combined impact of n-3PUFA, nutrition surveillance and physical activity.

Although fish oil in different clinical formulations has a high safety profile, we have been carefully analyzing this important parameter in our study. In fact, the n-3 PUFA supplementation in the present trial was safe and effective. This measure was necessary to ensure the security of n-3 capsules offered by Nutrisanté but have never been tested, and to meet Health Canada requirements.

In conclusion, the subgroup classified as severe in terms of steatosis exhibited a marked increase in transaminases and HOMA-IR, but it did not exhibit more MetS features than the moderate group. However, the intervention with n-3 PUFA is clearly beneficial not only for the alleviation of NAFLD, but also for the prevention of severe complications such as diabetes and CVD by targeting new biomarkers for NAFLD diagnosis, treatment and follow-up.

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SS was involved in the study concept and design, acquisition, analysis and interpretation of data, revision of the manuscript, technical support, statistical analysis and study supervision.

EL contributed to the study concept, design and supervision, drafting, writing and critical revision of the manuscript, for important intellectual content, obtained funding, and material support.

SS, FA and NA contributed to recruiting patients.

JD, RJ, AT, MP, KG and ED revised the manuscript

NP contributed to the study concept

All authors read and approved the final manuscript.

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REFERENCES

1. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global Epidemiology of Non-Alcoholic Fatty Liver Disease-Meta-Analytic Assessment of Prevalence, Incidence and Outcomes. Hepatology. 2015.
2. Molleston JP, White F, Teckman J, Fitzgerald JF. Obese children with steatohepatitis can develop cirrhosis in childhood. The American journal of gastroenterology. 2002;97(9):2460-2.
3. Oh MS, Kim S, Jang JH, Park JY, Kang HS, Lee MS, et al. Associations among the Degree of Nonalcoholic Fatty Liver Disease, Metabolic Syndrome, Degree of Obesity in Children, and Parental Obesity. Pediatr Gastroenterol Hepatol Nutr. 2016;19(3):199-206.
4. Ozturk K, Uygun A, Guler AK, Demirci H, Ozdemir C, Cakir M, et al. Nonalcoholic fatty liver disease is an independent risk factor for atherosclerosis in young adult men. Atherosclerosis. 2015;240(2):380-6.
5. Aller R, Izaola O, Gomez S, Tafur C, Gonzalez G, Berroa E, et al. Effect of silymarin plus vitamin E in patients with non-alcoholic fatty liver disease. A randomized clinical pilot study. European review for medical and pharmacological sciences. 2015;19(16):3118-24.
6. Della Corte C, Carpino G, De Vito R, De Stefanis C, Alisi A, Cianfarani S, et al. Docosahexanoic Acid Plus Vitamin D Treatment Improves Features of NAFLD in Children with Serum Vitamin D Deficiency: Results from a Single Centre Trial. PloS one. 2016;11(12):e0168216.
7. Ishii H, Horie Y, Ohshima S, Anezaki Y, Kinoshita N, Dohmen T, et al. Eicosapentaenoic acid ameliorates steatohepatitis and hepatocellular carcinoma in hepatocyte-specific Pten-deficient mice. Journal of hepatology. 2009;50(3):562-71.
8. Yan HM, Xia MF, Wang Y, Chang XX, Yao XZ, Rao SX, et al. Efficacy of Berberine in Patients with Non-Alcoholic Fatty Liver Disease. PloS one. 2015;10(8):e0134172.
9. Rana H, Yadav SS, Reddy HD, Singhal S, Singh DK, Usman K. Comparative Effect of Insulin Sensitizers and Statin on Metabolic Profile and Ultrasonographical Score in Non Alcoholic Fatty Liver Disease. J Clin Diagn Res. 2016;10(8):OC19-23.
10. Townsend SA, Newsome PN. Non-alcoholic fatty liver disease in 2016. Br Med Bull. 2016;119(1):143-56.

11. Ohki T, Isogawa A, Iwamoto M, Ohsugi M, Yoshida H, Toda N, et al. The effectiveness of liraglutide in nonalcoholic fatty liver disease patients with type 2 diabetes mellitus compared to sitagliptin and pioglitazone. ScientificWorldJournal. 2012;2012:496453.
12. Smits MM, Tonneijck L, Muskiet MH, Kramer MH, Pouwels PJ, Pieters-van den Bos IC, et al. Twelve week liraglutide or sitagliptin does not affect hepatic fat in type 2 diabetes: a randomised placebo-controlled trial. Diabetologia. 2016;59(12):2588-93.
13. Armstrong MJ, Gaunt P, Aithal GP, Barton D, Hull D, Parker R, et al. Liraglutide safety and efficacy in patients with non-alcoholic steatohepatitis (LEAN): a multicentre, double-blind, randomised, placebo-controlled phase 2 study. Lancet. 2016;387(10019):679-90.
14. Del Ben M, Polimeni L, Baratta F, Pastori D, Loffredo L, Angelico F. Modern approach to the clinical management of non-alcoholic fatty liver disease. World journal of gastroenterology : WJG. 2014;20(26):8341-50.
15. Nobili V, Manco M, Devito R, Ciampalini P, Piemonte F, Marcellini M. Effect of vitamin E on aminotransferase levels and insulin resistance in children with non-alcoholic fatty liver disease. Alimentary pharmacology & therapeutics. 2006;24(11-12):1553-61.
16. Lavine JE, Schwimmer JB, Van Natta ML, Molleston JP, Murray KF, Rosenthal P, et al. Effect of vitamin E or metformin for treatment of nonalcoholic fatty liver disease in children and adolescents: the TONIC randomized controlled trial. Jama. 2011;305(16):1659-68.
17. Schwimmer JB, Middleton MS, Deutsch R, Lavine JE. A phase 2 clinical trial of metformin as a treatment for non-diabetic paediatric non-alcoholic steatohepatitis. Alimentary pharmacology & therapeutics. 2005;21(7):871-9.
18. Vajro P, Mandato C, Franzese A, Ciccimarra E, Lucariello S, Savoia M, et al. Vitamin E treatment in pediatric obesity-related liver disease: a randomized study. Journal of pediatric gastroenterology and nutrition. 2004;38(1):48-55.
19. Pacifico L, Bonci E, Di Martino M, Versacci P, Andreoli G, Silvestri LM, et al. A double-blind, placebo-controlled randomized trial to evaluate the efficacy of docosahexaenoic acid supplementation on hepatic fat and associated cardiovascular risk factors in overweight children with nonalcoholic fatty liver disease. Nutrition, metabolism, and cardiovascular diseases : NMCD. 2015;25(8):734-41.

20. Alisi A, Bedogni G, Baviera G, Giorgio V, Porro E, Paris C, et al. Randomised clinical trial: The beneficial effects of VSL#3 in obese children with non-alcoholic steatohepatitis. Alimentary pharmacology & therapeutics. 2014;39(11):1276-85.
21. Spahis S, Alvarez F, Dubois J, Ahmed N, Peretti N, Levy E. Plasma fatty acid composition in French-Canadian children with non-alcoholic fatty liver disease: Effect of n-3 PUFA supplementation. Prostaglandins, leukotrienes, and essential fatty acids. 2015;99:25-34.
22. Nobili V, Alisi A, Della Corte C, Rise P, Galli C, Agostoni C, et al. Docosahexaenoic acid for the treatment of fatty liver: randomised controlled trial in children. Nutrition, metabolism, and cardiovascular diseases : NMCD. 2013;23(11):1066-70.
23. Capanni M, Calella F, Biagini MR, Genise S, Raimondi L, Bedogni G, et al. Prolonged n-3 polyunsaturated fatty acid supplementation ameliorates hepatic steatosis in patients with non-alcoholic fatty liver disease: a pilot study. Alimentary pharmacology & therapeutics. 2006;23(8):1143-51.
24. Argo CK, Patrie JT, Lackner C, Henry TD, de Lange EE, Weltman AL, et al. Effects of n-3 fish oil on metabolic and histological parameters in NASH: a double-blind, randomized, placebo-controlled trial. Journal of hepatology. 2015;62(1):190-7.
25. Cussons AJ, Watts GF, Mori TA, Stuckey BG. Omega-3 fatty acid supplementation decreases liver fat content in polycystic ovary syndrome: a randomized controlled trial employing proton magnetic resonance spectroscopy. The Journal of clinical endocrinology and metabolism. 2009;94(10):3842-8.
26. Dasarathy S, Dasarathy J, Khiyami A, Yerian L, Hawkins C, Sargent R, et al. Double-blind randomized placebo-controlled clinical trial of omega 3 fatty acids for the treatment of diabetic patients with nonalcoholic steatohepatitis. Journal of clinical gastroenterology. 2015;49(2):137-44.
27. Li YH, Yang LH, Sha KH, Liu TG, Zhang LG, Liu XX. Efficacy of poly-unsaturated fatty acid therapy on patients with nonalcoholic steatohepatitis. World journal of gastroenterology : WJG. 2015;21(22):7008-13.
28. Qin Y, Zhou Y, Chen SH, Zhao XL, Ran L, Zeng XL, et al. Fish Oil Supplements Lower Serum Lipids and Glucose in Correlation with a Reduction in Plasma Fibroblast Growth Factor 21 and Prostaglandin E2 in Nonalcoholic Fatty Liver Disease Associated with Hyperlipidemia: A Randomized Clinical Trial. PloS one. 2015;10(7):e0133496.

29. Parker HM, Johnson NA, Burdon CA, Cohn JS, O'Connor HT, George J. Omega-3 supplementation and non-alcoholic fatty liver disease: a systematic review and meta-analysis. Journal of hepatology. 2012;56(4):944-51.
30. Chen LH, Wang YF, Xu QH, Chen SS. Omega-3 fatty acids as a treatment for non-alcoholic fatty liver disease in children: A systematic review and meta-analysis of randomized controlled trials. Clinical nutrition. 2016.
31. Nobili V, Alkhouri N, Alisi A, Della Corte C, Fitzpatrick E, Raponi M, et al. Nonalcoholic fatty liver disease: a challenge for pediatricians. JAMA Pediatr. 2015;169(2):170-6.
32. Lacasse MCT, A.; Dubois, J.; Alvarez, F.; Spahis, S.; Chagnon, M.; Deschênes, S.; Levy, E. Effects of omega-3 supplementation on liver steatosis and carotid intima-media thickness:
a double-blind, one-way crossover randomized trial. Obesity Science & Practice 2017:11.
33. Shannon A, Alkhouri N, Carter-Kent C, Monti L, Devito R, Lopez R, et al. Ultrasonographic quantitative estimation of hepatic steatosis in children With NAFLD. Journal of pediatric gastroenterology and nutrition. 2011;53(2):190-5.
34. Bedogni G, Bellentani S, Miglioli L, Masutti F, Passalacqua M, Castiglione A, et al. The Fatty Liver Index: a simple and accurate predictor of hepatic steatosis in the general population. BMC gastroenterology. 2006;6:33.
35. Spahis S, Vanasse M, Belanger SA, Ghadirian P, Grenier E, Levy E. Lipid profile, fatty acid composition and pro- and anti-oxidant status in pediatric patients with attention-deficit/hyperactivity disorder. Prostaglandins, leukotrienes, and essential fatty acids. 2008;79(1-2):47-53.
36. Belanger SA, Vanasse M, Spahis S, Sylvestre MP, Lippe S, L'Heureux F, et al. Omega-3 fatty acid treatment of children with attention-deficit hyperactivity disorder: A randomized, double-blind, placebo-controlled study. Paediatr Child Health. 2009;14(2):89-98.
37. Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. The American journal of gastroenterology. 1999;94(9):2467-74.
38. Sviklane L, Olmane E, Dzerve Z, Kupcs K, Pirags VV, Sokolovska J. Fatty liver index and hepatic steatosis index predict non-alcoholic fatty liver disease in type 1 diabetes. Journal of gastroenterology and hepatology. 2017.

39. Lambert M, Paradis G, O'Loughlin J, Delvin EE, Hanley JA, Levy E. Insulin resistance syndrome in a representative sample of children and adolescents from Quebec, Canada. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity. 2004;28(7):833-41.
40. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. 1985;28(7):412-9.
41. Touboul PJ, Hennerici MG, Meairs S, Adams H, Amarenco P, Bornstein N, et al. Mannheim carotid intima-media thickness consensus (2004-2006). An update on behalf of the Advisory Board of the 3rd and 4th Watching the Risk Symposium, 13th and 15th European Stroke Conferences, Mannheim, Germany, 2004, and Brussels, Belgium, 2006. Cerebrovasc Dis. 2007;23(1):75-80.
42. Masson S, Marchioli R, Mozaffarian D, Bernasconi R, Milani V, Dragani L, et al. Plasma n-3 polyunsaturated fatty acids in chronic heart failure in the GISSI-Heart Failure Trial: relation with fish intake, circulating biomarkers, and mortality. Am Heart J. 2013;165(2):208-15 e4.
43. Antunes Bde M, Monteiro PA, Silveira LS, Brunholi Cde C, Lira FS, Freitas Junior IF. Macronutrient Intake Is Correlated with Dyslipidemia and Low-Grade Inflammation in Childhood Obesity but Mostly in Male Obese. Nutricion hospitalaria. 2015;32(3):997-1003.
44. Harris WS. The omega-3 index: from biomarker to risk marker to risk factor. Current atherosclerosis reports. 2009;11(6):411-7.
45. Harris WS, Von Schacky C. The Omega-3 Index: a new risk factor for death from coronary heart disease? Prev Med. 2004;39(1):212-20.
46. Hernaez R, Lazo M, Bonekamp S, Kamel I, Brancati FL, Guallar E, et al. Diagnostic accuracy and reliability of ultrasonography for the detection of fatty liver: a meta-analysis. Hepatology. 2011;54(3):1082-90.
47. Gastaldelli A, Cusi K, Pettiti M, Hardies J, Miyazaki Y, Berria R, et al. Relationship between hepatic/visceral fat and hepatic insulin resistance in nondiabetic and type 2 diabetic subjects. Gastroenterology. 2007;133(2):496-506.
48. Fabbrini E, Magkos F, Mohammed BS, Pietka T, Abumrad NA, Patterson BW, et al. Intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity.

Proceedings of the National Academy of Sciences of the United States of America. 2009;106(36):15430-5.

49. Sanyal AJ, Campbell-Sargent C, Mirshahi F, Rizzo WB, Contos MJ, Sterling RK, et al. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. Gastroenterology. 2001;120(5):1183-92.
50. Yki-Jarvinen H. Liver fat in the pathogenesis of insulin resistance and type 2 diabetes. Digestive diseases. 2010;28(1):203-9.
51. Zimmermann A, Zimmermann T, Schattenberg J, Pottgen S, Lotz J, Rossmann H, et al. Alterations in lipid, carbohydrate and iron metabolism in patients with non-alcoholic steatohepatitis (NASH) and metabolic syndrome. Eur J Intern Med. 2011;22(3):305-10.
52. Gaggini M, Morelli M, Buzzigoli E, DeFronzo RA, Bugianesi E, Gastaldelli A. Non-alcoholic fatty liver disease (NAFLD) and its connection with insulin resistance, dyslipidemia, atherosclerosis and coronary heart disease. Nutrients. 2013;5(5):1544-60.
53. Arulanandan A, Ang B, Bettencourt R, Hooker J, Behling C, Lin GY, et al. Association Between Quantity of Liver Fat and Cardiovascular Risk in Patients With Nonalcoholic Fatty Liver Disease Independent of Nonalcoholic Steatohepatitis. Clin Gastroenterol Hepatol. 2015;13(8):1513-20 e1.
54. Ballestri S, Lonardo A, Romagnoli D, Carulli L, Losi L, Day CP, et al. Ultrasonographic fatty liver indicator, a novel score which rules out NASH and is correlated with metabolic parameters in NAFLD. Liver international : official journal of the International Association for the Study of the Liver. 2012;32(8):1242-52.
55. Juarez-Rojas JG, Medina-Urrutia AX, Jorge-Galarza E, Gonzalez-Salazar C, Kimura-Hayama E, Cardoso-Saldana G, et al. Fatty liver increases the association of metabolic syndrome with diabetes and atherosclerosis. Diabetes care. 2013;36(6):1726-8.
56. Zhang T, Zhang Y, Zhang C, Tang F, Li H, Zhang Q, et al. Prediction of metabolic syndrome by non-alcoholic fatty liver disease in northern urban Han Chinese population: a prospective cohort study. PloS one. 2014;9(5):e96651.
57. Ryoo JH, Choi JM, Moon SY, Suh YJ, Shin JY, Shin HC, et al. The clinical availability of non alcoholic fatty liver disease as an early predictor of the metabolic syndrome in Korean men: 5-year's prospective cohort study. Atherosclerosis. 2013;227(2):398-403.

58. Lonardo A, Ballestri S, Marchesini G, Angulo P, Loria P. Nonalcoholic fatty liver disease: a precursor of the metabolic syndrome. Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver. 2015;47(3):181-90.
59. van der Poorten D, Milner KL, Hui J, Hodge A, Trenell MI, Kench JG, et al. Visceral fat: a key mediator of steatohepatitis in metabolic liver disease. Hepatology. 2008;48(2):449-57.
60. Finelli C, Tarantino G. Should visceral fat, strictly linked to hepatic steatosis, be depleted to improve survival? Hepatology international. 2013;7(2):413-28.
61. Eguchi Y, Eguchi T, Mizuta T, Ide Y, Yasutake T, Iwakiri R, et al. Visceral fat accumulation and insulin resistance are important factors in nonalcoholic fatty liver disease. J Gastroenterol. 2006;41(5):462-9.
62. Seppala-Lindroos A, Vehkavaara S, Hakkinen AM, Goto T, Westerbacka J, Sovijarvi A, et al. Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. The Journal of clinical endocrinology and metabolism. 2002;87(7):3023-8.
63. McMillan KP, Kuk JL, Church TS, Blair SN, Ross R. Independent associations between liver fat, visceral adipose tissue, and metabolic risk factors in men. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme. 2007;32(2):265-72.
64. Sands SA, Reid KJ, Windsor SL, Harris WS. The impact of age, body mass index, and fish intake on the EPA and DHA content of human erythrocytes. Lipids. 2005;40(4):343-7.
65. Parker HM, O'Connor HT, Keating SE, Cohn JS, Garg ML, Caterson ID, et al. Efficacy of the Omega-3 Index in predicting non-alcoholic fatty liver disease in overweight and obese adults: a pilot study. The British journal of nutrition. 2015;114(5):780-7.
66. Yamazaki Y, Kondo K, Maeba R, Nishimukai M, Nezu T, Hara H. Proportion of nervonic acid in serum lipids is associated with serum plasmalogen levels and metabolic syndrome. J Oleo Sci. 2014;63(5):527-37.
67. Lemaitre RN, Fretts AM, Sitlani CM, Biggs ML, Mukamal K, King IB, et al. Plasma phospholipid very-long-chain saturated fatty acids and incident diabetes in older adults: the Cardiovascular Health Study. The American journal of clinical nutrition. 2015;101(5):1047-54.

68. Forouhi NG, Koulman A, Sharp SJ, Imamura F, Kroger J, Schulze MB, et al. Differences in the prospective association between individual plasma phospholipid saturated fatty acids and incident type 2 diabetes: the EPIC-InterAct case-cohort study. Lancet Diabetes Endocrinol. 2014;2(10):810-8.
69. Janczyk W, Lebensztejn D, Wierzbicka-Rucinska A, Mazur A, Neuhoff-Murawska J, Matusik P, et al. Omega-3 Fatty acids therapy in children with nonalcoholic Fatty liver disease: a randomized controlled trial. The Journal of pediatrics. 2015;166(6):1358-63 e1-3.
70. Kim CH, Han KA, Yu J, Lee SH, Jeon HK, Kim SH, et al. Efficacy and Safety of Adding Omega-3 Fatty Acids in Statin-treated Patients with Residual Hypertriglyceridemia: ROMANTIC (Rosuvastatin-OMAcor iN residual hyperTrIglyCeridemia), a Randomized, Double-blind, and Placebo-controlled Trial. Clin Ther. 2018;40(1):83-94.
71. Barbosa MM, Melo AL, Damasceno NR. The benefits of omega-3 supplementation depend on adiponectin basal level and adiponectin increase after the supplementation: A randomized clinical trial. Nutrition. 2017;34:7-13.
72. Nobili V, Bedogni G, Alisi A, Pietrobattista A, Rise P, Galli C, et al. Docosahexaenoic acid supplementation decreases liver fat content in children with non-alcoholic fatty liver disease: double-blind randomised controlled clinical trial. Archives of disease in childhood. 2011;96(4):350-3.
73. Nogueira MA, Oliveira CP, Ferreira Alves VA, Stefano JT, Rodrigues LS, Torrinhas RS, et al. Omega-3 polyunsaturated fatty acids in treating non-alcoholic steatohepatitis: A randomized, double-blind, placebo-controlled trial. Clinical nutrition. 2016;35(3):578-86.
74. Zhu FS, Liu S, Chen XM, Huang ZG, Zhang DW. Effects of n-3 polyunsaturated fatty acids from seal oils on nonalcoholic fatty liver disease associated with hyperlipidemia. World journal of gastroenterology : WJG. 2008;14(41):6395-400.

Table 1: Baseline anthropometric and liver characteristics of young boy NAFLD patients

Variables	CTR (n=20)	mNAFLD (n=9)	sNAFLD (n=11)
Age (y)	13.85 ± 0.21	13.22 ± 1.02	14.33 ± 0.97
BMI (Kg/m2)	17.14 ± 0.26	32.09 ± 1.52***	32.00 ± 1.76***
ALT (U/L)	19.86 ± 0.75	20.67 ± 1.13	52.10 ± 4.45***###
AST (U/L)	15.86 ± 0.90	22.44 ± 1.54**	34.80 ± 1.61***###
ALT/AST	1.28 ± 0.05	0.93 ± 0.04	1.49 ± 0.09###
GGT (U/L)	11.80 ± 0.73	17.25 ± 1.70*	25.78 ± 3.59**#
Liver-kidney contrast (%)			
0 = Liver hypoechoic relative to kidney		20	0
1 = Mild hyperechoic liver relative to kidney		60	0
2 = Moderate hyperechoic liver relative to kidney		10	35
3 = Marked hyperechoic liver relative to kidney		10	65
Ultrasound deep attenuation (%)			
0 = No deep attenuation		75	45
1= Visible, blurred diaphragm		25	45
2 = Undistinguishable diaphragm		0	10
Vessel blurring (%)			
0 = No vessel blurring		90	0
1 = Narrowed and blurred vessels		10	90
Steatosis score (%)			
0		0	0
1-2		75	0
2-3		25	85
3-4		-	15
Mean		1.67±0.47	4.7±0.3###
Fatty liver index (%)			
Low (≤ 30)		80	30

High-very high (60-90)	20	70
Mean	52.11±6.25	71.60±8.85$^{\#}$

ALT: alanine transaminase; AST: aspartate aminotransferase; BMI: body mass index; CTR: controls (Healthy subjects); GGT: gamma-glutamyl transpeptidase; mNAFLD: moderate non-alcoholic fatty liver disease; sNAFLD: severe non-alcoholic fatty liver disease

All values are expressed as mean ± SEM. Data were analyzed by using Anova Tukey's multiple comparisons or student *t* test (if two groups). *P<0.05, **P<0.001, ***P<0.0001 vs CTR; $^{\#}$P<0.05, $^{\#\#\#}$P<0.0001 vs. mNAFLD group.

Table 2: Baseline metabolic biomarkers and lipid profile of young NAFLD patients

Variables	CTR (n=20)	mNAFLD (n=9)	sNAFLD (n=11)
Metabolic variables			
Fasting blood Glucose (mmol/L)	5.22 ± 0.08	5.23 ± 0.11	5.35 ± 0.09
Fasting Insulin (pmol/L)	29.03 ± 2.08	140.30 ± 6.85***	182.80 ± 15.53$^{***\#}$
HOMA-IR	0.86 ± 0.10	4.68 ± 0.23***	5.59 ± 0.52$^{***\#}$
SBP (mmHg)	106.33 ± 1.50	111.67 ± 3.85	116.27 ± 5.11*
DBP (mmHg)	58.15 ± 1.17	57.33 ± 1.66	59.73 ± 2.87
IMTL (mm)	ND	0.56 ± 0.03	0.59 ± 0.02
IMTR (mm)	ND	0.56 ± 0.03	0.58 ±0.02
Lipid profile			
TG (mmol/L)	0.63 ± 0.03	1.46 ± 0.18***	1.35 ± 0.14***
TC (mmol/L	4.23 ± 0.13	4.56 ± 0.41	4.03 ± 0.26
HDL-C (mmol/L)	1.60 ± 0.02	1.00 ± 0.08***	0.81 ± 0.06***
LDL-C (mmol/L)	2.34 ± 0.13	2.72 ± 0.32	2.60 ± 0.24
Apo AI (g/L)	1.35 ±0.02	1.22 ± 0.07*	1.10 ± 0.04***
Apo B (g/L)	0.63 ± 0.03	0.80 ± 0.02*	0.77 ± 0.05*
Apo C2 (ug/ml)	105.19 ± 10.90	137.35 ± 15.06	120.45 ± 8.09
Apo C3 (ug/ml)	147.02 ± 7.60	168.48 ± 15.64	117.47 ± 16.66*
Lipid ratios			
Apo C2/Apo C3	0.71 ± 0.06	0.73 ± 0.12**	1.38 ± 0.26$^{\#*}$
TC/HDL-C	2.66 ± 0.09	4.63 ± 0.45***	5.25 ± 0.51***
LDL-C/ HDL-C	1.47 ± 0.08	2.70 ± 0.25***	3.45 ± 0.45***
Apo B/Apo AI	0.47 ± 0.02	0.68 ± 0.05**	0.70 ± 0.05***
Apo B/TG	1.07 ± 0.08	0.63 ± 0.10*	0.63 ± 0.08*

Apo: apolipoprotein; CTR: control; DBP: diastolic blood pressure; HDL-C: high-density lipoprotein-cholesterol; IMTL: intima media thickness left; IMTR: intima media thickness right; LDL-C: low-density lipoprotein-cholesterol; ND: no data; SBP: systolic blood pressure; TC: total cholesterol; TG: triglyceride.

All values are expressed as mean ± SEM. Data were analyzed by using Anova Tukey's multiple comparisons. $^{*}P<0.05$, $^{**}P<0.001$, $^{***}P<0.0001$ vs. CTR; $^{\#}P<0.05$ vs. mNAFLD group.

Table 3: Baseline lipid peroxidation and inflammatory biomarkers of young NAFLD patients

Variables	CTRL (n=20)	mNAFLD (n=9)	sNAFLD (n=11)
MDA (pmol/L)	127.51 ± 7.39	212.17 ± 27.17**	187.47 ± 18.55*
oxLDL (U/L)	0.49 ± 0.02	50.26 ± 6.86***	53.50 ± 4.16***
hCRP (mg/L)	0.15 ± 0.02	2.58 ± 0.81***	2.15 ± 0.80***
IL-6 (pg/ml)	43.13 ± 5.12	92.36 ± 4.37***	93.35 ± 5.15***
Adiponectin (ng/ml)	8820.00 ± 670.00	2268.96 ± 559.05***	1624.70 ± 277.88***
Leptin (ng/ml)	1.90 ± 0.28	46.83 ± 7.07***	33.72 ± 5.04***
Acyl-ghrelin (pg/ml)	78.28 ± 5.04	151.80 ± 16.10***	156.56 ± 11.50***

CTR: control; hCRP: human C reactive protein; IL: interleukin; MDA: malondialdehyde; ox: oxidized.

All values are expressed as mean ± SEM. Data were analyzed by using an Anova Tukey's multiple comparisons. *P<0.05, **P<0.001, ***P<0.0001 vs. CTR.

Table 4: Baseline fatty acids composition in red blood cells in NAFLD patients

Fatty Acids (FA)	CTR (n=20)	mNAFLD (n=9) (%)	sNAFLD (n=11)	CTR (n=20)	mNAFLD (n=9) (µg/g Hb)	sNAFLD (n=11)
SFA						
PA (C16:0)	16.74 ± 0.72	25.57 ± 1.20***	27.16 ± 1.2***8	1392.14 ± 24.42	2668.77 ± 288.63***	3063.36 ± 232.46***
SA (C18:0)	16.02 ± 1.03	16.69 ± 0.29	15.43 ±1.58	1337.81 ± 85.35	1708.02 ± 99.52*	1765.72 ± 201.26**
MUFA						
POA (C16 :1n-7)	0.28 ± 0.02	1.18 ± 0.10***	1.22 ± 0.16***	23.33 ± 2.37	124.51 ± 17.79***	139.94 ± 20.09***
OA (C18:1n9)	16.38 ± 0.66	18.62 ± 0.61	18.91 ± 0.76*	1397.32 ± 93.00	1919.95 ± 150.94**	2121.02 ± 128.13***
EIA (C20:1n9)	0.58 ± 0.10	0.34 ± 0.01**	0.34 ± 0.01**	47.95 ± 7.82	34.41 ± 1.30	37.40 ± 1.45
ETA (C20:3n9)	0.09 ± 0.01	0.05 ± 0.00**	0.06 ± 0.00**	7.42 ± 0.84	5.02 ± 0.30*	6.29 ± 0.53
ERA (C22:1n9)	1.22 ± 0.04	0.76 ± 0.05**	0.97 ± 0.17*	102.77 ± 4.91	76.35 ± 4.32**	78.54 ± 5.03***
NA (C24:1n9)	5.89 ± 0.27	3.31 ± 0.20**	2.96 ± 0.19***	499.35 ± 30.22	333.21 ± 16.44**	324.09 ± 13.98***
PUFA						
n-3 series						
ALA (C18:3n3)	0.23 ± 0.02	0.13 ± 0.01**	0.13 ± 0.01**	19.31 ± 1.74	13.74 ± 1.87*	13.90 ± 0.87*
EPA (C20:5n3)	0.46 ± 0.06	0.39 ± 0.02	0.32 ± 0.02$^{*\#}$	40.15 ± 6.28	41.12 ± 3.00	34.13 ± 1.05$^{*\#}$
DPA (C22:5n3)	2.53 ± 0.38	1.41 ± 0.16	1.24 ± 0.14*	225.60 ± 37.06	139.02 ± 12.25	134.67 ± 12.30*
DHA (C22:6n3)	5.12 ± 0.61	3.50 ± 0.20*	2.67 ± 0.21$^{***\#}$	420.77 ± 35.20	322.95 ± 27.40	312.75 ± 23.97*
n-6 series						
LA (C18 :2n-6)	11.05 ± 0.88	6.53 ± 0.17**	6.48 ± 0.27**	955.87 ± 93.33	670.34 ± 46.54*	728.42 ± 47.61*
GLA (C18:3n6)	0.00 ± 0.00	0.33 ± 0.02***	0.32 ± 0.01***	0.00 ± 0.00	33.39 ± 1.57***	35.38 ± 1.89***
HGLA (C20:3n6)	2.40 ± 0.24	1.12 ± 0.07***	1.23 ±0.06**	209.95 ± 25.29	112.83 ± 6.89**	136.10 ± 5.08**
AA (C20:4n6)	3.83 ± 0.53	9.86 ± 0.37***	9.14 ± 0.46***	318.59 ± 38.95	1001.59 ± 49.94***	1024.17 ± 63.25***
DIA (C22:4n6)	4.01 ± 0.53	3.06 ± 0.25	2.76 ±0.34	353.06 ± 50.21	306.47 ± 19.17	300.54 ± 33.86
Total n-3 FA	8.51 ± 0.62	5.18 ± 0.35**	4.66 ± 0.39***	720.80 ± 58.03	524.04 ± 34.06*	509.45 ± 33.70**
Total n-6 FA	21.69 ± 1.50	21.11 ± 0.64	20.11 ± 0.68	1872.35 ± 159.52	2145.39 ± 102.20	2245.65 ± 100.93

Total n-7 FA	1.64 ± 0.07	2.28 ± 0.13**	2.43 ±0.12***	138.89 ± 8.03	238.74 ± 27.78	274.50 ± 20.40
Total n-9 FA	24.06 ± 0.68	23.08 ± 0.52	23.23 ± 0.68	2047.40 ± 116.86	2368.94 ± 156.36	2603.55 ± 134.13
Total SFA	44.01 ± 1.81	47.76 ± 0.86	48.85 ± 0.96*	3668.91 ± 89.14	4926.31 ± 376.95**	5502.08 ± 287.33***
Total MUFA	19.81 ± 0.69	25.31 ± 0.58**	25.61 ± 0.73**	1686.93 ± 102.76	2602.67 ± 182.33**	2871.76 ± 149.73**
Total PUFA	30.29 ± 1.37	26.33 ± 0.89	24.83 ± 1.00**	2600.56 ± 185.73	2674.45 ± 125.74	2761.39 ± 114.81
Trans FA	0.00 ± 0.00	0.60 ± 0.02***	0.59 ± 0.02***	0.00 ± 0.00	60.78 ± 3.55**	65.73 ± 3.83***
Total FA				8455.75 ± 314.35	9318.22 ± 191.64	10904.80 ± 375.01***#

AA :Arachidonic acid; ALA: Alpha linolenic acid; DHA: Docosahexanoic acid; DPA: Docosapentaenoic acid; DTA: Docosatetraenoic acid; EIA: Eicosenoic acid; EPA: eicosapentaenoic acid; ERA: Erucic acid; ETA: Eicosatrienoic acid; FA: Fatty acid; GLA: Gamma Linoleic acid; Hb: Hemoglobin; HGLA: Homogamma linolenic acid; LA: Linoleic acid; MA: Myristic acid; MUFA: Monounsaturated fatty acid; NA: Nervonic acid; OA: Oleic acid; PA: Palmitic acid; POA: Palmitoleic acid; PUFA: Polyunsaturated fatty acid; SA: Stearic acid; SFA: Saturated fatty acid. All values are expressed as mean ± SEM. Data were analyzed by using an Anova Tukey's multiple comparisons. Statistical significance was defined as *P<0.05; **P<0.001; ***P<0.0001 vs. CTRs; $^{\#}P$<0.05 vs. mNAFLD.

Table 5: Baseline fatty acids ratios in red blood cells in NAFLD patients

	CTR (n=20)	mNAFLD (n=9)	sNAFLD (n=11)
16:1n-7/18:2n-6	0.00 ± 0.00	0.18 ± 0.02***	0.21 ± 0.02***
18:2n-6/20:4n-6	0.17 ± 0.02	0.67 ± 0.03***	0.74 ± 0.06***
20:3n-9/20:4n-6	0.00 ± 0.00	0.01 ± 0.00***	0.01 ± 0.00***
Total n7/18:2n-6	0.01 ± 0.00	0.35 ± 0.02***	0.38 ± 0.03***
24:0/22:0	0.14 ± 0.01	2.85 ± 0.12***	2.69 ± 0.21***
PUFA/SFA	0.03 ± 0.00	0.55 ± 0.03***	0.51 ± 0.03***
EFA (LA+ALA)/NON-EFA	0.01 ± 0.00	0.07 ± 0.00***	0.07 ± 0.00***
EPA/DHA	0.01 ± 0.00	0.13 ± 0.01***	0.13 ± 0.01***
DHA/AA	0.09 ± 0.02	0.32 ± 0.02***	0.31 ± 0.03***
ALA/EPA	0.04 ± 0.01	0.35 ± 0.04***	0.38 ± 0.04***
ALA/LA	0.00 ± 0.00	0.02 ± 0.00**	0.02 ± 0.00**
n-6/n-3	0.14 ± 0.01	4.18 ± 0.21***	4.58 ± 0.35***
Δ5 (20:4n-6/20 :3n-6)	1.89 ± 0.38	9.01 ± 0.47***	7.64 ± 0.55***
Δ6 (20:3n-6/18:2n-6)	0.22 ± 0.02	0.17 ± 0.01**	0.19 ± 0.01**
Δ7 (16:1n-7/16:0)	0.02 ± 0.00	0.05 ± 0.00**	0.04 ± 0.00**
Δ9 (18:1n-9/18:0)	1.10 ± 0.11	1.12 ± 0.05	1.13 ± 0.05
Omega-3 Index : %(EPA+DHA)	5.58 ± 0.60	3.89 ± 0.22**	2.99 ± 0.22**#

AA: Arachidonic acid; ALA: Alpha linolenic acid; DHA: Docosahexanoic acid; EFA: Essential fatty acid; EPA: eicosapentaenoic acid; LA: Linoleic acid; PUFA: Polyunsaturated fatty acid; SFA: Saturated fatty acid. All values are expressed as mean ± SEM. Data were analyzed by using an Anova Tukey's multiple comparisons. Statistical significance was defined as ***P*<0.001; ****P*<0.0001 vs. CTRs; #*P*<0.05 vs. mNAFLD.

Table 6: Anthropometric and liver markers of young severe NAFLD patients following 6 month-treatments with n-3 PUFA

Variables	Before Tx	After Tx	*P*
BMI (Kg/m2)	32.00 ± 1.76	31.24 ± 1.53	***NS***
ALT (U/L)	52.10 ± 4.45	37.75 ± 5.74	***0.0065***
AST (U/L)	34.80 ± 1.61	32.13 ± 3.10	***NS***
ALT/AST	1.49 ± 0.09	1.14 ± 0.11	***0.0005***
GGT (U/L)	25.78 ± 3.59	24.89 ± 3.86	***NS***
Albumin	39.78 ± 0.46	40.56 ± 0.75	***NS***
FLI	71.60 ± 8.85	60.80 ± 10.97	***0.04***

ALT: alanine transaminase; AST: aspartate aminotransferase; BMI: Body mass index; FLI: fatty liver index; GGT: gamma-glutamyl transpeptidase; NS: not significant; PUFA: polyunsaturated fatty acids; Tx: treatment. All values are expressed as mean ± SEM. Data were analyzed by using a student *t* test.

Table 7: Metabolic biomarkers and lipid profile of young severe NAFLD patients following 6 month-treatments with n-3 PUFA

Variables	Before Tx	After Tx	*P*
Metabolic variables			
Fasting blood Glucose (mmol/L)	5.35 ± 0.09	5.37 ± 0.14	***NS***
Fasting Insulin (pmol/L)	182.80 ± 15.53	115.00 ± 12.89	***0.001***
HOMA-IR	5.59 ± 0.52	3.47 ± 0.09	***0.001***
SBP (mmHg)	116.27 ± 5.11	113.36 ± 4.12	***NS***
DBP (mmHg)	59.73 ± 2.87	58.27 ± 2.80	***NS***
IMTL (mm)	0.59 ± 0.02	0.54 ± 0.01	***0.01***
IMTR (mm)	0.58 ±0.02	0.57 ±0.02	***NS***
Lipid profile			
TG (mmol/L)	1.35 ± 0.14	1.07 ± 0.14	***0.0001***
TC (mmol/L)	4.03 ± 0.26	3.60 ± 0.21	***NS***
HDL-C (mmol/L)	0.81 ± 0.06	0.89 ± 0.05	***NS***
LDL-C (mmol/L)	2.60 ± 0.24	2.09 ± 0.18	***0.05***
Apo A1 (g/L)	1.10 ± 0.04	1.10 ± 0.06	***NS***
Apo B (g/L)	0.77 ± 0.05	0.60 ± 0.03	***0.05***
Apo C2 (ug/ml)	120.45 ± 8.09	111.47 ± 10.67	***NS***
Apo C3 (ug/ml)	117.47 ± 16.66	114.84 ± 22.68	***NS***
Ratios			
Apo C2/Apo C3	1.38 ± 0.26	1.35 ± 0.24	***NS***
TC/HDL-C	5.25 ± 0.51	3.97 ± 0.33	***0.05***
LDL-C/HDL-C	3.45 ± 0.45	2.34 ± 0.24	***0.05***
Apo B/Apo AI	0.70 ± 0.05	0.60 ± 0.05	***0.05***
Apo B/TG	0.63 ± 0.08	0.63 ± 0.08	***NS***

Apo: apolipoprotein; DBP: diastolic blood pressure; HDL-C: high-density lipoprotein-cholesterol; IMTL: intima media thickness left; IMTR: intima media thickness right; LDL-C: low-density lipoprotein-cholesterol; NS: not significant; PUFA: polyunsaturated fatty acids; SBP: systolic blood pressure; TC: total cholesterol; TG: triglyceride; Tx: treatment.

All values are expressed as mean ± SEM. Data were analyzed by using a student *t* test.

Table 8: Lipid peroxidation and inflammatory biomarkers of young severe NAFLD patients following 6 month-treatments with n-3 PUFA

Variables	Before Tx	After Tx	*P*
MDA (pmol/L)	187.45 ± 18.55	153.73 ± 15.60	***NS***
oxLDL (U/L)	53.50 ± 4.16	41.06 ± 2.01	***0.001***
CRP (mg/L)	2.15 ± 0.80	1.46 ± 0.60	***NS***
IL-6 (pg/ml)	93.35 ± 5.15	88.23 ± 4.23	***NS***
Adiponectin (ng/ml)	1624.70 ± 277.88	2487.00 ± 385.62	***0.001***
Leptin (ng/ml)	33.72 ± 5.04	26.40 ± 3.16	***0.05***
Acyl-ghrelin (pg/ml)	156.56 ± 11.50	115.64 ± 13.92	***0.05***

CRP: C reactive protein; IL: interleukin; MDA: malondialdehyde; NS: not significant; PUFA: polyunsaturated fatty acids; Tx: treatment. All values are expressed as mean ± SEM. Data were analyzed by using student *t* test.

Table 9: Fatty acids composition in red blood cells in severe NAFLD patients following 6-month treatments with n-3 PUFA

Fatty Acids	Baseline (%)	After treatment (%)	Baseline (µg/g Hb)	After treatment (µg/g Hb)
SFA				
PA (C16:0)	27.16 ± 1.28	22.36 ± 0.97**	3063.36 ± 232.46	2237.99 ± 112.83**
SA (C18:0)	15.43 ±1.58	14.63 ± 1.53	1765.72 ± 201.26	1502.24 ± 162.08*
MUFA				
POA (C16 :1n-7)	1.22 ± 0.16	1.08 ± 0.16	139.94 ± 20.09	110.20 ± 15.81
OA (C18:1n9)	18.91 ± 0.76	22.70 ± 0.51**	2121.02 ± 128.13	2273.05 ± 92.88
EIA (C20:1n9)	0.34 ± 0.01	0.35 ± 0.01	37.40 ± 1.45	34.51 ± 1.07
ETA (C20:3n9)	0.06 ± 0.00	0.05 ± 0.00	6.29 ± 0.53	4.99 ± 0.46**
ERA (C22:1n9)	0.97 ± 0.17	0.77 ± 0.04	78.54 ± 5.03	77.48 ± 5.24
NA (C24:1n9)	2.96 ± 0.19	2.97 ± 0.12	324.09 ± 13.98	297.51 ± 15.50
PUFA				
n-3 series				
ALA (C18:3n3)	0.13 ± 0.01	0.14 ± 0.01	13.90 ± 0.87	13.92 ± 1.12
EPA (C20:5n3)	0.32 ± 0.02	1.04 ± 0.10***	34.13 ± 1.05	102.81 ± 9.19***
DPA (C22:5n3)	1.24 ± 0.14	1.31 ± 0.13	134.67 ± 12.30	133.35 ± 16.76
DHA (C22:6n3)	2.67 ± 0.21	5.89 ± 0.31***	312.75 ± 23.97	589.68 ± 35.53***
n-6 series				
LA (C18 :2n-6)	6.48 ± 0.27	6.78 ± 0.34	728.42 ± 47.61	677.42 ± 38.06
GLA (C18:3n6)	0.32 ± 0.01	0.32 ± 0.02	35.38 ± 1.89	32.26 ± 1.81
HGLA (C20:3n6)	1.23 ±0.06	0.94 ± 0.06**	136.10 ± 5.08	95.26 ± 8.36***
AA (C20:4n6)	9.14 ± 0.46	8.29 ± 0.34	1024.17 ± 63.25	830.91 ± 42.28**
DIA (C22:4n6)	2.76 ±0.34	1.95 ± 0.20	300.54 ± 33.86	196.80 ± 21.93*
Total n-3 FA	4.66 ± 0.39	8.46 ± 0.33***	509.45 ± 33.70	847.63 ± 44.20***
Total n-6 FA	20.11 ± 0.68	18.48 ± 0.43	2245.65 ± 100.93	1850.93 ± 80.06**
Total n-7 FA	2.43 ±0.12	2.41 ± 0.16	274.50 ± 20.40	240.24 ± 15.92
Total n-9 FA	23.23 ± 0.68	26.84 ± 0.52	2603.55 ± 134.13	2687.54 ± 105.28

Total SFA	48.85 ± 0.96	43.18 ± 0.69***	5502.08 ± 287.33	4338.79 ± 203.34**
Total MUFA	25.61 ± 0.73	29.20 ± 0.48***	2871.76 ± 149.73	2922.79 ± 106.34
Total PUFA	24.83 ± 1.00	26.99 ± 0.42	2761.39 ± 114.81	2703.55 ± 104.48
Trans FA	0.59 ± 0.02	0.60 ± 0.02	65.73 ± 3.83	62.64 ± 3.12
Total FA			10904.80 ± 375.01	9949.52 ± 416.35*

AA :Arachidonic acid; ALA: Alpha linolenic acid; DHA: Docosahexanoic acid; DPA: Docosapentaenoic acid; DTA: Docosatetraenoic acid; EIA: Eicosenoic acid; EPA: eicosapentaenoic acid; ERA: Erucic acid; ETA: Eicosatrienoic acid; FA: Fatty acid; GLA: Gamma Linoleic acid; Hb: Hemoglobin; HGLA: Homogamma linolenic acid; LA: Linoleic acid; MA: Myristic acid; MUFA: Monounsaturated fatty acid; NA: Nervonic acid; OA: Oleic acid; PA: Palmitic acid; POA: Palmitoleic acid; PUFA: Polyunsaturated fatty acid; SA: Stearic acid; SFA: Saturated fatty acid. All values are expressed as mean ± SEM. Data were analyzed by using a Student's *t* test. Statistical significance was defined as *P<0.05; **P<0.001; ***P<0.0001 vs. sNAFLD before treatment.

Table 10: Fatty acids ratios in red blood cells in severe NAFLD patients following 6-month treatments with n-3 PUFA

RATIO	Baseline	After treatment
16:1n-7/18:2n-6	0.21 ± 0.02	0.18 ± 0.02
18:2n-6/20:4n-6	0.74 ± 0.06	0.84 ± 0.07*
20:3n-9/20:4n-6	0.01 ± 0.00	0.01 ± 0.00
Total n7/18:2n-6	0.38 ± 0.03	0.36 ± 0.03
24:0/22:0	2.69 ± 0.21	2.82 ± 0.12
PUFA/SFA	0.51 ± 0.03	0.63 ± 0.02**
EFA (LA+ALA)/NON-EFA	0.07 ± 0.00	0.08 ± 0.00
EPA/DHA	0.13 ± 0.01	0.18 ± 0.02*
DHA/AA	0.31 ± 0.03	0.72 ± 0.05****
ALA/EPA	0.38 ± 0.04	0.15 ± 0.02****
ALA/LA	0.02 ± 0.00	0.02 ± 0.00
n-6/n-3	4.58 ± 0.35	2.23 ± 0.12****
Δ5 (20:4n-6/20 :3n-6)	7.64 ± 0.55	9.05 ± 0.50**
Δ6 (20:3n-6/18:2n-6)	0.19 ± 0.01	0.14 ± 0.01**
Δ7 (16:1n-7/16:0)	0.04 ± 0.00	0.05 ± 0.01
Δ9 (18:1n-9/18:0)	1.13 ± 0.05	1.46 ± 0.07***
Omega-3 Index : %(EPA+DHA)	2.99 ± 0.22	6.93 ± 0.36***

AA: Arachidonic acid; ALA: Alpha linolenic acid; DHA: Docosahexanoic acid; EFA: Essential fatty acid; EPA: eicosapentaenoic acid; LA: Linoleic acid; PUFA: Polyunsaturated fatty acid; SFA: Saturated fatty acid. All values are expressed as mean ± SEM. Data were analyzed by using a Student's *t* test. Statistical significance was defined as **P*<0.05; ***P*<0.001; ****P*<0.0001 vs. sNAFLD before treatment.

Table 11: N-3 PUFA-associated cardiometabolic markers in NAFLD

Variables	R	*P*
Δ Omega-3 index		
Δ IMTR	-0.71	***0.05***
Δ HOMA-IR	-0.63	***0.05***
Δ LDL/HDL-C	-0.73	***0.001***
Δ CHOL/HDL-C	-0.78	***0.001***
Δ VLCFA; (C20:0, C22:0, C24:0)	0.86	***0.0001***
Δ n-6/n-3		
CE	0.41	***0.01***
FLI (at the end of Tx)	-0.74	***0.001***
Phospholipids	-0.65	***0.05***
Alpha-linolenic; C18:3n-3		
n-6/n-3 ratio (at the end of Tx)		
Omega 3 index	-0.93	***0.0001***

CE: cholesteryl ester; CHOL: cholesterol; Δ: changes values between the end of treatment and baseline; IMTL: intima media thickness left; FLI: fatty liver index; HDL-C: high-density lipoprotein-cholesterol; Tx: treatment; VLCF: very long chain fatty acids.

Data were analyzed using a univariate analysis correlation with a Spearman test for non-normally distributed variable.

Revue 1

«Metabolic Syndrome as a Multifaceted Risk Factor for Oxidative Stress»

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Spahis S a effectué la revue critique de la littérature, a compilé les données et crée les Figures et Tables qui accompagnent la revue. Elle a contribué à la rédaction, soumission et révision de la revue, sous la supervision du Directeur de recherche, Dr Levy E.

Borys JM a participé à la révision du manuscrit.

E. Levy a contribué à la rédaction et la révision du manuscrit.

Forum Review Article

METABOLIC SYNDROME AS A MULTIFACETED RISK FACTOR FOR OXIDATIVE STRESS

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ABSTRACT

Significance: Metabolic syndrome (MetS) is associated with a greater risk of diabetes and cardiovascular diseases. It is estimated that this multifactorial condition affects 20 to 30% of the world's population. A detailed understanding of MetS mechanisms is crucial for the development of effective prevention strategies and adequate intervention tools that could curb its increasing prevalence and limit its comorbidities, particularly in younger age groups. With advances in basic redox biology, oxidative stress (OxS) involvement in the complex pathophysiology of MetS has become widely accepted. Nevertheless, its clear association with and causative effects on MetS requires further elucidation. **Recent Advances**: Although a better understanding of the causes, risks and effects of MetS is essential, studies suggest that oxidant/antioxidant imbalance is a key contributor to this condition. OxS is now understood to be a major underlying mechanism for mitochondrial dysfunction, ectopic lipid accumulation and gut microbiota impairment. **Critical Issues**: Further studies, particularly in the field of translational research, are clearly required to understand and control the production of reactive oxygen species (ROS) levels, especially in the mitochondria, since the various therapeutic trials conducted to date have not targeted this major ROS-generating system or aimed to delay MetS onset or preventing its progression. **Future directions**: Multiple relevant markers need to be identified in order to clarify the role of ROS in the etiology of MetS. Future clinical trials should provide important proof of concept for the effectiveness of antioxidants as useful therapeutic approaches to simultaneously counteract mitochondrial OxS, alleviate MetS symptoms and prevent complications.

1. INTRODUCTION

Metabolic syndrome (MetS) is a major worldwide concern for the public health system. It now affects 20% to 30% of the population in developed countries. Its persistent increase worldwide represents a risk factor for type 2 diabetes (T2D), cardiovascular diseases (CVD) (85,125) and premature mortality (159). The key components of the MetS cluster are obesity (particularly abdominal body fat accumulation), impaired glucose metabolism, dyslipidemia, and hypertension (17,148) **(Fig.1)**. Growing evidence suggests a dominant pathogenic role for oxidative stress (OxS), a dominant event in cellular damage and dysfunction, given its strong relationship with various MetS combinations and resulting clinical complications (137,166). In fact, it has been proposed that MetS-induced OxS is an early event in all metabolic manifestations in view of the association of the end-products of free radical-mediated OxS with body mass index, insulin resistance (IR) state, hyperlipidemia and hypertension (56,88,218).

In this review, we will focus on the state-of-the-art knowledge pertaining to the role of OxS in the pathogenesis of dysregulated MetS parameters. A link with mitochondrial dysfunction and metabolic signaling pathway derangements will be emphasized. In particular, the impact of OxS-associated MetS on the insulin sensitivity of metabolic organs and crosstalk with low-grade inflammation will be evidenced, since OxS promotes inflammatory agents that conversely participate in reactive oxygen species (ROS) generation, thereby creating a vicious pathogenic circle that amplifies oxidative processes. Critical cellular and molecular determinants will be discussed in order to determine whether OxS is causal or simply related to MetS. Finally, whether OxS lowering represents an interesting target for MetS prevention and complications will be considered with particular focus on the impact of therapies aimed at restoring redox homeostasis and fighting MetS.

2. OXIDATIVE STRESS: A BRIEF OVERVIEW

OxS results from a state of disequilibrium between exaggerated ROS output and a limited biological capability to neutralize free radicals in living organisms. The discrepancy between excessive reactive molecules and weak endogenous defense results in damaged lipids, proteins, DNA and cellular structures **(Fig. 2)**, ultimately culminating in the pathogenesis of a broad range of diseases **(Fig. 3)**. OxS is generated in various dynamic intracellular organelles such as the endoplasmic reticulum, lysosomes and mitochondria as by-products of oxidative protein folding, dysfunctional autophagy, and mitochondrial respiration and detoxification. The main ROS

resulting from chemical reduction of O_2 include superoxide anion ($O_2^{-\bullet}$), hydroxyl radical (•OH), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hypochlorous radical, peroxinitrite radical (ONOO-), ozone and nitric oxide (NO) (166). The relevant sources of ROS are mitochondrial electron-transport system and the homologues of nicotinamide adenine dinucleotide phosphate oxidase (NOX1, NOX2 and NOX3), as well as cyclooxygenases, xanthine oxidase, lipooxygenases and uncoupled nitric oxide synthase (NOS) (137). The reactive molecules are formed by reduction–oxidation (redox) reactions in response to the activation of the several types of oxidases. In a first step, O_2 is reduced with the combination of additional electrons, thereby producing the powerful $O_2^{-\bullet}$ that reacts with other endogenous molecules to generate secondary oxidizing molecules, either directly or via reactions catalyzed by free metals (e.g., Cu, Fe, Cd and Pb) or metals-containing components (88). Thereafter, the reduction of $O_2^{-\bullet}$ by spontaneous or enzymatic dismutation reactions leads to the output of the stable byproduct H_2O_2, which actively participates in the formation of the highly reactive hydroxyl radical and thiol functional groups. Moreover, $O_2^{-\bullet}$ may react with NO to form the potent nitrating and oxidizing ONOO-. In contrast to the short-lived reactive species $O_2^{-\bullet}$ due to different reactions [transformation into quick and spontaneous dismutation to H_2O_2 or via rapid catalysis by superoxide dismutase (SOD)] (59), H_2O_2 is characterized by its long life span, relative stability, easy intra- and intercellular diffusion and tight regulation by endogenous and exogenous enzymes, which convert it into water and O_2 or possibly into different metabolites (22). It is important to mention that ROS signaling actions depend on the subcellular site of production, type of species generated, proximity to antioxidants, species half-life, cell membrane permeability, and local concentration of ROS (69,94). While H_2O_2 is particularly important in cellular signaling, both $O_2^{-\bullet}$ and H_2O_2 are able to oxidatively modify thiols on protein cysteine residues and, thereby, induce structural changes and trigger the signaling activation/inactivation of various molecules such as mitogen-activated protein kinases (modulating the activation of defense mechanisms following exposure to OxS), tyrosine kinases (central players in the post-translational activation of antioxidant enzymes) and transcription factors (controlling several biological processes such as cell growth, cell migration, the expression of pro-inflammatory genes and the biogenesis of extracellular matrix proteins (50,58,112). Importantly, the biological activity of a wide variety of proteins may be compromised not only by thiol oxidation but also by carbonylation, side-chain oxidation, fragmentation, unfolding and misfolding. In addition to damaging ROS during redox homeostasis instability, cells have to tackle organic analogs, including

reactive nitrogen species, nitrous oxide, ONOO-, peroxynitrous acid, nitryl anion, nitrosyl chloride, nitrosyl cation, nitrogen dioxide, dinitrogen trioxide and nitrous acid. The ROS responsible for protein modifications in plasma are: radical species produced by the activities of NOX, NOS and oxygenase, in addition to reactive nitrogen species from myeloperoxidase (MPO) and NOS activities, and hypochlorous acid from MPO.

Antioxidants provide a critical defense against OxS. The antioxidant defense system consists of several endogenous enzymes (catalase [CAT], GPX, thrioredoxin reductase, Cu Zn SOD [SOD1], Mn SOD [SOD2], extracellular SOD [SOD3], and glutathione reductase [GR)]) and various circulating biomolecules (bilirubin, coenzyme Q10, N-acetycysteine, melatonin, uric acid, glutathione, coenzyme, nitric oxide, pyruvate, albumin, and ceruloplasmin), as well as dietary components (vitamins A, C, E, folic acid, flavonoids, polyphenols, Zinc, and selenium) (37,97,133,157,204).

2.1 Oxidative stress and metabolic syndrome

MetS is characterized by an inappropriate rise of various factors, but particularly plasma-free fatty acids (FFA) (74) that activate ROS production (86). Even though the mechanism is still poorly understood, FFA increase has been shown to enhance •OH, $O_2^{-\bullet}$ and H_2O_2 in endothelial and vascular smooth muscle cells (VSMC) (86). Moreover, fat accumulation constitutes a trigger for ROS augmentation in adipose tissue, likely by stimulating NOX and lowering anti-oxidative enzymes (62). On the other hand, the exposure of adipose tissues to OxS under MetS conditions results in a decrease in anti-inflammatory adiponectin and an increase in inflammatory cytokines (154,188), both of which lead to compromised insulin signaling through the induction of insulin receptor phosphorylation and deterioration of glucose transporter 4 (GLUT4) translocation and gene transcription (15).

Additional studies have reported the regulatory role of transcription factor p53, a critical tumor suppressor and protector of the genome in the expression of glutathione peroxidase (GPX)1, the most profuse isoform of the GPX family capable of detoxifying H_2O_2 (195). Under pathophysiological conditions such as MetS, suppression of p53 down-regulates basal GPX1 and SOD2 expression, which triggers cellular ROS production, leading afterwards to oxidative damage, whereas reestablishing physiological levels of p53 enhances the antioxidant enzymes and lowers ROS formation (108,117,174) (**Fig. 4**). It is important to note that elevated ROS concentrations could inactivate tumor suppressor phosphatase and tensin homolog (PTEN) deleted on

chromosome 10, a member of the protein tyrosine phosphatase family, either in a forthright manner by oxidizing its cysteine residue or indirectly by stimulating its phosphorylation, thereby interfering with PTEN presence on the plasma membrane and resulting in the activation of the PI3K/Akt signaling cascade (98) (**Fig. 5**). Conversely, PTEN upregulation modulates PI3K/AKT signaling, which reduces cellular ROS generation (216). Collectively, these findings suggest the existence of multiple mechanisms of OxS elaboration by ROS in MetS. However, further research is needed to define the role of OxS-mediated PTEN and p53 in insulin target tissues and IR (**Fig. 5**).

2.2 Oxidative stress and obesity

Obesity, an excess accretion of adipose tissue, is a global pandemic (57). Its ever-increasing prevalence is related to the dysregulation of caloric intake and energy expenditure. A positive energy balance promotes OxS that is likely a mechanistic connexion between obesity and related complications, including IR. For example, FFAs that result from high fat and carbohydrate intake in obese rodents and humans may favor ROS production because of the saturation of the electron transport chain in the mitochondria (3,62). In turn, OxS may amplify fat accumulation by stimulating pre-adipocyte proliferation, adipocyte differentiation and mature adipocyte size (62,79,113). It has also been observed that OxS controls food intake and body weight by upholding various effects on hypothalamic neurons with a concomitant impact on satiety and hunger (82). When obesity sets in, adipose tissue becomes an ideal site capable of producing ROS by upregulating NOX activity (62,129).

Low levels of antioxidant molecules (146,207) along with high plasma advanced oxidation protein products, oxidized low-density lipoprotein (oxLDL), thiobarbituric acid reactive substances (42), and additional cellular/systemic OxS indicators have been reported in obese subjects. The activities of antioxidant enzymes, including GPX and CAT, have been found to be markedly decreased in obesity (155,173). Antioxidant defenses, which normally protect against infectious processes and contribute to normal functions such as proliferation, differentiation and signaling (19,32,61,92,171,194,215), when diminished, are unable to scavenge ROS. Although an inverse relationship between total antioxidant capacity and visceral fat was noted, regardless of other variables (35), an inverse relationship between body mass index/total body fat percentage could not be clearly demonstrated (23). One possible explanation is that different tissues attempt to increase their antioxidant defenses in order to counteract OxS.

NOXs in vascular cells constitute a major ROS site that can set off downstream components such as NO synthase and the mitochondria (136). Interestingly, elevated vascular ROS in mice overexpressing p22phox and under high-fat regimen-trigger obesity and even a MetS phenotype by raising inflammation, promoting adipogenesis and developing exercise intolerance (221). Fat accumulation-induced OxS also takes part in the pathogenesis of obesity-associated MetS through the deregulated synthesis of adipocytokines such as PAI-1, leptin, resistin, visfatin, adiponectin, tumor necrosis factor-α and interleukin-6 (71) (**Fig. 6**). While several groups agree that obesity can cause metabolic derangements and that it precedes and predisposes to the development of MetS via leptin resistance (221), the induction of leptin resistance by vascular ROS is able to disrupt the inhibition of insulin biogenesis and delivery from pancreatic β-cells occurring under normal conditions, thereby constituting a feed-forward cycle forcing weight gain (106). Further support of this concept may be reflected by the diffusion of NOX-generated ROS to the mitochondria to activate OxS, thereby causing mitochondrial dysfunction, limiting muscle exercise capacity and favoring weight gain (26,221). Accordingly, Mn-SOD knockout mice exhibited severe exercise disturbances (109).

2.3 Oxidative stress and impaired glucose metabolism/insulin resistance

IR plays a crucial role in interlinking the various constituents of the MetS cluster and in fortifying the syndrome's evolution. OxS has been shown to be associated with IR (54), a key feature of MetS (3). This association is not surprising, given the insulin action of glucose metabolism and cascade signaling (143), the abnormalities of which contribute to MetS (223). Indeed, insulin insensitivity leads to increase circulating glucose levels (as frequently noticed in the postprandial state) because of the inability to coordinately suppress hepatic glucose production and glycogen degradation, while enhancing glucose uptake into the muscle and adipose tissue via the mobilization of insulin dependent glucose transporter type-4 (GLUT4) from intracellular storage membrane vesicles to the cell surface (7,192). To better understand this aspect, investigators exposed skeletal muscle cells to fructose and noted diminished glucose uptake and sluggish GLUT4 translocation and disturbed insulin signaling in response to the ROS-interceded activation of intracellular c-Jun N-terminal kinase and extracellular signal–regulated kinase 1/2 (ERK1/2) pathways (89). Antioxidant mechanisms to shut off ROS-initiated signals prevented IR and its metabolic consequences. Similarly, the administration of antioxidants to fructose-fed rats lowered ROS production and precluded IR (189). Therefore, excessive ROS concentrations were capable of damaging cells by

oxidizing biomolecules and stimulating various stress sensitive intracellular pathways such as c-Jun N-terminal kinase, ERK1/2, and NF-κB, resulting in the development of IR through transcription factor and stress kinase-mediated chronic, low-grade inflammation (104,162). It is worth noting that IR affects GLUT1 (the transporter of glucose) expression and dehydroascorbic acid (the oxidized form of ascorbate), both of which function in antioxidant defense (75,177). This is important, since GLUT1 is largely abundant in glycolytically active cells and has been snapped up by vitamin C auxotrophs to preserve hepatic redox states through the transport of dehydroascorbate. Generation of the electron carrier nicotinamide adenine dinucleotide phosphate in the pentose phosphate pathway and the conversion of dehydroascorbic acid to ascorbate depress ROS levels: the former by donating electrons to antioxidant pathways such as GR and thioredoxin reductase and the latter by serving as an antioxidant.

ROS over-building and restricted antioxidant enzyme concentrations not only increase IR, but also affect the secretory capacity and viability of β-cells (96), a major component of MetS. Accordingly, IR-associated hyperglycemia is linked to the excessive generation of free radicals and superoxides via the proton electrochemical gradient, which overwhelms the antioxidant defense capacity of the various tissues (147,204). Persistence of hyperglycemia is thought to favor glucose autoxidation and NOS activation, which collectively enhance OxS (45,67), and culminate in polyol, hexosamine, advanced glycosylation end products and protein kinase C pathways (24), which collectively exacerbate the complications of T2D (14,38,53,102,122). Furthermore, elevated circulating and cellular OxS markers in patients with nascent MetS are associated with increased levels of oxLDL, nitrotyrosine, monocyte superoxide and NOX activity along with a decrease in the nuclear factor erythroid-derived 2 like 2 (Nrf2) antioxidant defense system (91). A decline in transcription factor function in response to the disequilibrium in the redox system results in imperfectly weak antioxidant defense, since Nrf2 cannot be freed from kelch-like ECH-associated protein, thereby preventing its translocation into the nucleus to bind to the antioxidant reactive element in the genes that encode antioxidant enzymes such as GR, aldo-keto reductase, heme oxygenase-1, and γ-glutamylcysteine synthetase (31).

2.4 Oxidative stress and dyslipidemia

Dyslipidemia is characterized by increased plasma triglycerides (TGs), ascribed to augmented synthesis of very-low-density lipoproteins (VLDLs), FFAs, small dense LDL particles and reduced high-density lipoprotein cholesterol (HDL-C) in MetS (52,72,170). It is now thought that small,

dense LDL particles, non-HDL-C and apolipoprotein B represent valuable markers for the diagnosis and severity of MetS (65,95,128,135). Interestingly, elevated OxS has been simultaneously associated with hypertriglyceridemia, deficient HDL antioxidant capacity and a chronic inflammatory state in patients with MetS (19). A link between OxS and lipid disorders in MetS has been reinforced by the administration of statins that improved not only atherogenic dyslipidemia, but also oxidant/antioxidant status in subjects with MetS (19). Similarly, inhibition of intestinal cholesterol absorption by ezetimibe was accompanied with an amelioration of IR by lowering ROS in obese mice (193). Additional evidence and potential mechanisms explain the relationship between OxS and lipoprotein levels in the context of dyslipidemia: (i) increased production of $O_2^{-\bullet}$ via the NADP oxidase pathway is detected during hypertriglyceridemia, hypertension and obesity (21,222); (ii) the susceptibility of small, dense LDL particles to oxidation is a function of hypertriglyceridemia degree (105); (iii) LDL oxidation intensifies with augmented waist circumference, C reactive protein concentration and the number of LDL particles (29,44,60,200,205,210); (iv) the lowering of bilirubin levels by MetS contribute to increased LDL oxidation and cardiovascular risk (140), whereas normal bilirubin concentrations can prevent lipid oxidation (215); (v) the aberrant expression of small noncoding RNAs disturbs mitochondrial and endoplasmic reticulum metabolic homeostasis, thereby impacting on lipid and lipoprotein metabolism in the MetS (34); and (vi) red meat consumption is associated with the occurrence of OxS, hypertriglyceridemia and MetS (36).

2.5 Oxidative stress and hypertension

The endothelium, interfacing with the blood stream, is not only a physical barrier tightly controlling vascular permeability such as the passage of luminal macromolecules into the tissue , but it also ensures vessel homeostasis via modulation of vascular tone, platelet aggregation and coagulation, inflammation and growth factors. For instance, barrier dysfunction occurs with high levels of angiopoietin 2 and vascular endothelial growth factor. On the contrary, NO as an endothelium-derived relaxing factor inhibits platelet aggregation, neutrophil attachment to endothelial cells, adhesion molecule expression and smooth muscle cell proliferation (64,138). Under NO deficiency, the onset of atherosclerosis can be observed. Interestingly, a controlled production of low concentrations of ROS, as signaling molecules, maintains vessel integrity by orchestrating endothelial function and vascular contraction/dilatation (199). However, an aberrant balance between free radicals and antioxidants results in endothelial dysfunction, enhanced contractility,

VSMC growth, monocyte intrusion, lipid peroxidation, inflammation, and marked deposition of extracellular matrix proteins, key components in hypertensive vascular injury (49,197) (**Fig. 2**). Available conclusive reports underscore the critical role of ROS in the development of vascular dysfunction and hypertension (130). This link between OxS and deep alterations in the vasculature can be due to rich providers of NOX in endothelial, adventitial, and vascular smooth muscle cells (111). Indeed, $O_2^{-\bullet}$ produced in high concentrations interacts with NO to produce the highly reactive intermediate ONOO− that acts as a vasoconstrictor and a cytotoxic molecule with the potential to cause oxidative damage to cellular constituents (219). ONOO− is also an inhibitor of prostacyclin synthase and endothelial NO synthase activities, which restrain the bioavailability of antioxidants and endothelial mediators with protective functions (12,110). This radical species, closely implicated in experimental and human hypertension, is now recognized as a mediator of vascular injury in cardiovascular pathologies (78,116,158,196). For instance, genetic animal models such as the spontaneously hypertensive rat (SHR) exhibit a number of polymorphisms in the gene promoter region of the NOX $p22^{phox}$ subunit with augmented protein expression and enhanced NOX activity (39). The resulting abundance of vascular $O_2^{-\bullet}$ generation reduced NO bioavailability and raised OxS, whereas the administration of antioxidant vitamins, NOX inhibitors, SOD mimetics, BH4 and Ang II type-1 receptor blockers concomitantly reduced vascular $O_2^{-\bullet}$ formation and markedly alleviated hypertension in these animals (158,171,184), which suggests that NOX upregulation and OxS increase precede the development of hypertension. This concept is strengthened by the elevated expression of $p47^{phox}$, another key component of NOX endowed with the ability to stimulate $O_2^{-\bullet}$ production (190) in the renal vasculature, macula densa and distal nephron from SHRs (61,103). Human studies also provide ROS by-product accumulation in hypertensive patients (40,167,217), probably arising from decreased SOD and CAT antioxidant activity in combination with low concentrations of ROS scavengers (vitamin E, glutathione) (167,209). Interestingly, the renin-angiotensin system appears as a potent contributor of NOX activation and ROS generation (49,111,198). In this context, it is appropriate to recall that the therapeutic BP-lowering actions of Ang II type-1 receptor blockers and angiotensin-converting enzyme inhibitors have been ascribed to NOX inhibition and decreased ROS production (66,220). Currently, hypertension is considered a primary risk factor for MetS. The association between hypertension and MetS highlights the production of ROS and the development of OxS as common causative links. It is possible that OxS produced in hypertension may lead to other components of

the MetS cluster. IR per se or via an increased plasma concentration of FFAs, a prominent attribute of the MetS, could increase ROS production through NOX activation, deteriorate vascular reactivity, alter the smooth muscle cell phenotype and enhance vascular growth (86,99,212,222), thereby partially accounting for the phenotypic modifications that result in hypertension. For example, IR in rats caused by a high-fructose feeding was closely linked to OxS characterized by an upsurge in the generation of $O_2^{-\bullet}$, culminating in raised plasma levels of lipid peroxidation markers. Elevated cardiac $O_2^{-\bullet}$ production is associated with an overexpression of $p22^{phox}$ with the parallel occurrence of IR and near the start of development of cardiac hypertrophy, mild hypertension and vascular alteration as pointed out by an elevation in the media/lumen ratio of mesenteric arteries (47). Therefore, amplification loops may take part in the intensification of OxS in MetS. The evidence indicating that antioxidant treatment ameliorates OxS and reduces blood pressure in various animal models, including the SHR (144,211), fortifies not only the perception that OxS is undeniably a fundamental element in defining the stage of endothelial dysfunction and blood pressure, but also the prognosis of patients with conventional MetS closely determined by the status of the pro-oxidant/antioxidant balance, which necessitates powerful therapeutic agents able to synergistically scavenge reactive free radicals from every MetS component.

3. MITOCHONDRIA-DERIVED OXIDATIVE STRESS

As mentioned before, ROS are adversely implicated in the deviant signaling and tissue harm detected in the pathophysiology of MetS. The failure to regulate properly redox-sensitive signaling pathways and the OxS-mediated abnormal changes in cellular components are major contributors to tissue dysfunctions. Mitochondria, as major sources of ROS, promote OxS and are clearly implicated in the development of MetS components and complications. Furthermore, mitochondria are targets of cellular ROS that severely affect mitochondrial functions and result in defects in mitochondrial electron transport, complex enzyme activities, adenosine triphosphate (ATP) diminution, caspase 3 liberation and mitochondrial DNA lessening (43,77,101,131,163), thereby contributing to MetS progression. In turn, mitochondrial dysfunction may have repercussions on its regulation of cellular functions, such as intermediary metabolism (77), redox signaling (43), calcium (Ca^{2+}) homeostasis (27,169), cell proliferation (6,153), development (134,178) and cell death (68,208).

3.1 Mitochondria-derived oxidative stress and obesity

A number of studies have stated that obesity causes mitochondrial OxS and dysfunction in different tissues. The vicious cycle between muscle depletion and the accretion of ectopic fat, termed sarcopenia, may result from an intricate interplay of various factors such as OxS and mitochondrial dysfunction. Apparently, high caloric intake raises plasma FFAs and glucose levels, which are closely associated with high ROS generation and obesity exacerbation (149). Conversely, calorie restriction alleviates sarcopenia by reducing mitochondrial abnormalities (124), which highlights the mechanistic implication of mitochondrial OxS and dysfunctions in obesity-comorbidities. The mitochondria of subcutaneous white adipose tissue from obese patients with or without T2D displayed a pronounced increase in protein carbonyls and lipid peroxidation products, reflecting an enhanced mitochondrial ROS production rate along with decreased activities of mitochondrial antioxidant enzymes like SOD and GPX, a phenomenon that is likely to cause metabolic abnormalities in adipose tissue during obesity (30).

In young patients, excess body weight impairs mitochondrial function in cardiomyocytes even in the absence of heart failure and diabetes (145). Mitochondrial dysfunction is characterized by proapoptotic Bax and Bcl-xS, reduced antiapoptotic Bcl-xL expression, cytochrome C release and partially activated caspase cascade, high protein carbonyl content and 8-hydroxy-2′-deoxyguanosine in cariomyocytes. In addition, the use of several experimental animal models of hyperglycemia and overweight/obesity allowed for the detection of mitochondrial ROS-induced OxS, uncoupling and lessened ATP production in the heart of rodent models, whereas the pharmacological approach targeting mitochondria could effectively scavenge mitochondrial ROS, improve cardiac hypertrophy, normalize mitochondrial energetics and insulin-stimulated glucose utilization, thus indicating that obesity and IR are linked to heart mitochondrial dysfunction and OxS (84). Obesity also results in mitochondrial dysfunction in skeletal muscle and adipose tissue in humans and mouse models (101). Given the decline in the number of mitochondria and the decrease in the expression of genes related to mitochondrial biogenesis in adipocytes, which occur concomitantly with aberrant mitochondrial morphology and oxidative phosphorylation functions (16,182), mitochondrial dysfunction may be considered a key factor in deleterious intra-adipocyte metabolism with a negative impact on non-adipose tissues. For example, it has been shown that adipose-tissue dysfunction in perilipin-1 null mice, which affects heart health and contributes to the development of metabolic cardiomyopathy through mitochondrial swollen forms, disrupted cristae and OxS in cardiomyocytes (118). Among the molecular mechanisms behind cardiac

dysfunction in obesity, there are alterations in intracellular Ca^{2+}-mediated regulation, which significantly impacts on contraction and relaxation frequency (13,90). Obesity is also a significant risk for hepatic lipid accumulation caused by the uptake of circulating FFA and induced *de novo* lipogenesis, forcing out lipotoxicity, mitochondrial dysfunction, OxS and IR (107), thereby culminating in the development of non-alcoholic fatty liver disease (73,141). Therefore, the alleviation of mitochondrial OxS and dysfunctions in excessive adipose tissue may be useful in shielding from obesity-induced hepatosteatosis and lipid accumulation in non-adipose tissues.

3.2 Mitochondria-derived oxidative stress and insulin resistance

Mitochondrial dysfunction related to mitochondrial biogenesis, reduced mitochondrial content and/or decreased protein content, along with oxidative protein activity can affect insulin sensitivity (41). The resulting decrease in substrate oxidation (e.g., FFA) leads to lipid accumulation and the accretion of metabolically active diacylglycerols and ceramides, which inhibit insulin receptors and protein kinase AKT (25,176,179). An aggravating factor is the reduced fuel oxidation that disturbs electron flow through the electron transport chain, leading to more electron leakage towards oxygen and the production of superoxide that, in combination with mitochondrial DNA lesions, protein aggregations and lipid peroxidation, gives rise to either mitophagy or apoptosis (131). Therefore, it is not surprising that dysfunctional mitochondria are observed in the context of glucose intolerance (100), largely accompanied by abnormally low mitochondrial NADH:O_2 oxidoreductase activity, small size, down-regulated biogenesis and oxidative phosphorylation pathways (101,132). The mechanism responsible for most of these defects was linked to the declined expression of Peroxisome proliferator-activated receptor gamma coactivator 1 α (PGC1 α), the master regulator of mitochondrial metabolism (101,132) as illustrated in human studies (76,83,168). Nevertheless, these investigations remain inconclusive as to whether the primary cause of these abnormalities is due to the mitochondrial number or actual metabolic changes within the mitochondria (11,20,81,161). Moreover, impaired mitochondrial function in IR is not unanimously accepted, since various groups have reported no correlation or even the absence of alterations in the markers of mitochondrial content and functions, while others have insisted on the offsetting augmentation of mitochondrial oxidative ability with raised lipid provision (46,63,87,175,191,201,202). For now, despite the associative studies of IR and mitochondrial dysfunction, we cannot definitively conclude whether modifications in mitochondrial function are a cause or consequence of IR, particularly in view of various differences in methodology and

experimental protocols. Although several groups have attempted to tackle this critical issue using transgenic approaches such as posttranslational modification of mitochondrial transcription factor A (controlling mitochondrial transcription), PGC1 α (regulating mitochondrial biogenesis and interacting with a great number of transcription factors and nuclear hormone receptors that are associated with mitochondrial function) and acetyl-CoA carboxylase 2 (the inhibition of which stimulates the access of long-chain fatty acids into mitochondria for β-oxidation), their findings are still unable to clearly demonstrate functional defects in mitochondria as upstream faults causing IR (1,33,80,114,151,181,206,213,214).

3.3 Mitochondria-derived oxidative stress and dyslipidemia

The cause that links hyperlipidemia to the pathogenesis of MetS is frequently attributed to the toxic action of lipids in various target tissues. Indeed, it is assumed that lipid overflow causes ectopic fat deposition, which impairs the function of various organs (48). Multiple biochemical mechanisms have been proposed indicating that lipid excess induces cell OxS and damage (180). Free cholesterol, oxLDL and glycated HDL are additional potential causes of mitochondrial dysfunction and/or apoptosis (164). For example, oxLDL affects mitochondrial functions, specifically oxidative phosphorylation in vascular endothelial cells and may increase ROS, resulting in mitochondrial-specific OxS, 8-oxo-deoxyguanine (183).

3.4 Mitochondria-derived oxidative stress and hypertension

Mitochondrial-dependent apoptotic pathway actively participates in vascular endothelium disruption triggered by oxLDL accumulation (120). In fact, oxLDL accretion promotes ROS production in endothelial cells, which leads to pro-apoptotic effects in association with substantial perturbations in mitochondrial ATP production and abnormally high permeability, culminating in cytochrome C discharge and the subsequent activation of executioner caspases (172). In turn, the deterioration of mitochondrial function raises ROS generation and exacerbates OxS in the atherogenic process (8). By acting on the underlining mechanisms (i.e. oxLDL-induced cellular apoptosis and mitochondrial dysfunction) through the mitochondrial-dependent apoptotic pathway and glycogen synthase kinase 3β/β-catenin signaling pathways, it is possible to fight vascular dysfunctions and atherosclerosis (115). Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is a key protein to control in order to prevent severe damage to the endothelium. It represents the major receptor responsible for the binding, internalization and degradation of oxLDL. LOX-1 is implicated in various pathophysiological events, including endothelial

dysfunction, hypertension and atherosclerosis (51). It is upregulated not only by oxLDL, but also by ROS and angiotensin II (119), and results in mitochondrial dysfunction (126). Importantly, full control of LOX-1 may suppress the induction of oxidative damage to mitochondrial DNA and proteins and may improve clinical conditions recognized to undergo vascular remodeling and tissue dysfunction, such as T2D, hypertension, and dyslipidemia (51).

4. MICROBIOTA AND METABOLIC SYNDROME TRAITS

Despite the importance of intestinal microbiota, there are only few data concerning its extra-intestinal metabolic role, namely in MetS. Currently, it is well established that this gut ecosystem not only influences intestinal functioning, but it may also confer metabolic endotoxemia and low-grade chronic inflammation because of the transfer of microbial metabolites into systemic circulation via an abnormally permeable intestinal barrier (142), which eventuates MetS (28). Our recent studies clearly showed that improvement of OxS, endotoxemia, inflammation and MetS features by polyphenols is associated with a robust modulation in the relative abundance of Akkermansia spp. in high fat/high sucrose-fed mice (4,5). Conversely, uncontrolled, prolonged generation of short chain fatty acids by dysbiotic microbiota promotes MetS in Tolk-like receptor 5 in knockout mice (185). Apparently, modification of the gut microbiota by nutritional status, improvement of intestinal integrity and induction of microbial metabolic output could positively impact on host health and may represent beneficial avenues for the management of MetS. So far, it has not been established whether microbiota produces beneficial effects on MetS components by regulating mitochondrial events.

5. EFFECTS OF ANTIOXIDANTS ON METABOLIC SYNDROME TRAITS

Many investigators believe that implementation of lifestyle changes (e.g., healthy diets and physical activity) are of the utmost importance for the treatment of MetS and its consequent complications (150,152). MetS is a constellation of interrelated conditions, including hypertension, dyslipidemia, IR, T2D and central obesity. Treatments that target individual components may fight MetS at the forefront of T2D and CVD. Therefore, the current therapy for obesity includes Orlistat, an inhibitor of gastrointestinal lipase activity for weight reduction, lorcaserin, phentermine-topiramate, buproprion/naltrexone and liraglutide in addition to lifestyle modification and bariatric surgery. The pharmacologic agents for abnormal glucose metabolism/IR and T2D are metformin and glucagon-like peptide 1 receptor agonists. The first line medication for elevated blood pressure includes amiloride and thiazide diuretics for unproblematic patients, angiotensin-converting

enzyme inhibitors or angiotensin receptor blockers for subjects with T2D, congestive heart failure or chronic kidney disease, or beta blockers for persons with angina (18,156,160). Finally, dyslipidemia is treated with HMG Co-A reductase statin inhibitors (LDL-cholesterol), fibrates (TG), niacins (to raise HDL-cholesterol and lower TG and LDL-cholesterol). Interestingly, short-term fenofibrate treatment may ameliorate not only dyslipidemia, but also IR, hypertension, inflammation and OxS markers in individuals with MetS, signifying that this drug can efficiently decrease the risk of arteriosclerosis via different pathways (203).

Nevertheless, lifestyle modifications and better diet control seem to be more effective than medications (123,139). Several clinical investigations have corroborated the benefits of consuming bioactive nutraceuticals from functional foods for the amelioration of hyperglycemia (9,10,32). Various nutritional antioxidants such as flavonoids, arginine, vitamin C, vitamin E, carotenoids, resveratrol and selenium can be used to prevent and treat health complications resulting from MetS (70). However, the underlying mechanisms are not fully elucidated for most of these natural compounds. Flavonoids and other phenolic compounds are widely used in view of their antioxidant content capable of neutralizing OxS or activating cytoprotective gene transcription, which keeps out oxidative impairment of macromolecules (2). Indeed, several natural compounds can stimulate the endogenous defense system by activating the nuclear factor Nrf2, a critical modulator of antioxidant response (194). Various studies reported its preventive effects on the development of MetS and related complications, including the transition from pre-T2D to T2D (93). In view of the significant contribution of mitochondria to ROS production, energy metabolism and MetS development, several therapeutic strategies have recently been designed to prevent mitochondrial dysfunction (55,121,186,187). For example, targeting the antioxidant ubiquinone MitoQ to mitochondria led to enhanced defense against pathological oxidative repercussions in MetS (127,165).

6. CONCLUSIONS

OxS is increasingly perceived as the root cause of the initiation and evolution of MetS and may constitute the major unifying mechanism of the obesity, dyslipidemia, IR and hypertension components associated with MetS. Although mitochondrial redox state and dysfunction are closely implicated in the pathogenesis of MetS, it remains unclear whether they may be the cause or consequence. Substantial evidence indicates ROS scavenging potential in normalizing mitochondrial biogenesis, energetics and functional capacity while preventing MetS complications

and severity, which provides additional mechanistic insight into the role of OxS in MetS development and subsequent complications. However, the therapeutic utility of ROS scavenging and particularly mitochondria-targeted antioxidants as a therapy for MetS-related disorders requires further investigation given the controversial findings. Further studies should be instrumental in determining whether mitochondria-targeted approaches improve mitochondrial structure, DNA integrity, ROS production, dynamics, mitophagy and function, before turning to the wide range of effects and mechanisms of action in MetS features.

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AUTHOR DISCLOSURE STATEMENT

The authors declare that they have no competing financial interests.

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
Ca^{2+}	Calcium
CVD	Cardiovascular diseases
FFA	Free fatty acids
GLUT	Glucose transporter
GPX	Glutathione peroxidase
GR	Glutathione reductase
H_2O_2	Hydrogen peroxide
HDL-C	High-density lipoprotein-cholesterol
IR	Insulin resistance
LOX-1	Lectin-like oxidized low-density lipoprotein receptor-1
MetS	Metabolic syndrome
MPO	Myeloperoxidase
NO	Nitric oxide
NOS	Endothelial nitric oxide synthase
NOX	nicotinamide adenine dinucleotide phosphate oxidases
Nrf2	Nuclear factor erythroid-derived 2 like 2
$O2^{-\bullet}$	Superoxide anion
•OH	Hydroxyl radical
ONOO−	Peroxynitrite
oxLDL	Oxidized low-density lipoprotein
OxS	Oxidative stress
PGC1 α	Peroxisome proliferator-activated receptor gamma coactivator 1 α
PTEN	Tumor suppressor phosphatase and tensin homolog
ROS	Reactive oxygen species
SOD	Superoxide dismutase
T2D	Type 2 diabetes
TG	Triglyceride
VLDL	Very-low-density lipoprotein
VSMC	Vascular smooth muscle cells

REFERENCES

1. Abu-Elheiga L, Matzuk MM, Abo-Hashema KA, Wakil SJ. Continuous fatty acid oxidation and reduced fat storage in mice lacking acetyl-CoA carboxylase 2. *Science* 291: 2613-6, 2001.
2. Alam MA, Subhan N, Rahman MM, Uddin SJ, Reza HM, Sarker SD. Effect of citrus flavonoids, naringin and naringenin, on metabolic syndrome and their mechanisms of action. *Adv Nutr* 5: 404-17, 2014.
3. Anderson EJ, Lustig ME, Boyle KE, Woodlief TL, Kane DA, Lin CT, Price JW, 3rd, Kang L, Rabinovitch PS, Szeto HH, Houmard JA, Cortright RN, Wasserman DH, Neufer PD. Mitochondrial H2O2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. *J Clin Invest* 119: 573-81, 2009.
4. Anhe FF, Pilon G, Roy D, Desjardins Y, Levy E, Marette A. Triggering Akkermansia with dietary polyphenols: a new weapon to combat the metabolic syndrome? *Gut Microbes*: 0, 2016.
5. Anhe FF, Roy D, Pilon G, Dudonne S, Matamoros S, Varin TV, Garofalo C, Moine Q, Desjardins Y, Levy E, Marette A. A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased Akkermansia spp. population in the gut microbiota of mice. *Gut* 64: 872-83, 2015.
6. Antico Arciuch VG, Elguero ME, Poderoso JJ, Carreras MC. Mitochondrial regulation of cell cycle and proliferation. *Antioxid Redox Signal* 16: 1150-80, 2012.
7. Armoni M, Harel C, Karnieli E. Transcriptional regulation of the GLUT4 gene: from PPAR-gamma and FOXO1 to FFA and inflammation. *Trends Endocrinol Metab* 18: 100-7, 2007.
8. Auge N, Maupas-Schwalm F, Elbaz M, Thiers JC, Waysbort A, Itohara S, Krell HW, Salvayre R, Negre-Salvayre A. Role for matrix metalloproteinase-2 in oxidized low-density lipoprotein-induced activation of the sphingomyelin/ceramide pathway and smooth muscle cell proliferation. *Circulation* 110: 571-8, 2004.
9. Bahadoran Z, Mirmiran P, Azizi F. Dietary polyphenols as potential nutraceuticals in management of diabetes: a review. *J Diabetes Metab Disord* 12: 43, 2013.

10. Bahadoran Z, Mirmiran P, Azizi F. Potential efficacy of broccoli sprouts as a unique supplement for management of type 2 diabetes and its complications. *J Med Food* 16: 375-82, 2013.
11. Bandyopadhyay GK, Yu JG, Ofrecio J, Olefsky JM. Increased malonyl-CoA levels in muscle from obese and type 2 diabetic subjects lead to decreased fatty acid oxidation and increased lipogenesis; thiazolidinedione treatment reverses these defects. *Diabetes* 55: 2277-85, 2006.
12. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* 271: C1424-37, 1996.
13. Bers DM. Cardiac excitation-contraction coupling. *Nature* 415: 198-205, 2002.
14. Bhatia S, Shukla R, Venkata Madhu S, Kaur Gambhir J, Madhava Prabhu K. Antioxidant status, lipid peroxidation and nitric oxide end products in patients of type 2 diabetes mellitus with nephropathy. *Clin Biochem* 36: 557-62, 2003.
15. Bloch-Damti A, Bashan N. Proposed mechanisms for the induction of insulin resistance by oxidative stress. *Antioxid Redox Signal* 7: 1553-67, 2005.
16. Bogacka I, Xie H, Bray GA, Smith SR. Pioglitazone induces mitochondrial biogenesis in human subcutaneous adipose tissue in vivo. *Diabetes* 54: 1392-9, 2005.
17. Bonomini F, Rodella LF, Rezzani R. Metabolic syndrome, aging and involvement of oxidative stress. *Aging Dis* 6: 109-20, 2015.
18. Borghi C, Force ST, Rossi F, Force SIFT. Role of the Renin-Angiotensin-Aldosterone System and Its Pharmacological Inhibitors in Cardiovascular Diseases: Complex and Critical Issues. *High Blood Press Cardiovasc Prev* 22: 429-44, 2015.
19. Bostan C, Yildiz A, Ozkan AA, Uzunhasan I, Kaya A, Yigit Z. Beneficial effects of rosuvastatin treatment in patients with metabolic syndrome. *Angiology* 66: 122-7, 2015.
20. Boushel R, Gnaiger E, Schjerling P, Skovbro M, Kraunsoe R, Dela F. Patients with type 2 diabetes have normal mitochondrial function in skeletal muscle. *Diabetologia* 50: 790-6, 2007.
21. Brasier AR, Recinos A, 3rd, Eledrisi MS. Vascular inflammation and the renin-angiotensin system. *Arterioscler Thromb Vasc Biol* 22: 1257-66, 2002.
22. Breton-Romero R, Lamas S. Hydrogen peroxide signaling in vascular endothelial cells. *Redox Biol* 2: 529-34, 2014.

23. Brown LA, Kerr CJ, Whiting P, Finer N, McEneny J, Ashton T. Oxidant stress in healthy normal-weight, overweight, and obese individuals. *Obesity (Silver Spring)* 17: 460-6, 2009.
24. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature* 414: 813-20, 2001.
25. Bruce CR, Risis S, Babb JR, Yang C, Kowalski GM, Selathurai A, Lee-Young RS, Weir JM, Yoshioka K, Takuwa Y, Meikle PJ, Pitson SM, Febbraio MA. Overexpression of sphingosine kinase 1 prevents ceramide accumulation and ameliorates muscle insulin resistance in high-fat diet-fed mice. *Diabetes* 61: 3148-55, 2012.
26. Cai H. NAD(P)H oxidase-dependent self-propagation of hydrogen peroxide and vascular disease. *Circ Res* 96: 818-22, 2005.
27. Cali T, Ottolini D, Brini M. Mitochondrial Ca(2+) as a key regulator of mitochondrial activities. *Adv Exp Med Biol* 942: 53-73, 2012.
28. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmee E, Cousin B, Sulpice T, Chamontin B, Ferrieres J, Tanti JF, Gibson GR, Casteilla L, Delzenne NM, Alessi MC, Burcelin R. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 56: 1761-72, 2007.
29. Chait A, Brazg RL, Tribble DL, Krauss RM. Susceptibility of small, dense, low-density lipoproteins to oxidative modification in subjects with the atherogenic lipoprotein phenotype, pattern B. *Am J Med* 94: 350-6, 1993.
30. Chattopadhyay M, Khemka VK, Chatterjee G, Ganguly A, Mukhopadhyay S, Chakrabarti S. Enhanced ROS production and oxidative damage in subcutaneous white adipose tissue mitochondria in obese and type 2 diabetes subjects. *Mol Cell Biochem* 399: 95-103, 2015.
31. Cheng X, Siow RC, Mann GE. Impaired redox signaling and antioxidant gene expression in endothelial cells in diabetes: a role for mitochondria and the nuclear factor-E2-related factor 2-Kelch-like ECH-associated protein 1 defense pathway. *Antioxid Redox Signal* 14: 469-87, 2011.
32. Chiu J, Khan ZA, Farhangkhoee H, Chakrabarti S. Curcumin prevents diabetes-associated abnormalities in the kidneys by inhibiting p300 and nuclear factor-kappaB. *Nutrition* 25: 964-72, 2009.
33. Choi CS, Befroy DE, Codella R, Kim S, Reznick RM, Hwang YJ, Liu ZX, Lee HY, Distefano A, Samuel VT, Zhang D, Cline GW, Handschin C, Lin J, Petersen KF,

Spiegelman BM, Shulman GI. Paradoxical effects of increased expression of PGC-1alpha on muscle mitochondrial function and insulin-stimulated muscle glucose metabolism. *Proc Natl Acad Sci U S A* 105: 19926-31, 2008.

34. Christian P, Su Q. MicroRNA regulation of mitochondrial and ER stress signaling pathways: implications for lipoprotein metabolism in metabolic syndrome. *Am J Physiol Endocrinol Metab* 307: E729-37, 2014.
35. Chrysohoou C, Panagiotakos DB, Pitsavos C, Skoumas I, Papademetriou L, Economou M, Stefanadis C. The implication of obesity on total antioxidant capacity in apparently healthy men and women: the ATTICA study. *Nutr Metab Cardiovasc Dis* 17: 590-7, 2007.
36. Cocate PG, Natali AJ, de Oliveira A, Alfenas Rde C, Peluzio Mdo C, Longo GZ, dos Santos EC, Buthers JM, de Oliveira LL, Hermsdorff HH. Red but not white meat consumption is associated with metabolic syndrome, insulin resistance and lipid peroxidation in Brazilian middle-aged men. *Eur J Prev Cardiol* 22: 223-30, 2015.
37. Codoner-Franch P, Pons-Morales S, Boix-Garcia L, Valls-Belles V. Oxidant/antioxidant status in obese children compared to pediatric patients with type 1 diabetes mellitus. *Pediatr Diabetes* 11: 251-7, 2010.
38. Colak E, Majkic-Singh N, Stankovic S, Sreckovic-Dimitrijevic V, Djordjevic PB, Lalic K, Lalic N. Parameters of antioxidative defense in type 2 diabetic patients with cardiovascular complications. *Ann Med* 37: 613-20, 2005.
39. Costa MA, Elesgaray R, Caniffi C, Fellet A, Mac Laughlin M, Arranz C. Role of nitric oxide as a key mediator on cardiovascular actions of atrial natriuretic peptide in spontaneously hypertensive rats. *Am J Physiol Heart Circ Physiol* 298: H778-86, 2010.
40. Cracowski JL, Baguet JP, Ormezzano O, Bessard J, Stanke-Labesque F, Bessard G, Mallion JM. Lipid peroxidation is not increased in patients with untreated mild-to-moderate hypertension. *Hypertension* 41: 286-8, 2003.
41. Crescenzo R, Bianco F, Mazzoli A, Giacco A, Liverini G, Iossa S. A possible link between hepatic mitochondrial dysfunction and diet-induced insulin resistance. *Eur J Nutr*, 2015.
42. D'Archivio M, Annuzzi G, Vari R, Filesi C, Giacco R, Scazzocchio B, Santangelo C, Giovannini C, Rivellese AA, Masella R. Predominant role of obesity/insulin resistance in oxidative stress development. *Eur J Clin Invest* 42: 70-8, 2012.

43. Daiber A. Redox signaling (cross-talk) from and to mitochondria involves mitochondrial pores and reactive oxygen species. *Biochim Biophys Acta* 1797: 897-906, 2010.
44. de Graaf J, Hak-Lemmers HL, Hectors MP, Demacker PN, Hendriks JC, Stalenhoef AF. Enhanced susceptibility to in vitro oxidation of the dense low density lipoprotein subfraction in healthy subjects. *Arterioscler Thromb* 11: 298-306, 1991.
45. de MBS, da Fonseca LJ, da SGG, Rabelo LA, Goulart MO, Vasconcelos SM. Oxidative stress as an underlying contributor in the development of chronic complications in diabetes mellitus. *Int J Mol Sci* 14: 3265-84, 2013.
46. de Wilde J, Mohren R, van den Berg S, Boekschoten M, Dijk KW, de Groot P, Muller M, Mariman E, Smit E. Short-term high fat-feeding results in morphological and metabolic adaptations in the skeletal muscle of C57BL/6J mice. *Physiol Genomics* 32: 360-9, 2008.
47. Delbosc S, Paizanis E, Magous R, Araiz C, Dimo T, Cristol JP, Cros G, Azay J. Involvement of oxidative stress and NADPH oxidase activation in the development of cardiovascular complications in a model of insulin resistance, the fructose-fed rat. *Atherosclerosis* 179: 43-9, 2005.
48. Despres JP, Lemieux I. Abdominal obesity and metabolic syndrome. *Nature* 444: 881-7, 2006.
49. Diep QN, Amiri F, Touyz RM, Cohn JS, Endemann D, Neves MF, Schiffrin EL. PPARalpha activator effects on Ang II-induced vascular oxidative stress and inflammation. *Hypertension* 40: 866-71, 2002.
50. Dikalov SI, Dikalova AE, Bikineyeva AT, Schmidt HH, Harrison DG, Griendling KK. Distinct roles of Nox1 and Nox4 in basal and angiotensin II-stimulated superoxide and hydrogen peroxide production. *Free Radic Biol Med* 45: 1340-51, 2008.
51. Ding Z, Liu S, Wang X, Dai Y, Khaidakov M, Romeo F, Mehta JL. LOX-1, oxidant stress, mtDNA damage, autophagy, and immune response in atherosclerosis. *Can J Physiol Pharmacol* 92: 524-30, 2014.
52. Einhorn D, Reaven GM, Cobin RH, Ford E, Ganda OP, Handelsman Y, Hellman R, Jellinger PS, Kendall D, Krauss RM, Neufeld ND, Petak SM, Rodbard HW, Seibel JA, Smith DA, Wilson PW. American College of Endocrinology position statement on the insulin resistance syndrome. *Endocr Pract* 9: 237-52, 2003.

53. El Boghdady NA, Badr GA. Evaluation of oxidative stress markers and vascular risk factors in patients with diabetic peripheral neuropathy. *Cell Biochem Funct* 30: 328-34, 2012.
54. Evans JL, Goldfine ID, Maddux BA, Grodsky GM. Are oxidative stress-activated signaling pathways mediators of insulin resistance and beta-cell dysfunction? *Diabetes* 52: 1-8, 2003.
55. Feillet-Coudray C, Fouret G, Ebabe Elle R, Rieusset J, Bonafos B, Chabi B, Crouzier D, Zarkovic K, Zarkovic N, Ramos J, Badia E, Murphy MP, Cristol JP, Coudray C. The mitochondrial-targeted antioxidant MitoQ ameliorates metabolic syndrome features in obesogenic diet-fed rats better than Apocynin or Allopurinol. *Free Radic Res* 48: 1232-46, 2014.
56. Fernandez-Real JM, McClain D, Manco M. Mechanisms Linking Glucose Homeostasis and Iron Metabolism Toward the Onset and Progression of Type 2 Diabetes. *Diabetes Care* 38: 2169-76, 2015.
57. Flegal KM, Carroll MD, Ogden CL, Curtin LR. Prevalence and trends in obesity among US adults, 1999-2008. *JAMA* 303: 235-41, 2010.
58. Flohe L, Toppo S, Cozza G, Ursini F. A comparison of thiol peroxidase mechanisms. *Antioxid Redox Signal* 15: 763-80, 2011.
59. Fridovich I. Superoxide anion radical (O2-.), superoxide dismutases, and related matters. *J Biol Chem* 272: 18515-7, 1997.
60. Frohlich M, Imhof A, Berg G, Hutchinson WL, Pepys MB, Boeing H, Muche R, Brenner H, Koenig W. Association between C-reactive protein and features of the metabolic syndrome: a population-based study. *Diabetes Care* 23: 1835-9, 2000.
61. Fujii S, Zhang L, Igarashi J, Kosaka H. L-arginine reverses p47phox and gp91phox expression induced by high salt in Dahl rats. *Hypertension* 42: 1014-20, 2003.
62. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* 114: 1752-61, 2004.
63. Garcia-Roves P, Huss JM, Han DH, Hancock CR, Iglesias-Gutierrez E, Chen M, Holloszy JO. Raising plasma fatty acid concentration induces increased biogenesis of mitochondria in skeletal muscle. *Proc Natl Acad Sci U S A* 104: 10709-13, 2007.

64. Garg UC, Hassid A. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J Clin Invest* 83: 1774-7, 1989.

65. Gazi I, Tsimihodimos V, Filippatos T, Bairaktari E, Tselepis AD, Elisaf M. Concentration and relative distribution of low-density lipoprotein subfractions in patients with metabolic syndrome defined according to the National Cholesterol Education Program criteria. *Metabolism* 55: 885-91, 2006.

66. Ghiadoni L, Magagna A, Versari D, Kardasz I, Huang Y, Taddei S, Salvetti A. Different effect of antihypertensive drugs on conduit artery endothelial function. *Hypertension* 41: 1281-6, 2003.

67. Giacco F, Brownlee M. Oxidative stress and diabetic complications. *Circ Res* 107: 1058-70, 2010.

68. Giorgi C, Baldassari F, Bononi A, Bonora M, De Marchi E, Marchi S, Missiroli S, Patergnani S, Rimessi A, Suski JM, Wieckowski MR, Pinton P. Mitochondrial Ca(2+) and apoptosis. *Cell Calcium* 52: 36-43, 2012.

69. Go YM, Jones DP. Redox biology: interface of the exposome with the proteome, epigenome and genome. *Redox Biol* 2: 358-60, 2014.

70. Gregorio BM, De Souza DB, de Morais Nascimento FA, Pereira LM, Fernandes-Santos C. The potential role of antioxidants in metabolic syndrome. *Curr Pharm Des*, 2015.

71. Grundy SM, Brewer HB, Jr., Cleeman JI, Smith SC, Jr., Lenfant C, American Heart A, National Heart L, Blood I. Definition of metabolic syndrome: Report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. *Circulation* 109: 433-8, 2004.

72. Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, Gordon DJ, Krauss RM, Savage PJ, Smith SC, Jr., Spertus JA, Costa F, American Heart A, National Heart L, Blood I. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. *Circulation* 112: 2735-52, 2005.

73. Gusdon AM, Song KX, Qu S. Nonalcoholic Fatty liver disease: pathogenesis and therapeutics from a mitochondria-centric perspective. *Oxid Med Cell Longev* 2014: 637027, 2014.

74. Haffner S, Taegtmeyer H. Epidemic obesity and the metabolic syndrome. *Circulation* 108: 1541-5, 2003.

75. Hanukoglu I, Rapoport R. Routes and regulation of NADPH production in steroidogenic mitochondria. *Endocr Res* 21: 231-41, 1995.

76. Heilbronn LK, Gan SK, Turner N, Campbell LV, Chisholm DJ. Markers of mitochondrial biogenesis and metabolism are lower in overweight and obese insulin-resistant subjects. *J Clin Endocrinol Metab* 92: 1467-73, 2007.

77. Herrmann JM, Longen S, Weckbecker D, Depuydt M. Biogenesis of mitochondrial proteins. *Adv Exp Med Biol* 748: 41-64, 2012.

78. Higashi Y, Sasaki S, Nakagawa K, Matsuura H, Oshima T, Chayama K. Endothelial function and oxidative stress in renovascular hypertension. *N Engl J Med* 346: 1954-62, 2002.

79. Higuchi M, Dusting GJ, Peshavariya H, Jiang F, Hsiao ST, Chan EC, Liu GS. Differentiation of human adipose-derived stem cells into fat involves reactive oxygen species and Forkhead box O1 mediated upregulation of antioxidant enzymes. *Stem Cells Dev* 22: 878-88, 2013.

80. Hoehn KL, Turner N, Swarbrick MM, Wilks D, Preston E, Phua Y, Joshi H, Furler SM, Larance M, Hegarty BD, Leslie SJ, Pickford R, Hoy AJ, Kraegen EW, James DE, Cooney GJ. Acute or chronic upregulation of mitochondrial fatty acid oxidation has no net effect on whole-body energy expenditure or adiposity. *Cell Metab* 11: 70-6, 2010.

81. Holloway GP, Thrush AB, Heigenhauser GJ, Tandon NN, Dyck DJ, Bonen A, Spriet LL. Skeletal muscle mitochondrial FAT/CD36 content and palmitate oxidation are not decreased in obese women. *Am J Physiol Endocrinol Metab* 292: E1782-9, 2007.

82. Horvath TL, Andrews ZB, Diano S. Fuel utilization by hypothalamic neurons: roles for ROS. *Trends Endocrinol Metab* 20: 78-87, 2009.

83. Hwang H, Bowen BP, Lefort N, Flynn CR, De Filippis EA, Roberts C, Smoke CC, Meyer C, Hojlund K, Yi Z, Mandarino LJ. Proteomics analysis of human skeletal muscle reveals novel abnormalities in obesity and type 2 diabetes. *Diabetes* 59: 33-42, 2010.

84. Ilkun O, Wilde N, Tuinei J, Pires KM, Zhu Y, Bugger H, Soto J, Wayment B, Olsen C, Litwin SE, Abel ED. Antioxidant treatment normalizes mitochondrial energetics and

myocardial insulin sensitivity independently of changes in systemic metabolic homeostasis in a mouse model of the metabolic syndrome. *J Mol Cell Cardiol* 85: 104-16, 2015.

85. Ingelsson E, Sullivan LM, Murabito JM, Fox CS, Benjamin EJ, Polak JF, Meigs JB, Keyes MJ, O'Donnell CJ, Wang TJ, D'Agostino RB, Sr., Wolf PA, Vasan RS. Prevalence and prognostic impact of subclinical cardiovascular disease in individuals with the metabolic syndrome and diabetes. *Diabetes* 56: 1718-26, 2007.
86. Inoguchi T, Li P, Umeda F, Yu HY, Kakimoto M, Imamura M, Aoki T, Etoh T, Hashimoto T, Naruse M, Sano H, Utsumi H, Nawata H. High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C--dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes* 49: 1939-45, 2000.
87. Iossa S, Lionetti L, Mollica MP, Crescenzo R, Barletta A, Liverini G. Effect of long-term high-fat feeding on energy balance and liver oxidative activity in rats. *Br J Nutr* 84: 377-85, 2000.
88. Jacob C, Jamier V, Ba LA. Redox active secondary metabolites. *Curr Opin Chem Biol* 15: 149-55, 2011.
89. Jaiswal N, Maurya CK, Pandey J, Rai AK, Tamrakar AK. Fructose-induced ROS generation impairs glucose utilization in L6 skeletal muscle cells. *Free Radic Res* 49: 1055-68, 2015.
90. James P, Inui M, Tada M, Chiesi M, Carafoli E. Nature and site of phospholamban regulation of the Ca2+ pump of sarcoplasmic reticulum. *Nature* 342: 90-2, 1989.
91. Jialal I, Devaraj S, Adams-Huet B, Chen X, Kaur H. Increased cellular and circulating biomarkers of oxidative stress in nascent metabolic syndrome. *J Clin Endocrinol Metab* 97: E1844-50, 2012.
92. Jiang Y, Bao H, Ge Y, Tang W, Cheng D, Luo K, Gong G, Gong R. Therapeutic targeting of GSK3beta enhances the Nrf2 antioxidant response and confers hepatic cytoprotection in hepatitis C. *Gut* 64: 168-79, 2015.
93. Jimenez-Osorio AS, Gonzalez-Reyes S, Pedraza-Chaverri J. Natural Nrf2 activators in diabetes. *Clin Chim Acta* 448: 182-92, 2015.
94. Jones DP. Redefining oxidative stress. *Antioxid Redox Signal* 8: 1865-79, 2006.

95. Julius U, Dittrich M, Pietzsch J. Factors influencing the formation of small dense low-density lipoprotein particles in dependence on the presence of the metabolic syndrome and on the degree of glucose intolerance. *Int J Clin Pract* 61: 1798-804, 2007.

96. Kajimoto Y, Kaneto H. Role of oxidative stress in pancreatic beta-cell dysfunction. *Ann N Y Acad Sci* 1011: 168-76, 2004.

97. Kalinina EV, Chernov NN, Saprin AN. Involvement of thio-, peroxi-, and glutaredoxins in cellular redox-dependent processes. *Biochemistry (Mosc)* 73: 1493-510, 2008.

98. Kang KH, Lemke G, Kim JW. The PI3K-PTEN tug-of-war, oxidative stress and retinal degeneration. *Trends Mol Med* 15: 191-8, 2009.

99. Kashiwagi A, Shinozaki K, Nishio Y, Okamura T, Toda N, Kikkawa R. Free radical production in endothelial cells as a pathogenetic factor for vascular dysfunction in the insulin resistance state. *Diabetes Res Clin Pract* 45: 199-203, 1999.

100. Kelley DE, Goodpaster B, Wing RR, Simoneau JA. Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *Am J Physiol* 277: E1130-41, 1999.

101. Kelley DE, He J, Menshikova EV, Ritov VB. Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes* 51: 2944-50, 2002.

102. Kesavulu MM, Rao BK, Giri R, Vijaya J, Subramanyam G, Apparao C. Lipid peroxidation and antioxidant enzyme status in Type 2 diabetics with coronary heart disease. *Diabetes Res Clin Pract* 53: 33-9, 2001.

103. Kishi T, Hirooka Y, Kimura Y, Ito K, Shimokawa H, Takeshita A. Increased reactive oxygen species in rostral ventrolateral medulla contribute to neural mechanisms of hypertension in stroke-prone spontaneously hypertensive rats. *Circulation* 109: 2357-62, 2004.

104. Klaunig JE, Kamendulis LM, Hocevar BA. Oxidative stress and oxidative damage in carcinogenesis. *Toxicol Pathol* 38: 96-109, 2010.

105. Knopp RH, Paramsothy P. Oxidized LDL and abdominal obesity: a key to understanding the metabolic syndrome. *Am J Clin Nutr* 83: 1-2, 2006.

106. Koh KK, Park SM, Quon MJ. Leptin and cardiovascular disease: response to therapeutic interventions. *Circulation* 117: 3238-49, 2008.

107. Koppe SW. Obesity and the liver: nonalcoholic fatty liver disease. *Transl Res* 164: 312-22, 2014.

108. Kruiswijk F, Labuschagne CF, Vousden KH. p53 in survival, death and metabolic health: a lifeguard with a licence to kill. *Nat Rev Mol Cell Biol* 16: 393-405, 2015.

109. Kuwahara H, Horie T, Ishikawa S, Tsuda C, Kawakami S, Noda Y, Kaneko T, Tahara S, Tachibana T, Okabe M, Melki J, Takano R, Toda T, Morikawa D, Nojiri H, Kurosawa H, Shirasawa T, Shimizu T. Oxidative stress in skeletal muscle causes severe disturbance of exercise activity without muscle atrophy. *Free Radic Biol Med* 48: 1252-62, 2010.

110. Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE, Harrison DG. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest* 111: 1201-9, 2003.

111. Lassegue B, Clempus RE. Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am J Physiol Regul Integr Comp Physiol* 285: R277-97, 2003.

112. Lassegue B, Sorescu D, Szocs K, Yin Q, Akers M, Zhang Y, Grant SL, Lambeth JD, Griendling KK. Novel gp91(phox) homologues in vascular smooth muscle cells : nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. *Circ Res* 88: 888-94, 2001.

113. Lee H, Lee YJ, Choi H, Ko EH, Kim JW. Reactive oxygen species facilitate adipocyte differentiation by accelerating mitotic clonal expansion. *J Biol Chem* 284: 10601-9, 2009.

114. Leone TC, Lehman JJ, Finck BN, Schaeffer PJ, Wende AR, Boudina S, Courtois M, Wozniak DF, Sambandam N, Bernal-Mizrachi C, Chen Z, Holloszy JO, Medeiros DM, Schmidt RE, Saffitz JE, Abel ED, Semenkovich CF, Kelly DP. PGC-1alpha deficiency causes multi-system energy metabolic derangements: muscle dysfunction, abnormal weight control and hepatic steatosis. *PLoS Biol* 3: e101, 2005.

115. Lin P, Liu J, Ren M, Ji K, Li L, Zhang B, Gong Y, Yan C. Idebenone protects against oxidized low density lipoprotein induced mitochondrial dysfunction in vascular endothelial cells via GSK3beta/beta-catenin signalling pathways. *Biochem Biophys Res Commun* 465: 548-55, 2015.

116. Lip GY, Edmunds E, Nuttall SL, Landray MJ, Blann AD, Beevers DG. Oxidative stress in malignant and non-malignant phase hypertension. *J Hum Hypertens* 16: 333-6, 2002.

117. Liu B, Chen Y, St Clair DK. ROS and p53: a versatile partnership. *Free Radic Biol Med* 44: 1529-35, 2008.

118. Liu S, Geng B, Zou L, Wei S, Wang W, Deng J, Xu C, Zhao X, Lyu Y, Su X, Xu G. Development of hypertrophic cardiomyopathy in perilipin-1 null mice with adipose tissue dysfunction. *Cardiovasc Res* 105: 20-30, 2015.

119. Lu J, Mitra S, Wang X, Khaidakov M, Mehta JL. Oxidative stress and lectin-like ox-LDL-receptor LOX-1 in atherogenesis and tumorigenesis. *Antioxid Redox Signal* 15: 2301-33, 2011.

120. Lum H, Roebuck KA. Oxidant stress and endothelial cell dysfunction. *Am J Physiol Cell Physiol* 280: C719-41, 2001.

121. Mao G, Kraus GA, Kim I, Spurlock ME, Bailey TB, Zhang Q, Beitz DC. A mitochondria-targeted vitamin E derivative decreases hepatic oxidative stress and inhibits fat deposition in mice. *J Nutr* 140: 1425-31, 2010.

122. Martin-Gallan P, Carrascosa A, Gussinye M, Dominguez C. Biomarkers of diabetes-associated oxidative stress and antioxidant status in young diabetic patients with or without subclinical complications. *Free Radic Biol Med* 34: 1563-74, 2003.

123. Maruthur NM, Ma Y, Delahanty LM, Nelson JA, Aroda V, White NH, Marrero D, Brancati FL, Clark JM, Diabetes Prevention Program Research G. Early response to preventive strategies in the Diabetes Prevention Program. *J Gen Intern Med* 28: 1629-36, 2013.

124. Marzetti E, Lees HA, Wohlgemuth SE, Leeuwenburgh C. Sarcopenia of aging: underlying cellular mechanisms and protection by calorie restriction. *Biofactors* 35: 28-35, 2009.

125. McNeill AM, Katz R, Girman CJ, Rosamond WD, Wagenknecht LE, Barzilay JI, Tracy RP, Savage PJ, Jackson SA. Metabolic syndrome and cardiovascular disease in older people: The cardiovascular health study. *J Am Geriatr Soc* 54: 1317-24, 2006.

126. Mercer JR, Cheng KK, Figg N, Gorenne I, Mahmoudi M, Griffin J, Vidal-Puig A, Logan A, Murphy MP, Bennett M. DNA damage links mitochondrial dysfunction to atherosclerosis and the metabolic syndrome. *Circ Res* 107: 1021-31, 2010.

127. Mercer JR, Yu E, Figg N, Cheng KK, Prime TA, Griffin JL, Masoodi M, Vidal-Puig A, Murphy MP, Bennett MR. The mitochondria-targeted antioxidant MitoQ decreases features of the metabolic syndrome in ATM+/-/ApoE-/- mice. *Free Radic Biol Med* 52: 841-9, 2012.

128. Mikhailidis DP, Elisaf M, Rizzo M, Berneis K, Griffin B, Zambon A, Athyros V, de Graaf J, Marz W, Parhofer KG, Rini GB, Spinas GA, Tomkin GH, Tselepis AD, Wierzbicki AS, Winkler K, Florentin M, Liberopoulos E. "European panel on low density lipoprotein (LDL) subclasses": a statement on the pathophysiology, atherogenicity and clinical significance of LDL subclasses: executive summary. *Curr Vasc Pharmacol* 9: 531-2, 2011.

129. Mlinar B, Marc J. New insights into adipose tissue dysfunction in insulin resistance. *Clin Chem Lab Med* 49: 1925-35, 2011.

130. Montezano AC, Dulak-Lis M, Tsiropoulou S, Harvey A, Briones AM, Touyz RM. Oxidative stress and human hypertension: vascular mechanisms, biomarkers, and novel therapies. *Can J Cardiol* 31: 631-41, 2015.

131. Montgomery MK, Turner N. Mitochondrial dysfunction and insulin resistance: an update. *Endocr Connect* 4: R1-R15, 2015.

132. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 34: 267-73, 2003.

133. Moskaug JO, Carlsen H, Myhrstad MC, Blomhoff R. Polyphenols and glutathione synthesis regulation. *Am J Clin Nutr* 81: 277S-283S, 2005.

134. Moyes CD, Hood DA. Origins and consequences of mitochondrial variation in vertebrate muscle. *Annu Rev Physiol* 65: 177-201, 2003.

135. Mudd JO, Borlaug BA, Johnston PV, Kral BG, Rouf R, Blumenthal RS, Kwiterovich PO, Jr. Beyond low-density lipoprotein cholesterol: defining the role of low-density lipoprotein heterogeneity in coronary artery disease. *J Am Coll Cardiol* 50: 1735-41, 2007.

136. Mueller CF, Laude K, McNally JS, Harrison DG. ATVB in focus: redox mechanisms in blood vessels. *Arterioscler Thromb Vasc Biol* 25: 274-8, 2005.

137. Munzel T, Gori T, Bruno RM, Taddei S. Is oxidative stress a therapeutic target in cardiovascular disease? *Eur Heart J* 31: 2741-8, 2010.

138. Munzel T, Sinning C, Post F, Warnholtz A, Schulz E. Pathophysiology, diagnosis and prognostic implications of endothelial dysfunction. *Ann Med* 40: 180-96, 2008.

139. Narayan KM, Imperatore G, Benjamin SM, Engelgau MM. Targeting people with pre-diabetes. *BMJ* 325: 403-4, 2002.

140. Nascimento H, Alves AI, Coimbra S, Catarino C, Gomes D, Bronze-da-Rocha E, Costa E, Rocha-Pereira P, Aires L, Mota J, Ferreira Mansilha H, Rego C, Santos-Silva A, Belo L. Bilirubin is independently associated with oxidized LDL levels in young obese patients. *Diabetol Metab Syndr* 7: 4, 2015.

141. Nassir F, Ibdah JA. Role of mitochondria in nonalcoholic fatty liver disease. *Int J Mol Sci* 15: 8713-42, 2014.

142. Neves AL, Coelho J, Couto L, Leite-Moreira A, Roncon-Albuquerque R, Jr. Metabolic endotoxemia: a molecular link between obesity and cardiovascular risk. *J Mol Endocrinol* 51: R51-64, 2013.

143. Newsholme P, Cruzat V, Arfuso F, Keane K. Nutrient regulation of insulin secretion and action. *J Endocrinol* 221: R105-20, 2014.

144. Ng CF, Koon CM, Cheung DW, Lam MY, Leung PC, Lau CB, Fung KP. The anti-hypertensive effect of Danshen (Salvia miltiorrhiza) and Gegen (Pueraria lobata) formula in rats and its underlying mechanisms of vasorelaxation. *J Ethnopharmacol* 137: 1366-72, 2011.

145. Niemann B, Chen Y, Teschner M, Li L, Silber RE, Rohrbach S. Obesity induces signs of premature cardiac aging in younger patients: the role of mitochondria. *J Am Coll Cardiol* 57: 577-85, 2011.

146. Nikolaidis MG, Kerksick CM, Lamprecht M, McAnulty SR. Redox biology of exercise. *Oxid Med Cell Longev* 2012: 407978, 2012.

147. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 404: 787-90, 2000.

148. Nunez-Cortes JM, Morato TM, Toro R, Millan-Perez JJ, Rojas AM, Members Of The Scientific Committee Of The Hypertriglyceridemia Registry Of The Spanish Society Of Arteriosclerosis S. CARDIOMETABOLIC RISK RELATED to the ASSOCIATION of HYPERTRIGLYCERIDEMIA-LOW HDLc. *Curr Pharm Des*, 2015.

149. O'Keefe JH, Bell DS. Postprandial hyperglycemia/hyperlipidemia (postprandial dysmetabolism) is a cardiovascular risk factor. *Am J Cardiol* 100: 899-904, 2007.

150. O'Keefe JH, Gheewala NM, O'Keefe JO. Dietary strategies for improving post-prandial glucose, lipids, inflammation, and cardiovascular health. *J Am Coll Cardiol* 51: 249-55, 2008.

151. Olson DP, Pulinilkunnil T, Cline GW, Shulman GI, Lowell BB. Gene knockout of Acc2 has little effect on body weight, fat mass, or food intake. *Proc Natl Acad Sci U S A* 107: 7598-603, 2010.

152. Onat A. Metabolic syndrome: nature, therapeutic solutions and options. *Expert Opin Pharmacother* 12: 1887-1900, 2011.

153. Osteryoung KW, Nunnari J. The division of endosymbiotic organelles. *Science* 302: 1698-704, 2003.

154. Otani H. Oxidative stress as pathogenesis of cardiovascular risk associated with metabolic syndrome. *Antioxid Redox Signal* 15: 1911-26, 2011.

155. Ozgen IT, Tascilar ME, Bilir P, Boyraz M, Guncikan MN, Akay C, Dundaroz R. Oxidative stress in obese children and its relation with insulin resistance. *J Pediatr Endocrinol Metab* 25: 261-6, 2012.

156. Padwal R, Lin M, Eurich DT. The Comparative Effectiveness of Angiotensin-Converting Enzyme Inhibitors and Angiotensin II Receptor Blockers in Patients With Diabetes. *J Clin Hypertens (Greenwich)*, 2015.

157. Papp LV, Holmgren A, Khanna KK. Selenium and selenoproteins in health and disease. *Antioxid Redox Signal* 12: 793-5, 2010.

158. Park JB, Touyz RM, Chen X, Schiffrin EL. Chronic treatment with a superoxide dismutase mimetic prevents vascular remodeling and progression of hypertension in salt-loaded stroke-prone spontaneously hypertensive rats. *Am J Hypertens* 15: 78-84, 2002.

159. Patel TP, Rawal K, Bagchi AK, Akolkar G, Bernardes N, Dias DD, Gupta S, Singal PK. Insulin resistance: an additional risk factor in the pathogenesis of cardiovascular disease in type 2 diabetes. *Heart Fail Rev*, 2015.

160. Paulis L, Foulquier S, Namsolleck P, Recarti C, Steckelings UM, Unger T. Combined Angiotensin Receptor Modulation in the Management of Cardio-Metabolic Disorders. *Drugs*, 2015.

161. Phielix E, Schrauwen-Hinderling VB, Mensink M, Lenaers E, Meex R, Hoeks J, Kooi ME, Moonen-Kornips E, Sels JP, Hesselink MK, Schrauwen P. Lower intrinsic ADP-stimulated mitochondrial respiration underlies in vivo mitochondrial dysfunction in muscle of male type 2 diabetic patients. *Diabetes* 57: 2943-9, 2008.

162. Pickup JC. Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. *Diabetes Care* 27: 813-23, 2004.

163. Porto ML, Rodrigues BP, Menezes TN, Ceschim SL, Casarini DE, Gava AL, Pereira TM, Vasquez EC, Campagnaro BP, Meyrelles SS. Reactive oxygen species contribute to dysfunction of bone marrow hematopoietic stem cells in aged C57BL/6 J mice. *J Biomed Sci* 22: 97, 2015.

164. Puddu P, Puddu GM, Galletti L, Cravero E, Muscari A. Mitochondrial dysfunction as an initiating event in atherogenesis: a plausible hypothesis. *Cardiology* 103: 137-41, 2005.

165. Pung YF, Rocic P, Murphy MP, Smith RA, Hafemeister J, Ohanyan V, Guarini G, Yin L, Chilian WM. Resolution of mitochondrial oxidative stress rescues coronary collateral growth in Zucker obese fatty rats. *Arterioscler Thromb Vasc Biol* 32: 325-34, 2012.

166. Ray PD, Huang BW, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signal* 24: 981-90, 2012.

167. Redon J, Oliva MR, Tormos C, Giner V, Chaves J, Iradi A, Saez GT. Antioxidant activities and oxidative stress byproducts in human hypertension. *Hypertension* 41: 1096-101, 2003.

168. Ritov VB, Menshikova EV, He J, Ferrell RE, Goodpaster BH, Kelley DE. Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes. *Diabetes* 54: 8-14, 2005.

169. Rizzuto R, De Stefani D, Raffaello A, Mammucari C. Mitochondria as sensors and regulators of calcium signalling. *Nat Rev Mol Cell Biol* 13: 566-78, 2012.

170. Rodriguez A, Delgado-Cohen H, Reviriego J, Serrano-Rios M. Risk factors associated with metabolic syndrome in type 2 diabetes mellitus patients according to World Health Organization, Third Report National Cholesterol Education Program, and International Diabetes Federation definitions. *Diabetes Metab Syndr Obes* 4: 1-4, 2011.

171. Rodriguez-Iturbe B, Zhan CD, Quiroz Y, Sindhu RK, Vaziri ND. Antioxidant-rich diet relieves hypertension and reduces renal immune infiltration in spontaneously hypertensive rats. *Hypertension* 41: 341-6, 2003.

172. Roy Chowdhury SK, Sangle GV, Xie X, Stelmack GL, Halayko AJ, Shen GX. Effects of extensively oxidized low-density lipoprotein on mitochondrial function and reactive oxygen species in porcine aortic endothelial cells. *Am J Physiol Endocrinol Metab* 298: E89-98, 2010.

173. Ruperez AI, Olza J, Gil-Campos M, Leis R, Mesa MD, Tojo R, Canete R, Gil A, Aguilera CM. Are catalase -844A/G polymorphism and activity associated with childhood obesity? *Antioxid Redox Signal* 19: 1970-5, 2013.

174. Sablina AA, Budanov AV, Ilyinskaya GV, Agapova LS, Kravchenko JE, Chumakov PM. The antioxidant function of the p53 tumor suppressor. *Nat Med* 11: 1306-13, 2005.

175. Samocha-Bonet D, Campbell LV, Mori TA, Croft KD, Greenfield JR, Turner N, Heilbronn LK. Overfeeding reduces insulin sensitivity and increases oxidative stress, without altering markers of mitochondrial content and function in humans. *PLoS One* 7: e36320, 2012.

176. Samuel VT, Petersen KF, Shulman GI. Lipid-induced insulin resistance: unravelling the mechanism. *Lancet* 375: 2267-77, 2010.

177. Savini I, Catani MV, Duranti G, Ceci R, Sabatini S, Avigliano L. Vitamin C homeostasis in skeletal muscle cells. *Free Radic Biol Med* 38: 898-907, 2005.

178. Schatten H, Prather RS, Sun QY. The significance of mitochondria for embryo development in cloned farm animals. *Mitochondrion* 5: 303-21, 2005.

179. Schmitz-Peiffer C, Craig DL, Biden TJ. Ceramide generation is sufficient to account for the inhibition of the insulin-stimulated PKB pathway in C2C12 skeletal muscle cells pretreated with palmitate. *J Biol Chem* 274: 24202-10, 1999.

180. Schonfeld P, Wojtczak L. Fatty acids as modulators of the cellular production of reactive oxygen species. *Free Radic Biol Med* 45: 231-41, 2008.

181. Sczelecki S, Besse-Patin A, Abboud A, Kleiner S, Laznik-Bogoslavski D, Wrann CD, Ruas JL, Haibe-Kains B, Estall JL. Loss of Pgc-1alpha expression in aging mouse muscle potentiates glucose intolerance and systemic inflammation. *Am J Physiol Endocrinol Metab* 306: E157-67, 2014.

182. Semple RK, Crowley VC, Sewter CP, Laudes M, Christodoulides C, Considine RV, Vidal-Puig A, O'Rahilly S. Expression of the thermogenic nuclear hormone receptor coactivator PGC-1alpha is reduced in the adipose tissue of morbidly obese subjects. *Int J Obes Relat Metab Disord* 28: 176-9, 2004.

183. Shen GX. Mitochondrial dysfunction, oxidative stress and diabetic cardiovascular disorders. *Cardiovasc Hematol Disord Drug Targets* 12: 106-12, 2012.

184. Shokoji T, Nishiyama A, Fujisawa Y, Hitomi H, Kiyomoto H, Takahashi N, Kimura S, Kohno M, Abe Y. Renal sympathetic nerve responses to tempol in spontaneously hypertensive rats. *Hypertension* 41: 266-73, 2003.

185. Singh V, Chassaing B, Zhang L, San Yeoh B, Xiao X, Kumar M, Baker MT, Cai J, Walker R, Borkowski K, Harvatine KJ, Singh N, Shearer GC, Ntambi JM, Joe B, Patterson AD, Gewirtz AT, Vijay-Kumar M. Microbiota-Dependent Hepatic Lipogenesis Mediated by Stearoyl CoA Desaturase 1 (SCD1) Promotes Metabolic Syndrome in TLR5-Deficient Mice. *Cell Metab* 22: 983-96, 2015.

186. Smith RA, Hartley RC, Cocheme HM, Murphy MP. Mitochondrial pharmacology. *Trends Pharmacol Sci* 33: 341-52, 2012.

187. Smith RA, Hartley RC, Murphy MP. Mitochondria-targeted small molecule therapeutics and probes. *Antioxid Redox Signal* 15: 3021-38, 2011.

188. Soares AF, Guichardant M, Cozzone D, Bernoud-Hubac N, Bouzaidi-Tiali N, Lagarde M, Geloen A. Effects of oxidative stress on adiponectin secretion and lactate production in 3T3-L1 adipocytes. *Free Radic Biol Med* 38: 882-9, 2005.

189. Song D, Hutchings S, Pang CC. Chronic N-acetylcysteine prevents fructose-induced insulin resistance and hypertension in rats. *Eur J Pharmacol* 508: 205-10, 2005.

190. Sorescu GP, Song H, Tressel SL, Hwang J, Dikalov S, Smith DA, Boyd NL, Platt MO, Lassegue B, Griendling KK, Jo H. Bone morphogenic protein 4 produced in endothelial cells by oscillatory shear stress induces monocyte adhesion by stimulating reactive oxygen species production from a nox1-based NADPH oxidase. *Circ Res* 95: 773-9, 2004.

191. Sreekumar R, Unnikrishnan J, Fu A, Nygren J, Short KR, Schimke J, Barazzoni R, Nair KS. Impact of high-fat diet and antioxidant supplement on mitochondrial functions and gene transcripts in rat muscle. *Am J Physiol Endocrinol Metab* 282: E1055-61, 2002.

192. Stefan N, Kantartzis K, Haring HU. Causes and metabolic consequences of Fatty liver. *Endocr Rev* 29: 939-60, 2008.

193. Sugizaki T, Watanabe M, Horai Y, Kaneko-Iwasaki N, Arita E, Miyazaki T, Morimoto K, Honda A, Irie J, Itoh H. The Niemann-Pick C1 like 1 (NPC1L1) inhibitor ezetimibe

improves metabolic disease via decreased liver X receptor (LXR) activity in liver of obese male mice. *Endocrinology* 155: 2810-9, 2014.

194. Surh YJ, Kundu JK, Na HK. Nrf2 as a master redox switch in turning on the cellular signaling involved in the induction of cytoprotective genes by some chemopreventive phytochemicals. *Planta Med* 74: 1526-39, 2008.

195. Tan M, Li S, Swaroop M, Guan K, Oberley LW, Sun Y. Transcriptional activation of the human glutathione peroxidase promoter by p53. *J Biol Chem* 274: 12061-6, 1999.

196. Tanito M, Nakamura H, Kwon YW, Teratani A, Masutani H, Shioji K, Kishimoto C, Ohira A, Horie R, Yodoi J. Enhanced oxidative stress and impaired thioredoxin expression in spontaneously hypertensive rats. *Antioxid Redox Signal* 6: 89-97, 2004.

197. Taniyama Y, Griendling KK. Reactive oxygen species in the vasculature: molecular and cellular mechanisms. *Hypertension* 42: 1075-81, 2003.

198. Touyz RM. Reactive oxygen species in vascular biology: role in arterial hypertension. *Expert Rev Cardiovasc Ther* 1: 91-106, 2003.

199. Touyz RM, Yao G, Schiffrin EL. c-Src induces phosphorylation and translocation of p47phox: role in superoxide generation by angiotensin II in human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 23: 981-7, 2003.

200. Trevisan M, Browne R, Ram M, Muti P, Freudenheim J, Carosella AM, Armstrong D. Correlates of markers of oxidative status in the general population. *Am J Epidemiol* 154: 348-56, 2001.

201. Turner N, Bruce CR, Beale SM, Hoehn KL, So T, Rolph MS, Cooney GJ. Excess lipid availability increases mitochondrial fatty acid oxidative capacity in muscle: evidence against a role for reduced fatty acid oxidation in lipid-induced insulin resistance in rodents. *Diabetes* 56: 2085-92, 2007.

202. Turner N, Hariharan K, TidAng J, Frangioudakis G, Beale SM, Wright LE, Zeng XY, Leslie SJ, Li JY, Kraegen EW, Cooney GJ, Ye JM. Enhancement of muscle mitochondrial oxidative capacity and alterations in insulin action are lipid species dependent: potent tissue-specific effects of medium-chain fatty acids. *Diabetes* 58: 2547-54, 2009.

203. Ueno H, Saitoh Y, Mizuta M, Shiiya T, Noma K, Mashiba S, Kojima S, Nakazato M. Fenofibrate ameliorates insulin resistance, hypertension and novel oxidative stress markers in patients with metabolic syndrome. *Obes Res Clin Pract* 5: e267-360, 2011.

204. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 39: 44-84, 2007.

205. Van Guilder GP, Hoetzer GL, Greiner JJ, Stauffer BL, Desouza CA. Influence of metabolic syndrome on biomarkers of oxidative stress and inflammation in obese adults. *Obesity (Silver Spring)* 14: 2127-31, 2006.

206. Vernochet C, Mourier A, Bezy O, Macotela Y, Boucher J, Rardin MJ, An D, Lee KY, Ilkayeva OR, Zingaretti CM, Emanuelli B, Smyth G, Cinti S, Newgard CB, Gibson BW, Larsson NG, Kahn CR. Adipose-specific deletion of TFAM increases mitochondrial oxidation and protects mice against obesity and insulin resistance. *Cell Metab* 16: 765-76, 2012.

207. Viroonudomphol D, Pongpaew P, Tungtrongchitr R, Phonrat B, Supawan V, Vudhivai N, Schelp FP. Erythrocyte antioxidant enzymes and blood pressure in relation to overweight and obese Thai in Bangkok. *Southeast Asian J Trop Med Public Health* 31: 325-34, 2000.

208. Wang C, Youle RJ. The role of mitochondria in apoptosis*. *Annu Rev Genet* 43: 95-118, 2009.

209. Ward NC, Hodgson JM, Puddey IB, Mori TA, Beilin LJ, Croft KD. Oxidative stress in human hypertension: association with antihypertensive treatment, gender, nutrition, and lifestyle. *Free Radic Biol Med* 36: 226-32, 2004.

210. Weinbrenner T, Schroder H, Escurriol V, Fito M, Elosua R, Vila J, Marrugat J, Covas MI. Circulating oxidized LDL is associated with increased waist circumference independent of body mass index in men and women. *Am J Clin Nutr* 83: 30-5; quiz 181-2, 2006.

211. Welch WJ, Mendonca M, Blau J, Karber A, Dennehy K, Patel K, Lao YS, Jose PA, Wilcox CS. Antihypertensive response to prolonged tempol in the spontaneously hypertensive rat. *Kidney Int* 68: 179-87, 2005.

212. West N, Guzik T, Black E, Channon K. Enhanced superoxide production in experimental venous bypass graft intimal hyperplasia: role of NAD(P)H oxidase. *Arterioscler Thromb Vasc Biol* 21: 189-94, 2001.

213. Wredenberg A, Freyer C, Sandstrom ME, Katz A, Wibom R, Westerblad H, Larsson NG. Respiratory chain dysfunction in skeletal muscle does not cause insulin resistance. *Biochem Biophys Res Commun* 350: 202-7, 2006.

214. Wredenberg A, Wibom R, Wilhelmsson H, Graff C, Wiener HH, Burden SJ, Oldfors A, Westerblad H, Larsson NG. Increased mitochondrial mass in mitochondrial myopathy mice. *Proc Natl Acad Sci U S A* 99: 15066-71, 2002.

215. Wu TW, Fung KP, Wu J, Yang CC, Weisel RD. Antioxidation of human low density lipoprotein by unconjugated and conjugated bilirubins. *Biochem Pharmacol* 51: 859-62, 1996.

216. Xu J, Tian W, Ma X, Guo J, Shi Q, Jin Y, Xi J, Xu Z. The molecular mechanism underlying morphine-induced Akt activation: roles of protein phosphatases and reactive oxygen species. *Cell Biochem Biophys* 61: 303-11, 2011.

217. Yasunari K, Maeda K, Nakamura M, Yoshikawa J. Oxidative stress in leukocytes is a possible link between blood pressure, blood glucose, and C-reacting protein. *Hypertension* 39: 777-80, 2002.

218. Yida Z, Imam MU, Ismail M, Ismail N, Ideris A, Abdullah MA. High fat diet-induced inflammation and oxidative stress are attenuated by N-acetylneuraminic acid in rats. *J Biomed Sci* 22: 96, 2015.

219. Yokozawa T, Ishida A, Kashiwada Y, Cho EJ, Kim HY, Ikeshiro Y. Coptidis Rhizoma: protective effects against peroxynitrite-induced oxidative damage and elucidation of its active components. *J Pharm Pharmacol* 56: 547-56, 2004.

220. Yoshida J, Yamamoto K, Mano T, Sakata Y, Nishikawa N, Nishio M, Ohtani T, Miwa T, Hori M, Masuyama T. AT1 receptor blocker added to ACE inhibitor provides benefits at advanced stage of hypertensive diastolic heart failure. *Hypertension* 43: 686-91, 2004.

221. Youn JY, Siu KL, Lob HE, Itani H, Harrison DG, Cai H. Role of vascular oxidative stress in obesity and metabolic syndrome. *Diabetes* 63: 2344-55, 2014.

222. Zalba G, San Jose G, Moreno MU, Fortuno MA, Fortuno A, Beaumont FJ, Diez J. Oxidative stress in arterial hypertension: role of NAD(P)H oxidase. *Hypertension* 38: 1395-9, 2001.

223. Zhang H, Zhang T, Li S, Li Y, Hussain A, Fernandez C, Harville E, Bazzano LA, He J, Chen W. Long-term Impact of Childhood Adiposity on Adult Metabolic Syndrome Is Modified by Insulin Resistance: The Bogalusa Heart Study. *Sci Rep* 5: 17885, 2015.

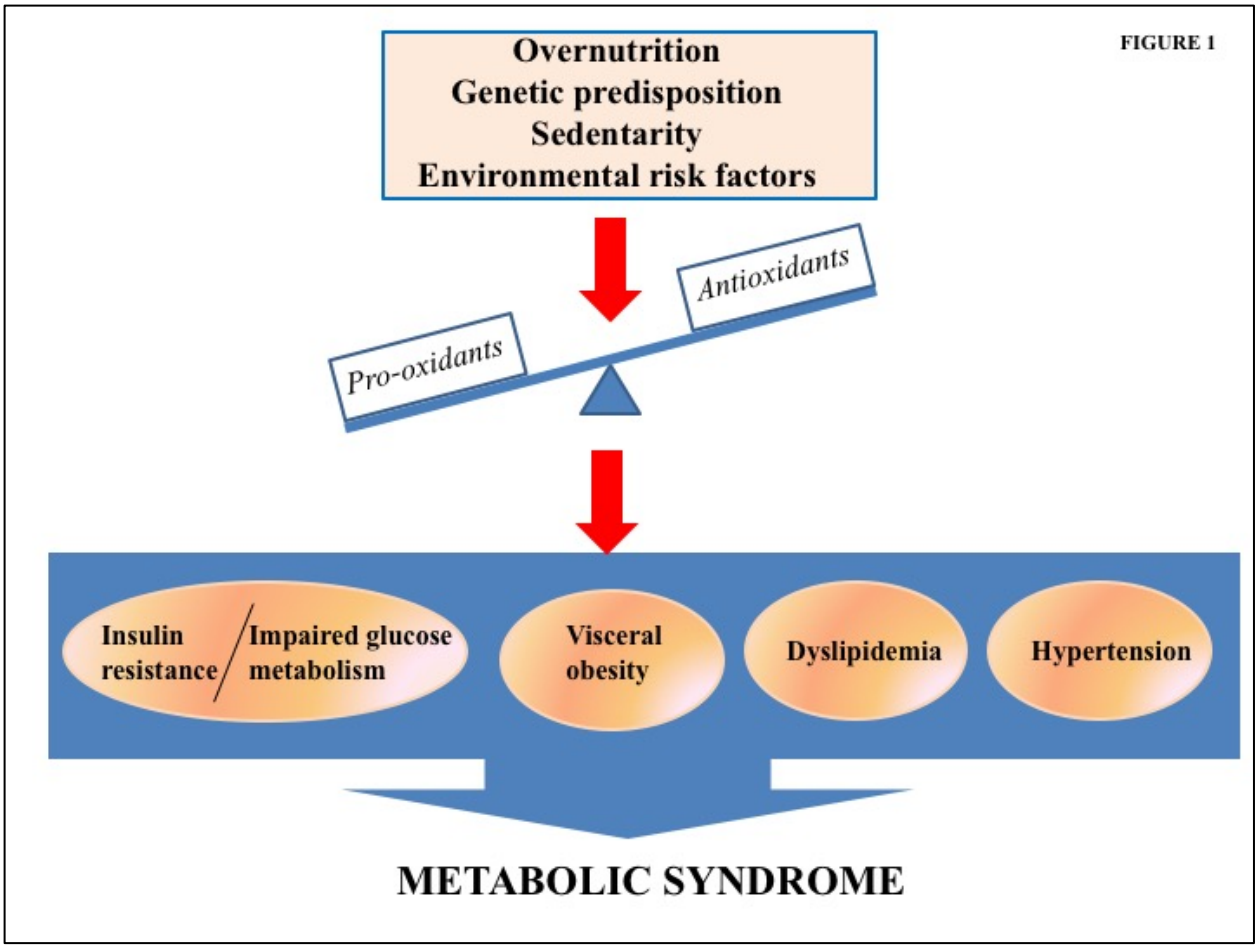

Figure 1. Diagrammatic summary of metabolic syndrome causes.

Insulin resistant states, body (and especially abdominal) fat excess, dyslipidemia and hypertension are identifiable conditions of Metabolic Syndrome (MetS). They may be due to an imbalance of antioxidants and pro-oxidants, triggered by the convergence of inadequate over-nutrition, genetic predisposition, sedentarity and environmental risk factors.

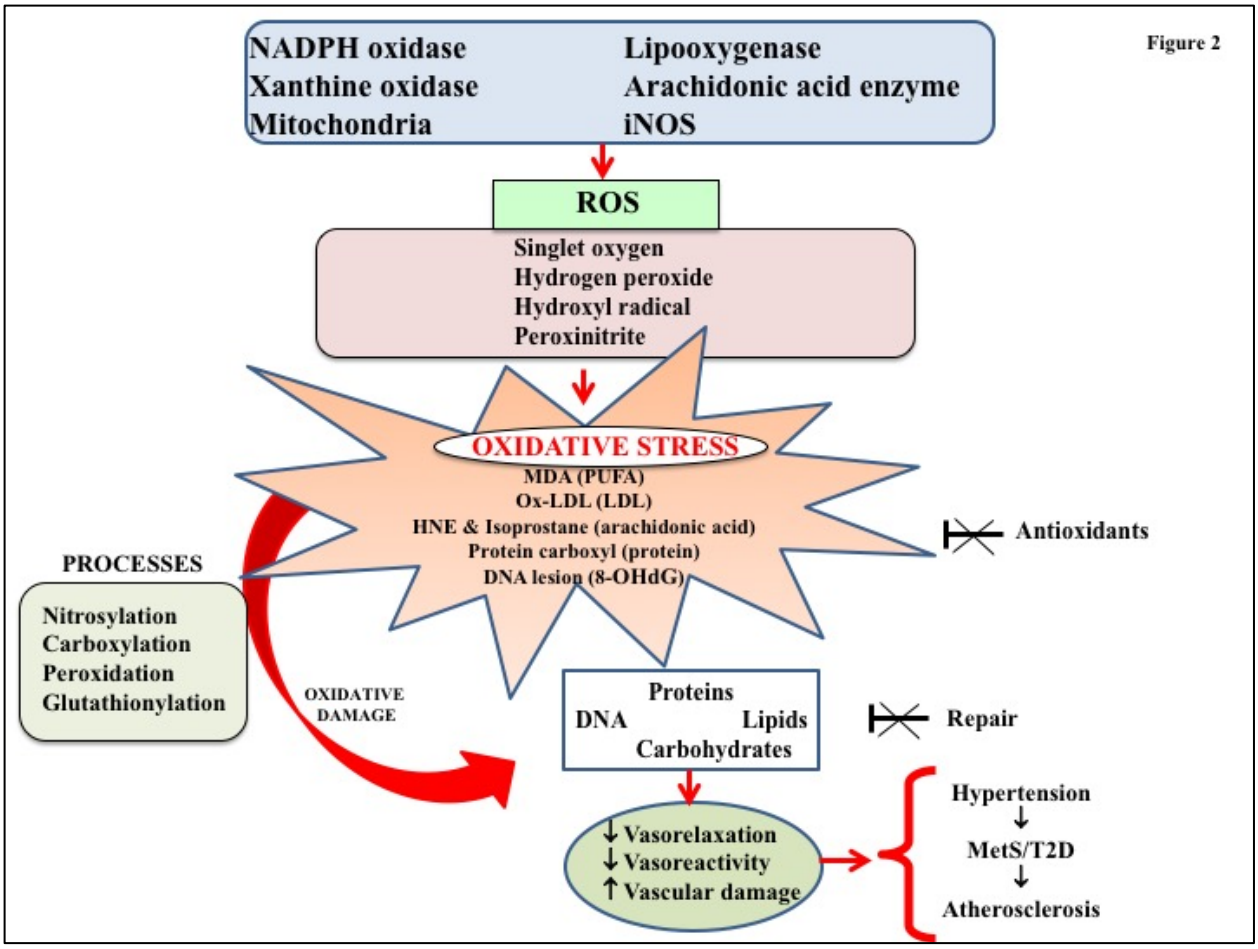

Figure 2. Cellular oxidative damage to biological molecules.

Various sources, including NADP oxidase, xanthine oxidase, lipoxygenase, iNOS, arachidonic enzyme and mitochondria, may contribute to the enhancement of ROS generation. Consequently, antioxidant defense is unable to detoxify free radicals, which alters the normal cell redox state and favors extensive OxS. Exposure of lipids, proteins and DNA to ROS and nitrogen species (RNS) results in chlorination, nitrosylation, carboxylation, peroxidation and glutathionylation with toxic effects on lipids (e.g., malondialdehyde, MDA), proteins (e.g., HNE-protein adducts) and DNA (e.g., 8-hydroxy-2-deoxyguanosine, 8-OHdG). Reduced nitric oxide bioavailability, caused by augmentation ROS production, leads to vascular alterations and endothelial dysfunction, thereby culminating in hypertension that promotes the risk of developing metabolic syndrome (MetS), type 2 diabetes (T2D) and atherosclerosis.

ROS: reactive oxygen species; PUFA: polyunsaturated fatty acids; ox-LDL: oxidized LDL

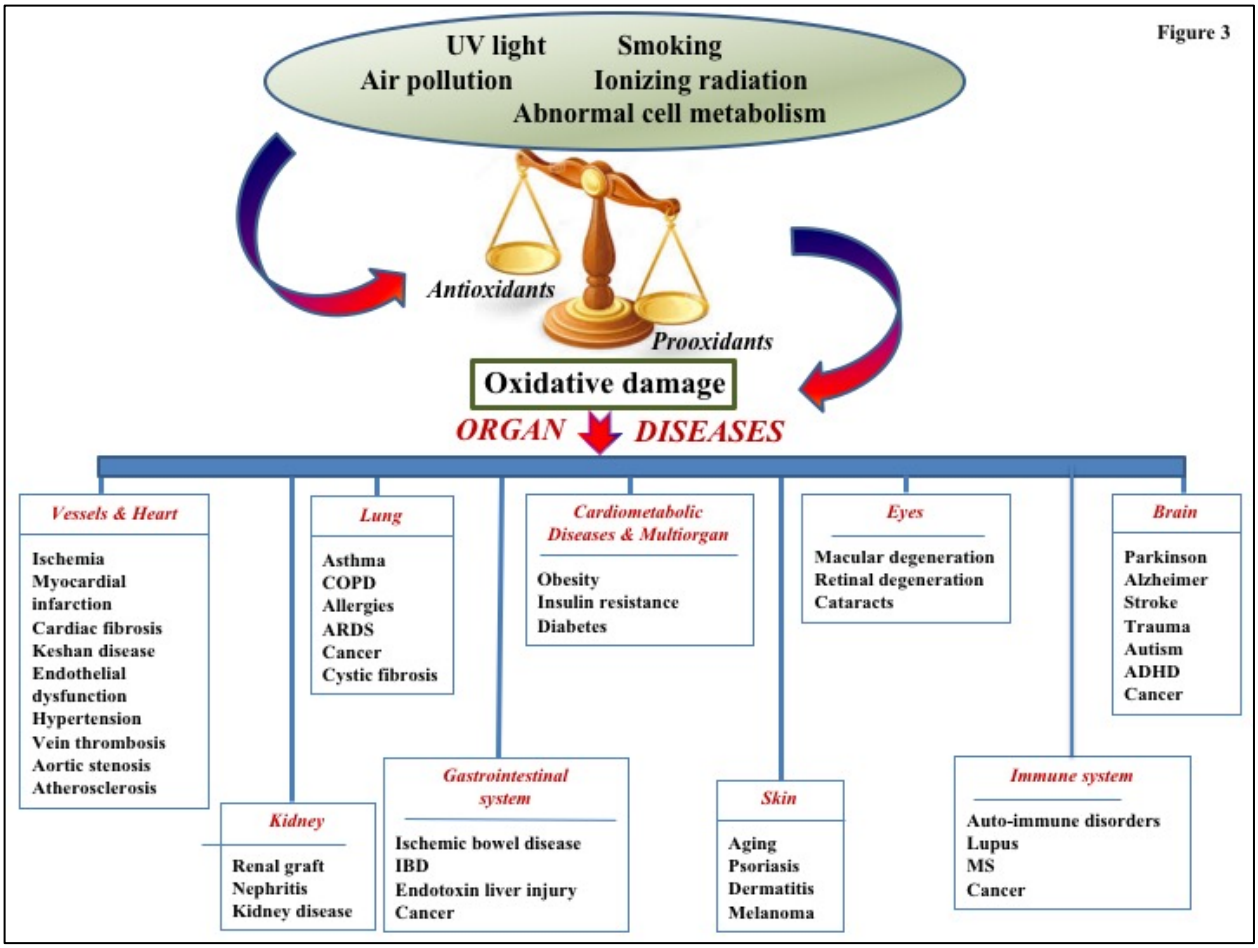

Figure 3. Link of oxidative stress to various diseases.

Numerous agents (UV light, air pollution, smoking, ionizing radiation and abnormal metabolism among others) may act as oxidants or catalysts of reactions that produce reactive oxygen and nitrogen species in addition to other oxidants. The increased levels of these pro-oxidants can overwhelm the antioxidant defense system's capacity and contribute to the initiation and development of many pathophysiological events through the activation of multiple intracellular signaling pathways, which induce inflammation, apoptosis or cell overgrowth and lead to organ dysfunction.

COPD: chronic obstructive pulmonary disease; ARDS: acute respiratory distress syndrome**;** ADHD: Attention deficit hyperactivity disorder**;** IBD: inflammatory bowel diseases; MS: multiple sclerosis.

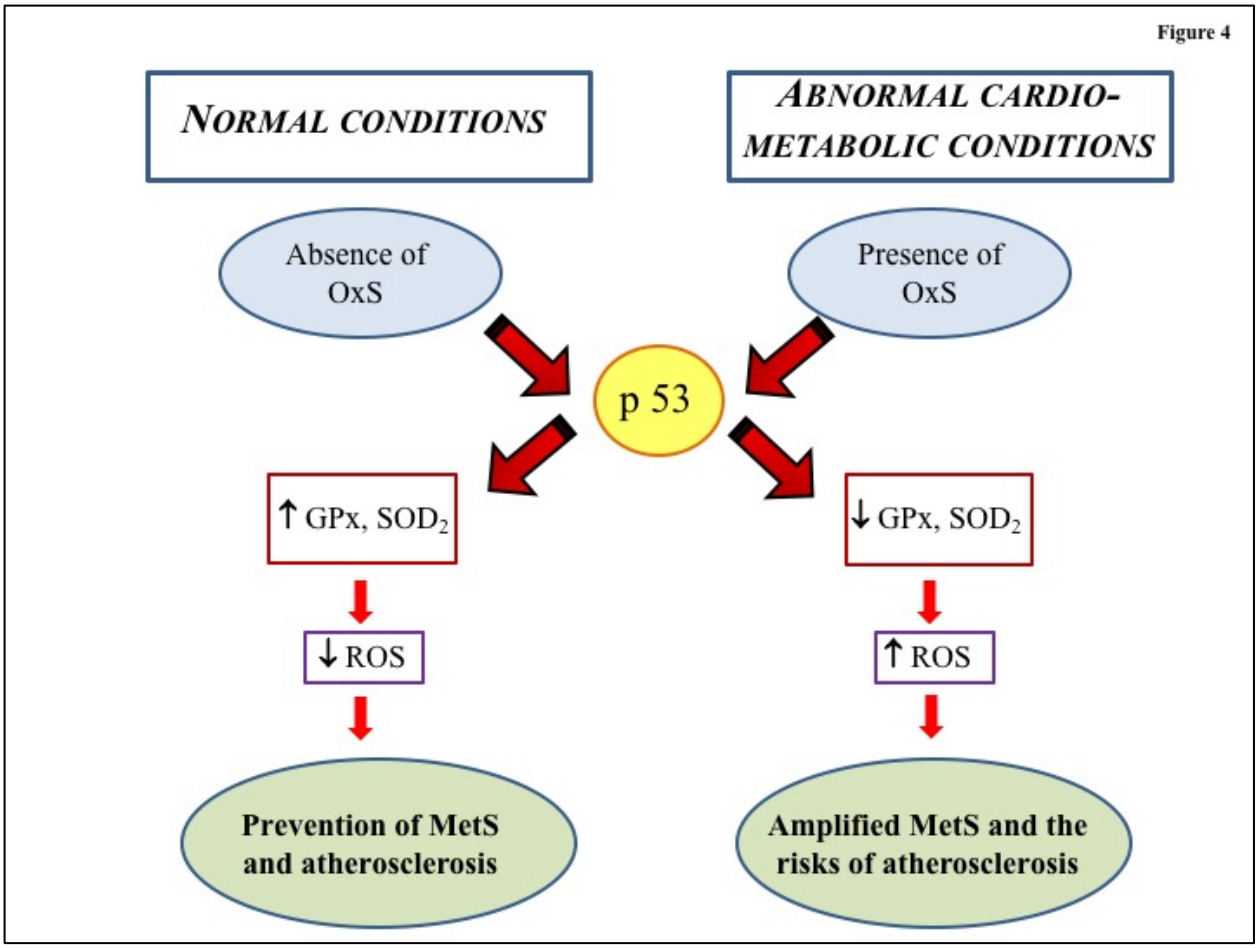

Figure 4. p53 and redox homeostasis.

p53 has broad effects on health and disease, since its expression is regulated by alterations in transcription, translation and mRNA splicing events, as well as by posttranslational modifications such as phosphorylation, acetylation, methylation, glycosylation, farnesylation, hydroxylation and ADP-ribosylation. These posttranslational modifications, together with the interaction of p53 with various protein-binding partners, help modulate subcellular localization, stability and conformation of p53. Depending on these changes, p53 can lower or heighten oxidative stress and, thereby, impact on the control or progression of obesity, metabolic syndrome (MetS) and diabetes. Importantly, p53 can function as a sensitizer and an activator of oxidative stress (OxS). In this context, ROS can **induce** p53, which promotes antioxidant activity under normal conditions and pro-oxidant activity under intense OxS.

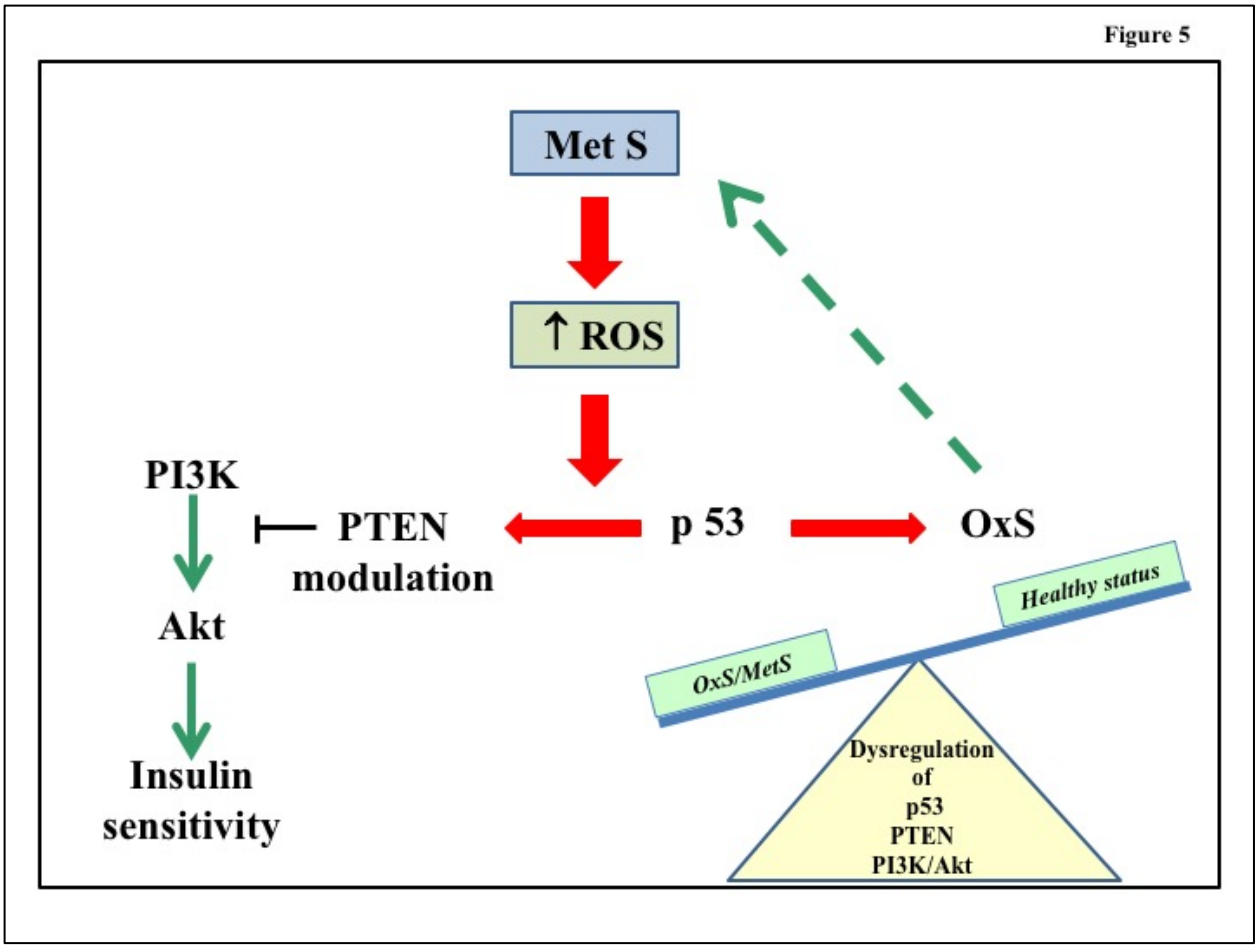

Figure 5. Vicious cycle among metabolic syndrome, p53/PTEN and insulin resistance.

Induction of p53 in Metabolic Syndrome (MetS) may activate PTEN and, thereby, impact on PI3K/Akt signaling and insulin sensitivity. Simultaneously, p53 induction enhances OxS, which amplifies MetS.

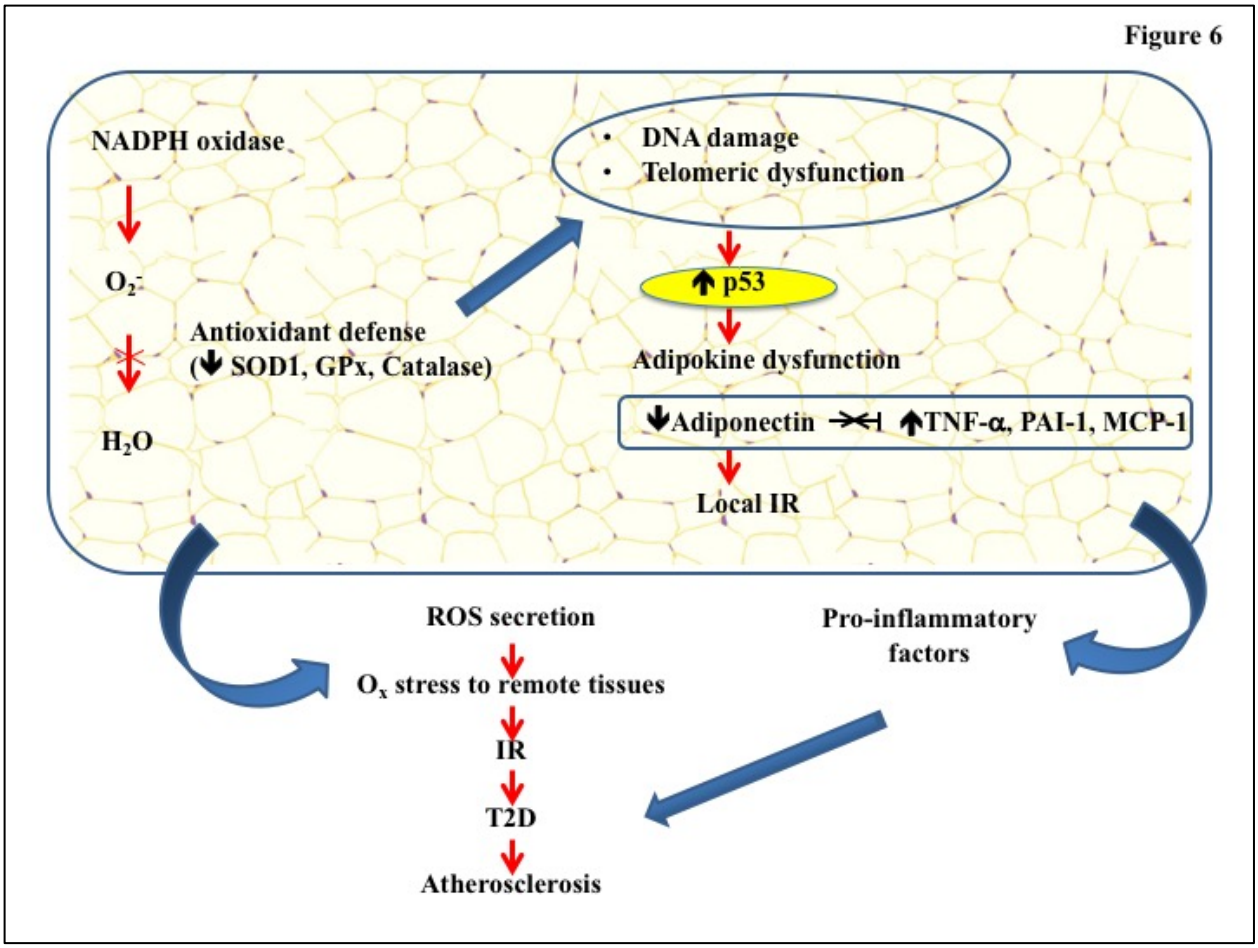

Figure 6. Mechanistic role of oxidative stress in adipose tissue insulin resistance and cardiometabolic disorders.

Fat accumulation induces reactive oxygen species (ROS) production, which affects genomic integrity and alters p53 expression, leading to adipose tissue inflammation, local insulin resistance and cardiometabolic disorders.

Revue 2

« Oxidative Stress as a Critical Factor in Non-alcoholic Fatty Liver Disease Pathogenesis»

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CONTRIBUTION DES AUTEURS

Spahis S a effectué la revue critique de la littérature, a compilé les données et crée les Figures et Tables qui accompagnent la revue. Elle a contribué à la rédaction, soumission et révision de la revue, sous la supervision du Directeur de recherche, Dr Levy E.

Delvin E et Borys JM ont participé à la révision du manuscrit.

Levy E a contribué à la rédaction et la révision du manuscrit.

Forum Review Article

OXIDATIVE STRESS AS A CRITICAL FACTOR IN NONALCOHOLIC FATTY LIVER DISEASE PATHOGENESIS

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ABSTRACT

Significance: Nonalcoholic fatty liver disease (NAFLD), characterized by liver triacylglycerol build-up, has been growing in the global world in concert with the raised prevalence of cardiometabolic disorders, including obesity, diabetes and hyperlipemia. Redox imbalance has been suggested to be highly relevant to NAFLD pathogenesis. **Recent advances:** As a major health problem, NAFLD progresses to the more severe non-alcoholic steatohepatitis (NASH) condition and predisposes susceptible individuals to liver and cardiovascular disease. Although NAFLD represents the predominant cause of chronic liver disorders, the mechanisms of its development and progression remain incompletely understood, even if various scientific groups ascribed them to the occurrence of insulin resistance, dyslipidemia, inflammation and apoptosis. Nevertheless, oxidative stress (OxS) more and more appears as the most important pathological events during NAFLD development and the hallmark between simple steatosis and NASH manifestation. **Critical issues:** The purpose of this review is to summarize recent developments in the understanding of NAFLD, essentially focusing on OxS as a major pathogenetic mechanism. Various attempts to translate reactive oxygen species (ROS) scavenging by antioxidants into experimental and clinical studies have yielded mostly encouraging results. **Future directions:** Although augmented concentrations of ROS and faulty antioxidant defense have been associated to NAFLD and related complications, mechanisms of action and proofs of principle should be highlighted in order to support the causative role of OxS and to translate its concept into the clinic.

1. INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a complex progressive liver disease controlled by multiple factors and still remains a major public health issue (18,25). Its prevalence continues to progress worldwide in parallel with the epidemic of obesity (57), reaching 20%-30% in general population, 80-90% of obese adults, 30-50% subjects with type 2 diabetes (T2D) and up to 90% in subjects with lipid disorders (25,107). In childhood, the estimated prevalence of NAFLD in obese children is rising to 40-70% (18,188). NAFLD presents a wide diversity of symptoms ranging from a simple steatosis to the concurrent histological appearence of inflammation and ballooning, which defines nonalcoholic steatohepatitis (NASH) with a prevalence of 10% to 56% in the obese population (246). Up to 20% of the patients who develop NASH can progress to fibrosis, cirrhosis and hepatocellular carcinoma (10). NAFLD and NASH are considered by the scientific community as hepatic expressions of the metabolic syndrome (MetS) since they are associated to obesity, insulin resistance (IR) and dyslipidaemia (24) along with chronic systemic oxidative stress (OxS) (48) and inflammation especially in NASH (124). Whereas the mechanisms for their pathogenesis are still enigmatic, the ‘’two hit hypothesis’’ is the widespread theory for the development of NAFLD (44). The first hit leading to steatosis results from IR, which leads to hepatic *de novo* lipogenesis and impaired fatty acid (FA) export. The “second hit“consists of elevated output of reactive oxygen species (ROS) that increase OxS capable of mediating inflammation and cytotoxicity, thereby culminating in NASH and fibrosis (8). A “third hit” has also been suggested, based on the fact that exaggerated OxS produces gradual hepatocyte death, lessens replication of mature hepatocytes and favors progenitor cell expansion, ensuing in liver cirrhosis and hepatocellular carcinoma (55,90). Overall, fat accumulation in the liver (1^{st} hit) augments vulnerability to OxS (2^{nd} hit), which triggers inflammation, endoplasmic reticulum (ER) stress, mitochondrial dysfunction and the incapacity of hepathocytes to synthesize endogenous antioxidants (209) (**Fig. 1**). Hense, even if NAFLD and NASH are multifactorial diseases, OxS appears to be nearly the most significant mechanisms that convey liver injury in NAFLD (43,222) with a branching of several intracellular events (e.g. mitochondrial dysfunction and ER stress) in combination with extracellular factors such iron accretion and gut flora. Therefore, delineation of the OxS mechanisms is fundamental for understanding NAFLD development (174) and represents the main goal of this review. The interconnection with the dynamics of antioxidant actions will be discussed largely in order to to glimpse the effective nutritional therapeutic avenues for NAFLD.

2. OXIDATIVE STRESS IN NAFLD AND NASH

As aforementioned, OxS plays a key role in the initiation and progression of NAFLD from simple steatosis to NASH even if the cause-effect relationship between OxS and the pathogenesis has not yet been established robustly (201). OxS occurs via elevated formation of ROS, which initiates lipid peroxidation by targeting the double bonds of polyunsaturated Fas (PUFA). The subsequent formation of extremely reactive aldehyde components, namely 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA), causes an intracellular damage. Many circulating biomarkers of lipid peroxidation were observed in NAFLD/NASH patients (86,148,165) (**Table 1**) and their high concentrations are associated with the intensity of liver disease (111). Concomitantly, a decline was noticed in antioxidant compounds such as catalase, glutathione (GSH), GSH S-transferase, superoxide dismutase (SOD), coenzyme Q (CoQ) and Cu-Zn SOD (60,223) (**Table 1**). Other investigators emphasized the increase in urinary 8-iso-prostaglandin F2α and serum NOX2-derived peptide, a NADPH oxidase isoform (49,50,148). Besides, the expression of hemeoxygenase-1, acting as a cellular protector against OxS in normal conditions, augments in NASH patients probably as an adapting response (120).

Various mechanisms have been reported to cause lipid peroxidation. Pro-oxidant systems such as cytochrome P450, lipoxygenase and cyclooxygenase along with free radical products have been alone or synergitically implicated in the emergence of OxS in NAFLD (201). Similarly, raised hepatic expression of CYP2E (isoform of cytochrome P450) appears to be behind ROS production in NASH (13,153). PUFA metabolic pathways are also singled out since their derived eicosanoids, including hydroxy-eicosatetraenoic, hydroxy-octadecadenoic and oxo-octadecadenoic acids have been found augmented in patients with NASH (67). Conversely, their inhibition contributes to the improvement of the disease (248). On the other hand, intensification of hepatic levels of 8-hydroxy-2-deoxyguanosine (8-OHdG), a reliable marker of mitochondrial oxidized DNA, is observed in NAFLD and NASH patients (68), which suggests an overproduction of oxidative DNA damage-targetting mitochondia dysfunction. Intra-hepatocyte mechanisms for the development of OxS will be documented in the following paragraphs.

3. MITOCHONDRIA DYSFUNCTION, OXIDATIVE STRESS AND LIVER STEATOSIS

According to some groups, OxS is not only a primary cause of liver fat accumulation (161), but it is also implicated in fibrosis development (149,193) in patients with NAFLD. The mitochondrion is a critical player in ROS overproduction (214), which results in abnormal respiration and

dysfunction of mitochondria, culminating in NAFLD development and expansion (97,189,211). Some groups of researchers even say that mitochondrial ROS and dysfunction in patients with NASH are directly implicated in NAFLD and NASH pathogenesis (140). It is quite understandable to detect mitochondrial dysfunctions in patients with NAFLD since inflated ROS may damage lipids, mitochondrial proteins, and DNA. In addition to the reported impairment of mitochondrial respiratory chain, ultrastructural mitochondrial alterations (29,104), hepatic ATP synthesis deficiency (19,41) and apoptosis (6,202) are reported in NAFLD probably as a consequence of massive OxS and inefficient antioxidant defense (79). The mechanisms for redox imbalance-mediated mitochondrial dysfunctions can be summarized as follows (**Fig. 2**): i) Excessive mitochondrial ROS are produced with enhanced electron leakage, which in turn elevates OxS and oxidative damage to mitochondria, thereby favoring a vicious cycle that leads to impairment of various defective mitochondrial (mt)DNA-encoded protein subunits and abnormal mitochondrial functions; ii) increased MDA and 4-HNE levels, emanating from lipid oxidation, inhibit cytochrome c oxidase and induce uncoupling protein 2 leading to mitochondrial membrane damage (102,160); iii) Increased ROS production and reduced hepatic ATP synthesis correlate positively with ultrastructural irregularities in hepatic subcellular mitochondria (e.g. distended and rounded, with deficiency of cristae and presence of paracristalline inclusions), thereby affecting mitochondrial functions (29,171,186); (iv) OxS is associated to the reduced expression of the transcription coactivator peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), the most important regulator of mitochondrial biogenesis, in NAFLD/NASH with an obvious negative impact on mitochondrial biogenesis process (1); (v) As PGC-1α coordinates numerous genes needed for mitochondrion production by mastering the activity of several nuclear transcription factors, its low expression in NAFLD/NASH disadvantages its interaction with both nuclear factor erythroid 1 and 2 (Nrf1 and Nrf2) to induce genes connected to the respiratory chain and mitochondrial import machinery, and transcription factors of mtDNA (TFAM, TFB1M, TFB2M), which govern mitochondrial functions (1,133,229); (vi) the down-regulation of PGC-1α in NAFLD/NASH may lead to the inactivation of antioxidant defenses, including SOD, catalase and gluthatione peroxidase (197). All together, mtOxS is able to raise lipid peroxides, alter mitochondrial morphology, impair mitochondrial bioenergetics, and affect mitochondrial functions. These changes damage hepatocytes, trigger inflammation, and contribute to IR. However, efforts should be depoloyed to deepen the mechanisms of action using cellular and

molecular sophisticated tools.

4. OXIDIZED LDL, NAFLD/NASH AND ATHEROSCLEROSIS

OxS is able to raise cardiovascular risks in patients with NAFLD via significant contribution to atherosclerosis risk factors and the induction of endothelial dysfunction (123). Elevated levels of ROS may lead to low-density lipoprotein oxidation (oxLDL) and macrophages transformation into foam cells, which represent the key stage in the development of the atherosclerotic damage (123). Interestingly, the postprandial increase in oxLDL independently predicted the severity of liver histology in subjects with NAFLD, thus providing a biological mechanism for the epidemiological association between liver disease and atherosclerosis in NASH (132,207). Validation was obtained in high fat diet (HFD)-fed mice that were administrated oxLDL (244). This experimental model could mimic human NASH, in which inflammatory events occur, including hepatocellular damage and inflammatory cell penetration (244).

OxLDL, as a product of ROS, is not only able to initiate intracellular OxS in Kupffer cells but also to cause hepatic inflammation through the process of oxLDL uptake (21,69). To examine whether oxLDL constitutes the basis for NASH pathogenesis, the recognition of oxLDL by Kupfer cells was inhibited by immunization of $ldlr^{-/-}$ (20). Consequently, a significant decline in hepatic inflammation was recorded as reflected by reduced macrophage, neutrophil and T-cell infiltration, and reduced gene expression of tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1β, monocyte chemoattractant protein-1 and fibrosis related genes. In an elegant study, oxLDL enhances lectin-like oxLDL receptor 1 expression and induces injury through ROS formation, nuclear factor kappaB (NF-κB) activation and eNOS downregulation, endothelin 1 and caveolin 1 upregulation and defenestration in human liver sinusoidal endothelial cells (251). Despite the relationship between oxLDL and liver steatosis, further studies are clearly needed to assess the clinical adequacy of plasma oxLDL as a non-invasive biomarker for NASH and to provide a basis for therapeutic strategies to improve NASH and related metabolic risk factors that conduct to T2D, cardiovascular diseases and liver deterioration (225).

5. MYELOPEROXIDASE, NAFLD/NASH AND ATHEROSCLEROSIS

Various groups investigated the role of neutrophils in NAFLD since they are essential responders to infections and release the enzyme myeloperoxidase (MPO) endowed with substantial oxidizing potential in the liver (95). Increased MPO-positive Kupfer cells were observed in the liver of obese NASH patients in close link with raised plasma MPO levels (170). In fact, MPO activity may

catalyze nitration of protein tyrosyl groups and may stimulate lipid peroxidation during hepatic steatosis (85) via accrement of HOCl-modified proteins and nitrated proteins in association with increased hepatic chemokine expression in NASH conditions (170). Conversely, MPO deficiency attenuates the development of NASH supporting an important role for neutrophil MPO-mediated OxS in its pathogenesis (169).

6. CROSS TALK BETWEEN OXIDATIVE STRESS AND ENDOPLASMIC RETICULUM STRESS

Concretely, OxS and ER stress are two processes linked together. For example, protein folding in ER is connected to ROS since after accepting electrons from protein disulphide isomerase, ER oxidoreductase-1 transfers them to molecular oxygen and produces H_2O_2 to folding, modification and trafficking of proteins (181). Additionally, RNA-dependent protein kinase-like ER eukaryotic initiation factor-2 α kinase phosphorylates Nrf2, which contributes to the dissociation of Nrf2–Keap 1 complex and promotes the gene expression containing antioxidant response element, reducing OxS by inducing the expression of antioxidant genes such as hemeoxygenase-1 (42). Conversely, the disturbance in GSH/ GSSH ratio in ER lumen leads to misfolding of proteins and increased ROS generation (12,31,121). In fact, Ashraf's review summarises the evidences and suggests that ER stress and OxS form a vicious cycle in the pathogenesis of NASH (12).

7. MICRO RNA REGULATION IN NAFLD/NASH

microRNAs (miRNA) have been revealed to be differentially expressed in NASH patients. Their modulation seems to be beneficial to NAFLD. It has been described that the inhibition of the transcriptional activity of p53 via down-regulation of miRNA34a in the liver of mice fed with HFD diminished OxS, apoptosis and hepatic steatosis (53). An imbalance in nitric oxide levels potentiates a dysregulation in ROS metabolism resulting in OxS. The enzyme nitric oxide synthases (NOS) is a key player in cardiometabolic disorders and cardiovascular diseases and its expression is downregulated at the post-transcriptional level by miRNAs, including miR-222/221and miR-92a (235) whereas miR-21 upregulates eNOS activity (230). Currently, beneficial effect of miRNAs is proposed as an acceptable tool to lower OxS related disorders, including NAFLD and MetS (11).

8. GENETIC MUTATIONS AND OXIDATIVE STRESS IN NAFLD/NASH

To assess the role of cellular OxS in the development of NAFLD and NASH, numerous studies have focused on genetic variants (**Table 2**) that might reduce the ability of hepatocytes to protect against OxS. When the rs4880 polymorphism of *SOD2* gene, coding for the mitochondrion-resident

enzyme manganese superoxide dismutase (MnSOD) was assessed, ineffective MnSOD mitochondrial targeting and reduced activity were recorded, suggesting that this variant is a good candidate modifier of NAFLD severity (4). In fact, a steady connection was observed between the functional SNP of *SOD2* and fibrosis severity in NAFLD, which evidences that mitochondria-derived OxS is significant in the pathogenesis of advanced NAFLD. Similarly, moderate to severe steatosis and inflammation resulted from the -55CT variant of uncoupling protein (*UCP)3* coding for the mitochondrial proton carrier uncoupling 3, a protein considered to be protective against OxS from β-oxidation of FA (7). A clear association was noted between NASH and the 129C/T polymorphism of glutamate-cystein ligase, the first and limiting enzyme in the formation of GSH (152). On the other hand, there was a protection against NAFLD by the 211G>A genotype of *uridine-5'-diphosphoglucuronosyltransferase 1A1* coding for the enzyme synthesizing bilirubin (110), that exhibits potent antioxidant properties (233).

9. FUNCTIONAL EVIDENCE ILLUSTRATING THE CAUSAL LINK BETWEEN OXIDATIVE STRESS AND NAFLD/NASH

In addition to environmental influences, the onset, incidence and severity of the NAFLD pathological state is significantly influenced by different interacting genetic factors as noted above. As NAFLD is inextricably connected with OxS, unsurprisingly various researchers have first addressed the functional role of specific genes related to cellular redox activity. Over the last years, genetic engineering technology in animals was used in order to improve molecular events, diagnostics and therapeutics related to NAFLD and NASH.

For example, the development of *SOD1* knockout (KO) mice shows a hepatic increase in FA synthesis (227) and a decrease in apolipoprotein B-100 levels (216), thereby leading to a lipid accumulation in the liver. In addition, *SOD1* KO mice display an increment of iron in hepatocytes (187,198), which raises OxS via Fenton reaction and the risk of liver disease progression, including fibrosis and NASH (177).

As Nrf2 empowers cells to tackle OxS by inducing cytoprotective genes, down-regulates the expression of genes involved in FA synthesis (94,234,242) and counteracts inflammation (168), it is highly interesting to determine its connection to NAFLD/NASH. Global KO of *Nrf2* strongly enhances the sensitivity of mice to develop NASH when they are administered a methionine- and choline-deficient regimen (38,200). In contrast, genetic activation of *Nrf2* by knockdown of Keap1 decreases their susceptibility to NASH caused by the choline-deficient diet (253). To approach the

mechanisms, some researchers used mice with loss of Nrf2 and on high-fat diets. These animals develop a significant induction of lipogenic genes, a reduced expression of β-oxidation genes, a decline of AMP-activated protein kinase concentrations, and a low activity of acetyl coenzyme A carboxylase compared to the wild-type livers, which is compatible with a higher FA biogenesis and fat accumulation characterizing NAFLD (127).

The KO of the senescence marker protein-30 (*SMP30*) gene, located in the p11.3-q11.2 segment of the X chromosome, results in aberrantly broadened mitochondria with blurred cristae and unusual accumulations of neutral lipids and phospholipids (87). Similarly, SMP30 decline in response to aging, extreme lipid alimentation or genetic deficiency, leads to a failure of Ca(2+) pumping activity, which prejudices acute insulin discharge by pancreatic β-cells and exacerbates inflammation, OxS and ER stress in NASH (99). The repercussion of SMP30 reduction on OxS and liver steatosis is largely explained by the function of SMP30 as an active enzyme in the antioxidant vitamin C biosynthesis (98). SMP30 may therefore represent a new target to restore glucose metabolism and ameliorate NAFLD.

The renin-angiotensin system (angiotensin (Ang)-converting enzyme (ACE)/Ang II/ AT1) has been identified as an important pathway that contributes to the development of NAFLD (239). Indeed, Ang II is able to cause the expansion of NAFLD in the transgenic Ren2 rat model (with elevated tissue Ang II) by increasing hepatic ROS (231).

Other investigators have stressed the role of alcohol dehydrogenase 5 (Class III), chi polypeptide (ADH3) in the production of OxS and liver steatosis. This enzyme is also known as glutathione-dependent formaldehyde dehydrogenase, thereby contributing to the neutralization of exogenous formaldehyde (51) and protecting cells from the genotoxicity elicited by endogenous formaldehyde (175). In animals, the absence of ADH3 provoked the fast advancement of NASH, which illustrates that ADH3 favors a strong reaction against lipid accumulation by cooperating with the KEAP1-NRF2 system (73).

In the setting of NAFLD in mice with whole-body PEPCK knockdown (while being placed on a standard diet with 10% calories from fat), there is a constitutive oxidative metabolism capable to lead to collateral OxS and inflammation processes that intensify IR and hepatocellular injury (185).

On the other hand and contrary to expectations, mice with the deletion of glutamate cysteine ligase, the rate-limiting enzyme for *de novo* synthesis of GSH, did not exhibit an enhancement of the progression of NAFLD to NASH with severe hepatic injury (78). Instead, the authors found that

despite their very low GSH levels, Gclm null mice were to a great extent protected against the development of NASH induced by the choline-deficient diet given their ability to develop compensatory mechanisms that protect the hepatocyte against metabolic abnormalities.

Although the identity of certain genes and impact on OxS certainly lead to significant pieces of information, functional studies are still required to delineate the mechanisms by which these risk factors cause the onset and worsening of NAFLD. Mouse models certainly constitute important tools for delivering novel understanding of the biology and genetics of NAFLD, which may clarify the mechanisms and means to develop and improve diagnostics and therapeutics.

limiting enzyme in the formation of glutathione (GSH), an impor-

tant endogen antioxidant.

10. NUTRITIONAL ANTI-OXIDANT INTERVENTIONS

10.1 Vitamin E

OxS has now been recognized as a central mechanism contributing to the improvment of liver lesions as it accelerates the transition from simple steatosis to NASH (79). Therefore, different groups targeted OxS in animal and human studies using vitamin E (**Table 3**), a free radical scavenger, a chain-breaking antioxidant in lipid peroxidation chain, and an anti-inflammatory agent (84). To examine the hepatoprotective activities of vitamin E, *ob/ob* mice were fed diets containing α- or γ-tocopherol before being injected lipolysaccharides (LPS) to produce hepatic damage and NASH. The 5-week dietary supplementation of α- or γ-tocopherol significantly lowered hepatic LPS-elicited lipid peroxidation (MDA), inflammation (TNF-α), and liver injury as noted by decreases in serum alanine aminotransferase (ALT) activity and serum lipids [free FA and triglycerides (TG)] without affecting body mass. Therefore, vitamin E preserved from pathogenic processes conducting to liver impairment and NASH in virtue of its antioxidant and anti-inflammatory efficiency, but not to any anti-obesity effects. The authors also concluded that vitamin E actions were directed against the “second-hit” of NASH that is characterized by worsened hepatic injury, inflammation and lipid peroxidation (45). This study with genetic *ob/ob* mice confirmed the data in rats and mice fed a methionine-choline deficient diet to provoke NASH, in which α-tocopherol supplementation reduced histologic signs of liver steatosis, inflammation and OxS (138,164). Likewise, subcutaneous administration of α-tocopherol to Zucker rats concomitantly raised α-tocopherol and lowered lipid peroxidation in mitochondria while improving liver injury (194).

Data from numerous human clinical trials are consistent with animal observations. In a study with 10 subjects with NAFLD and 10 patients with NASH, a one year of α-tocopherol treatment at a dose of 300 IU/day improved serum transaminases [ALT, aspartate aminotransferase (AST), and gamma glutamyltransferase (GGT)] levels, hepatic inflammation histological grade and fibrosis of only NASH patients (82). Confirmation was obtained employing a more elevated dose (400 IU/day) of α-tocopherol in 11 subjects with NASH, suggesting that α-tocopherol was safe as no side effects were noted (182). Vitamin E (400-1200 IU/day during 2-4 months) could reduce transaminase levels in the serum of children with NAFLD regardless of changes in body mass index or the echogenicity of the liver observed by dual energy X-ray absorptiometry or ultrasound (103). In the TONIC trial, vitamin E resulted in histologic improvement and NASH resolution along with amelioration in non-high-density lipoprotein (HDL)-cholesterol, LDL and total cholesterol levels in 56 children among the total 173 participants, suggesting an alleviation in cardiovascular risks in children with NASH (40). The PIVENS trial with 247 adults with NASH and without T2D showed that vitamin E at dose 800 IU daily during 96 weeks led to marked improvement in histologic features (43%) and significant resolution of NASH (47%) (182). Vitamin E with 1000 IU was more effective when combined with vitamin C (1000 mg/day) in improving fibrosis without changes in liver inflammatory factors over a period of 6 months (81). In contrast, a randomized, double-blind placebo-controlled clinical trial reported that vitamin E (400 IU/day) combined with metformin (800 mg/day) was more effective in achieving the main objectif consisting in lessening ALT level and IR in pediatric NAFLD patients (3). Overall, findings of human clinical trials have so far been inconsistent since most of them are retrospective studies, with insufficient sample size, and without control groups and liver biopsy documenting histological improvement. Even if current guidelines recommend vitamin E (at a dose of 800 IU/day) as the first-line pharmacotherapy for NASH (33), attention must be paid to the harmful concentrations of vitamin E reported in various studies (5). Therefore, the vitamin E therapeutic option in liver steatosis should be investigated in rigorously performed, randomized, controlled trials especially since (i) vitamin E does not act against the oxidation elicited by endogenous enzymes and non-radical oxidants such as hypochlorite (143); and (ii) the benefits of vitamin E have not been validated in patients with T2D as well as those with cirrhosis (184); and (iii) a meta-analysis revealed increased mortality with vitamin E administration (129).

10.2 Vitamin D

Growing evidence indicates the anti-inflammatory, immunomodulatory and antifibrotic properties of vitamin D (93), as well as its inverse association with MetS components such as body mass index, body fat content, hypertension, IR and T2D (37,77,199). These observations encouraged investigators to study the impact of vitamin D on fatty liver, especially given that i) the liver is a major organ in vitamin D production; ii) vitamin D deficiency is frequently reported in chronic liver disease (101); and iii) vitamin D levels are associated to NAFLD in adults and children (208). Earlier data in rats underlined the effectiveness of lipid transfer, metabolic proteins and vitamin D3 levels as a biomarkers for non-invasive diagnosis of steatosis progress and the usefulness of phototherapy as an appropriate supplementary treatment for NASH (135). Subsequently, it was reported that the development and progression of NAFLD by HFD and high fructose corn syrup diet (following 10 weeks of exposure) was exacerbated by vitamin D deficiency due to the induction of inflammation (IL-1β, IL-6 mRNA in the liver; IL-1β and IL-6 in the serum) and elevated OxS (HO-1 mRNA in the liver) (176). The authors proposed the activation of tolk-like receptors (TLR2 and TLR4) as the mechanism of action by way of CD14/LPS-binding protein with the induction of downstream inflammatory signaling molecules exaggerating steatosis and inflammation. Other investigators found that vitamin D may exhibit anti-proliferative and anti-fibrotic effects in hepatic stellate cells, thereby contributing to liver fibrosis (15). They reached this conclusion in view of actions of vitamin D on: i) inhibition of cyclin D1 expression and stellate cell proliferation; ii) inhibition of collagen Iα1 promoter activity, mRNA and protein expression along with an antifibrotic effect; iii) induction of matrix metalloproteinase enzymatic activity, which degraded extracellular matrix proteins; and iv) inhibition of tissue inhibitor of metalloproteinase 1 mRNA expression levels, whereas vitamin D receptor (VDR) silencing impaired the effect of vitamin D on cyclin D1 and collagen Iα1 expression.

In a human cross-sectional design, scientists evaluated the VDR expression in the liver of patients with NASH (15,163). VDR expression was negatively related to the significant worsening of liver histology and, once again, vitamin D/VDR system seemed to play a role in the progression of metabolic chronic liver injury in NASH. In other human studies, low serum $25(OH)D_3$ concentrations were recorded in subjects affected by the NAFLD (59), which probably promotes endotoxemia and thus activation of the innate immune system (122). Altogether, these observations provided a basis for designing clinical trials. When 27 patients with NAFLD in a parallel, double-blind, placebo-controlled study received either oral pearl consisting of 50,000 IU vitamin D3 every

14 day for 120 days, they displayed beneficial effects on serum MDA and hs-C reactive protein (CRP) levels without any changes in the serum levels of HOMA-IR index, liver enzymes and grades of hepatic lesions (192). Therefore, the authors suggested that supplementation with vitamin D might be considered as an adjunctive therapy to reduce inflammation and lipid peroxidation alongside other treatments for NAFLD patients. Currently, thorough planned trials are needed to determine whether vitamin D supplementation may constitute a new therapeutic alternative in NASH care.

10.3 Coenzyme Q

CoQ is an endogenous lipid-soluble benzoquinone compound that exists in a reduced (ubiquinol, CoQ10H2) as well as in an oxidized (ubiquinone, CoQ10) form. The redox activity enables CoQ10 to behave as an antioxidant capable of preventing lipid peroxidation and peroxidative damage to membrane phospholipids (130). It acts as an essential cofactor in oxidative phosphorylation in mitochondria because it operates in the mitochondrial inner membrane to transfer electrons from complexes I (NADH coenzyme Q reductase) and II (succinate dehydrogenase) to complex III (cytochrome bc1 complex), which are closely involved in OXPHOS (80). It also functions as an inductor of ATP formation in tissues with high-energy turnover (heart, liver and muscle) (23). It also serves as a cofactor and as a requisite cofactor of mitochondrial uncoupling proteins whose activation lessens free radical formation from mitochondria (17,91). As OxS is closely linked to hepatic lipid accumulation and since IR impoverishes intracellular amounts of natural cellular antioxidants (74), CoQ10-mediated antioxidant activity is able to alleviate ROS concentrations, thereby allowing NAFLD alleviation. In HFD-induced rat model of NAFLD, CoQ administration led to an increase in both apolipoprotein (apo) B synthesis, favoring the rise of the number and size of very low-density lipoprotein (VLDL) particles, which ultimately caused the secretion of larger VLDL and lessened hepatic steatosis (30). However, the changes in apo B48 concentrations elicited by dietary CoQ may also be due to diminished degradation of large VLDL that may represent atherogenic particles. Similarly, diet containing 57% of energy from fat induces the characteristic features of NAFLD in rats, including IR, hypertriglyceridemia, hepatic steatosis and liver damage, combined with altered CoQ10 metabolism (23). Besides, when 44 NAFLD patients were included in a randomized double-blind placebo controlled trial and supplemented with 100 mg/day CoQ10 capsules, they improved various anthropometric and biochemical parameters in NAFLD, including serum AST and various adipose-tissue derived cytokines (64). Similarly, a randomized, double-

blind, placebo-controlled trial showed that NAFLD participants receiving CoQ10 capsules (100 mg/day) exhibited a significant decrease in liver aminotransferases (AST, GGT, hs-CRP), inflammation (TNF-α) and the grades of liver steatosis, as well as raised levels of adiponectin (65).

10.4 Polyphenols

Since epidemiological investigations revealed that diets rich in vegetables and fruits could diminish the incidence of cardiometabolic disorders, their bioactive components are gaining more interest in improving health risks in view of their low toxicity and few side effects. Currently, polyphenol-rich plants and fruits are not only used to improve human health and promote quality life, but also to fight diseases, including obesity, T2D, cardiovascular disease and cancer (156). For food experts and nutritionists, discovering appropriate functional nutrients and developing a dietary therapy are important strategies in avoiding NAFLD (**Table 3**). For example, blueberries highly recommended in view of the phenolic potential health benefits of their compounds, have high anthocyanin content (112) that is beneficial in reducing the risk of MetS conditions, such as obesity, hyperglycemia, hyperinsulinemia, and hyperlipidemia (89,190). Phenolic content of wild Chinese blueberries displays good efficacy in impeding steatosis in HepG2 cells (113). In db/db mice, feeding a polyphenolic extract of lotus root for 3 weeks protects them from hepatic steatosis via the decline of lipogenic enzymes, such as fatty acid synthase and malic enzyme (213). Red wine comprises several polyphenols with likely biological actions (108), particularly resveratrol and catechins (14), but only few other components from red wine have been tested. Similarly, ellagitannin blend extracted from European oak bark utilized in red wine maturation exhibits both cardiac and liver protection and markedly improves metabolic profile in high-carbohydrate, HFD-fed Wistar rats (155). Attenuation is observed in hepatic steatosis and fibrosis with infiltration of inflammatory cells in liver, as well as in plasma activities of ALT and AST. As various investigations worldwide propose that the valuable actions of green tea are due to their capacity to regulate cell signaling and cell cycle (28), their epigallocatechin gallate is being used to treat diseases that are related to chronic inflammation. In Sprague–Dawley rats fed with HFD for 8 weeks to develop NAFLD, treatment with (-)-epigallocatechin-3-gaimproves hepatic histology (given the of decrease fatty liver score as well as necrotic and inflammatory foci), reduces liver injury (e.g. ALT/AST ratio) and attenuates fibrosis (sirius red and synaptophysin-positive stain) along with downregulation in the expressions of key pathological oxidative (e.g. nitrotyrosine formation) and pro-inflammatory markers (e.g. iNOS, Cyclooxygenase-2, and TNF-α) via transforming growth factors/SMAD,

PI3K/Akt/ forkhead box O1 and NF-κB pathways (236). In human studies, the consumption of bayberries (250 mL twice daily for 4 weeks) containing high levels of polyphenols beneficially alters the levels of oxidative (protein carbonyl groups), inflammatory (TNF-α, IL-8, hs-CRP), apoptotic biomarkers (tissue polypeptide-specific antigen, cytokeratin-18 fragment M3) and liver enzymes (ALT, AST) in young individuals with features of NAFLD. Other researchers turn towards resveratrol because of its protection against OxS and inflammation that are involved in NAFLD pathogenesis. The design of placebo-controlled, double blind randomized clinical trial has permitted to document improvement of conditions (ALT, liver steatosis, markers of inflammation, and hepatocellular apoptosis) in patients with NAFLD (63) or NASH (224) pathogenesis.

Interestingly, flavonoids are often employed in NAFLD models given their beneficial effects and especially their antioxidant power (173). The advantage of flavonoids may be related to their activation of peroxisome proliferator-activated receptor (PPAR)γ without serious side effects seen with the use of full agonists (34,105,128,203). More clinical are required to explore whether the antioxidant and antiinflammatory actions displayed by polyphenols are likely to make a significant contribution to the amelioration of NAFLD.

10.4.1 Berberine

Berberine is an isoquinoline alkaloid, which is present in the bark, roots and stems, as well as plants of the genus Berberis. It displays various pharmacological activities and has been investigated for treatment of cancer, obesity, T2D, inflammation, atherosclerosis, Alzheimer's disease, rheumatoid arthritis and cardiovascular diseases (2,154,228). This compound is able to fight MetS components such as abnormal glucose metabolism, dyslipidemia and hypertension (100,252). Since it has been able to suppress ROS production and to reduce inflammation (56,144), it appeared reasonable to test its effectiveness in NAFLD. Using both *in vitro* and *in vivo* models, it has been shown that berberine is capable of inhibiting cholesterol and TG biogenesis by down-regulating acetyl-CoA carboxylase via the activation of AMP-activated protein kinase (AMPK), thereby resulting in lower liver fat content (26). Berberine is also capable to prevent liver fibrosis through the regulation of antioxidants (SOD) and lipid peroxidation (MDA and HNE) (249). In diabetic rats, berberine alleviates the pathological evolution of liver and reverts the raised hepatic glycogen and TG to close the control levels by modulating PPARα/δ expression in the liver (255). Similarly, berberine exhibits the capacity to improve lipid dysregulation and fatty liver in db/db and ob/ob mice via peripheral AMPK activation and neural signalling (92). Another action on experimental NAFLD

is the down-regulation of liver UCP2 (mRNA and protein expression) in NAFLD rats. It can lessen hepatocyte fat accumulation while improving lipid metabolism disorder in NAFLD rat model (241). Using berberine-containing Chinese medicine, Zucker rats display sustained glucose-lowering effects, attenuated IR, lowered fatty degeneration in association with activation of AMPK, Akt, and insulin-like growth factor-binding protein pathways, with downregulation of miR29-b and induction of a gene network implicated in cell cycle and NADPH metabolism (254). Importantly, mitochondrial dysfunctions (membrane potential, oxygen consumption and ATP production) are improved in the liver in link with an increased activity of the mitochondrial sirtuin 3 in HFD-fed rats by berberin (210). Confirmation is obtained by demethyleneberberine, a natural mitochondria-targeted antioxidant, which exerts significant inhibition of mitochondrial dysfunction, OxS, and steatosis in fatty liver disease mouse model (250).

A double blind, crossover designed trial versus placebo shows that 8 week-treatment of a combined nutraceutical containing berberine, chlorogenic acid and tocotrienols significantly improves anthropometric and biochemical parameters (39). In particular, total cholesterol, LDL-cholesterol, TGs, non-HDL cholesterol, fasting insulin, HOMA-IR and steatosis are decreased in the nutraceutical group. In a randomized, parallel controlled, open-label clinical trial, life style intervention in combination with berberine (0.5g tid) has ameliorated NAFLD and related metabolic disorders (240). Given the limited number of human investigations, additional studies are needed to prove the effectiveness of berberine in preventing and treating NAFLD.

10.4.2 Resveratrol

Emerging evidence has pointed out the advantageous physiological actions of resveratrol in promoting health and treating diseases, including NAFLD (35,106,114,247). This 3,5,4′-trihydroxy-*trans*-stilbene, derived mainly from plants, grapes and berries, is a robust antioxidant and anti-inflammatory compound (158,159). It is also capable of negatively modulating IR, glucose intolerance and dyslipidemia (16). Through anti-lipidogenic effects, resveratrol may reverse steatosis by fighting *de novo* lipogenesis in various cell lines (72,191,226). In animal models, the administration of resveratrol resulted in preventive and therapeutic effects on hepatic steatosis (**Table 3**) via mechanisms leading to OxS reduction (27,191). Despite the early promise of experimental studies with resveratrol, to date very limited effort was made to uncover the impact of this natural polyphenolic compound on humans. In a small cohort of healthy obese males, resveratrol was able to decrease plasma ALT concentration and intrahepatic lipid content (212).

However, additional thorough studies in human subjects are still needed over long time periods to determine the efficiency of resveratrol in alleviating fatty liver diseases.

10.4.3 Silybin/Sylimarin

Silymarin and silibinin are other natural polyphenolic flavonoids contained in the herb milk thistle (*Silybum marianum*). Silymarin represents the crude extract of milk thistle seeds and is a complex of biological compounds that include silibinin or silybin, which is endowed with potent antioxidant, immunomodulatory, antifibrotic, antiproliferative, and antiviral activities (70,166). Silibinin is most commonly employed for gastrointestinal clinics to treat liver disorders such as NASH and cirrhosis (115). When a four-week daily dose (20 mg/kg i.p.) of silibinin is administrated to db/db mice fed a methionine-choline deficient diet, a model combining the features of the MetS with the histological pattern of NASH (**Table 3**), there is a marked antisteatotic effect accompanied by an antioxidant action of silybin, which is confirmed by decreased levels of hepatic OxS (isoprostanes, 8-OHdG and nitrites/nitrate) as well as inflammation (TNF-α and IL-6 mRNA) via reduced JNK phosphorylation (179). In the same animal model, silibinin treatment counteracts the evolution of liver damage by controlling lipid homeostasis and overturning OxS–mediated lipotoxicity and NF-κB activation in experimental NASH (178). A clinical trial of 179 NAFLD patients reveals that 12 months-treatment with silibinin, combined with phosphatidylcholine and vitamin E, is associated with amelioration of IR, liver enzymes and liver histology compared to placebo (115). Despite the huge number of findings collected from experimental models, one needs large human trials since the current translation of the evidence in clinical setting is far to be conclusive.

10.5 Polyunsaturated fatty acids

Scientific evidence showed that saturated lipids and fructose are more prospective to stimulate hepatic fat accumulation and progression of NAFLD, whereas unsaturated lipids seem to have a more preventive effect (46,224). Especially n-3 PUFA are capable of significantly reducing plasma and liver TG accumulation, which hampers NAFLD expansion via various metabolic pathways (126). Growing evidence, essentially based on cardioprotective properties (54,71), suggests the effectiveness of n-3 PUFA to prevent of treat NAFLD. Eicosapentaenoic (EPA), docohexaenoique (DHA) and α-linolenic acids are the three main long-chain n-3 PUFAs. Given their sluggish biogenesis in humans, EPA and DHA are considered essential PUFA and must therefore be obtained from diet (22), mainly from fatty fish. Their number of double bonds, the length of the

chain and the presence of the first double bond provide n-3 PUFA with unique properties, which distinguish them from other unsaturated fats (47).

The n-3 PUFA affect several steps of the pathophysiological cascade and can prevent or inhibit the progression of NAFLD (**Table 3**). The n-3 PUFA lead to i) decreased IR resulting from excessive caloric intake and lipid accumulation in adipose tissue; ii) increased circulating adiponectin levels in response to obesity, which in turn diminishes fat accumulation in the liver; iii) reduced adipose tissue-mediated exaggerated FA release, a condition contributing to dyslipidemia; iv) lowering of blood lipid levels and raising n-3/n-6 ratio in cell membrane phospholipids; v) enhancing the number of insulin receptors on hepatic cell membrane, a process improving insulin sensitivity; vi) altering the transcription of liver genes involved in lipid metabolism regulation via PPARα, a major nuclear factor that controls enzymes involved in mitochondrial β-oxidation, promotes degradation of free FAs through stimulation of β-oxidation preventing hepatic TG accumulation; vii) down-regulating expression of sterol regulatory element binding protein-1 (SREBP1c), a key transcription factor involved in TG synthesis suppress its activity, thereby averting liver TG accretion; viii) impeding mitochondrial ROS production and OxS occurrence; ix) limiting ER stress, which lessens hepatic lipotoxicity; x) thwarting the synthesis of pro-inflammatory eicosanoids and cytokines in hepatocytes or downregulation of transcription of proinflammatory cytokines via their n-3 anti-inflammatory derivatives (protectins, resolvins); xi) reducing hepatic fibrosis and NAFLD progression towards NASH and cirrhosis "second hits" (22); and xii) controlling glucose homeostasis and impeding IR development, thereby slowing down NAFLD progression (131,220) by negatively regulating carbohydrate-responsive element-binding protein expression (52) .

The decrease in hepatic n-3 PUFA concentrations may contribute to exacerbating hepatic lesions. This hypothesis has first been tested in NAFLD animal models in which n-3 PUFA improves histological lesions, restores adiponectin secretion, induces PPARα expression, and hampers TNF-α and SREBP1c production (22,205,237). In this context, n-3 PUFA is able to decrease inflammation (36) via probably inactivation of the NF-κB pathway (36), resulting from inhibition of IκB-α phosphorylation/degradation and preventing NF-κB translocation to the nucleus for the induction of pro-inflammatory cytokines. Further evidence also shows that n-3 PUFA may attenuate the generation of free radicals (220) and lipid peroxidation process (66).

In obese mice fed a HFD, the n-3 PUFA supplementation improves insulin sensitivity, while reducing OxS and inflammation associated with NAFLD (220). In a recent trial, the daily supplementation of NAFLD subjects with fish oil for 6 months ameliorates lipid profiles, decreases the discharge of pro-inflammatory cytokines from the liver, while inhibiting the redox imbalance characterized by increased lipid peroxidation and free radical activity (220). In a study with young patients with NAFLD, the administration of different doses (250 mg and 500 mg/day) of DHA markedly decrease hepatic lipid content without apparent side effects, suggesting that n-3 PUFA doses were safe and effective (145,146). Moreover, the increase of EPA to a dose of 2700 mg/day significantly improves levels of liver transaminases, free FAs and cholesterol in patients with NAFLD (206). Although n-3 PUFA supplementation is a promising approach in the treatment of NAFLD patients, more randomized studies with large cohorts are necessary to validate their effectiveness and safety.

10.6 Pre-Probiotics

Probiotic may restrain the role of bacterial pathogens in NAFLD in many animal models of HFD-induced NAFLD/NASH as is largely shown with the most characterized probiotic VSL#3 mixture, (62,109,119). For example, treatment with the probiotic mixture VSL#3 displays a reduction of antioxidative and anti-inflammatory effects in NAFLD ob/ob mice (109), a model of NAFLD induced by an HFD (134) and a model of hepatic natural killer T-cell depletion in HFD-fed animals (119). Administration of VSL#3 to an experimental model of NAFLD/NASH attenuates fibrosis (**Table 3**) by reducing transforming growth factor-β, and collagen, α- smooth muscle actin and matrix metalloproteinase-1 expression (221). Despite the numerous preclinical investigations carried out to evaluate the use of probiotics in the treatment of fatty liver disease, there are only a limited number of trials concerning their efficacy in human NAFLD. By testing a mixture of probiotics associated with prebiotics and vitamins in 10 patients with biopsy-proven NASH during 2 months of treatment, one can observe a substantial amelioration of hepatic injury and functional tests, as well as a partial sustained effect after the end of treatment (118). Another study carried out to evaluate the effects of VSL#3 probiotic therapy for 3 months in 22 NAFLD patients shows an amelioration of OxS and inflammation (116). Although these promising data are strongly indicative of a great potential for the use of probiotics in the prevention and treatment of NAFLD, there is a need of further clinical studies to better define this innovative strategy.

11. CONCLUSIONS

The prevalence of NAFLD raised rapidly in association with obesity and IR. As a metabolic stress-involved in liver disease, NAFLD has become a worldwide health problem with increased risks of T2D, cardiovascular events and mortality. Although the mechanisms remain poorly understood, OxS appears to play a major role in its pathogenesis and complications. The high production of ROS causes lipid peroxidation, followed by inflammation, activation of hepatic stellate cells leading to fibrogenesis, necrosis, cirrhosis and carcinoma. This review summarizes the plausible evidences documenting the contribution of OxS originating from mitochondria to the progression of NAFLD and its aggravation to NASH. Various natural compounds represent potential therapeutic candidates essentially due to their anti-oxidant, anti-inflammatory and anti-fibrotic properties. In spite of the various data obtained in experimental models, further breakthrough related to OxS signalling and mechanisms are still warranted to understand the NAFLD pathogenesis. In addition, despite the available clinical studies, there is clearly a significant requirement for robust and well-controlled trials using large cohorts to translate the evidence in clinical setting and to prove intervention efficacy.

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AUTHOR DISCLOSURE STATEMENT

The authors declare that they have no competing financial interests.

LIST OF ABBREVIATIONS

4-HNE,	4-hydroxy-2-nonenal
8-OHdG,	8-hydroxy-2-deoxyguanosine
ALT,	Alanine aminotransferase
AMPK,	AMP-activated protein kinase
Apo,	Apolipoprotein
AST,	Aspartate aminotransferase
CoQ,	Coenzyme Q
CRP,	C reactive protein
DHA,	Docohexaenoique
EPA,	Eicosapentaenoic
ER,	Endoplasmic reticulum
FA,	Fatty acid
GGT,	Gamma glutamyltransferase
GSH,	Glutathione
HDL,	High-density lipoprotein
HFD,	High fat diet
IR	Insulin resistance
LPS,	Lipolysaccharides
MDA,	Malondialdehyde
miRNA,	MicroRNA
MnSOD,	Manganese-dependent superoxide dismutase
MPO,	Myeloperoxidase
NAFLD,	Nonalcoholic fatty liver disease
NASH,	Non-alcoholic steatohepatitis
NF-KB,	Nuclear factor kappaB
NOS,	Nitric oxide synthases
Nrf2,	Nuclear factor erythroid 2
oxLDL,	Oxidized low-density lipoprotein
OxS,	Oxidative stress
PGC-1α,	Peroxisome proliferator-activated receptor-γ coactivator-1α

PPAR,	Peroxisome proliferator-activated receptor
PUFA,	Polyunsaturated FA
ROS,	Reactive oxygen species
SOD,	Superoxide dismutase
SREBP1c	Sterol regulatory element binding protein-1
T2D,	Type 2 diabetes
TLR,	Tolk-like receptors
TNF-α,	Tumor necrosis factor-α
UCP,	Uncoupling protein
VDR,	Vitamin D receptor
VLDL,	Very low-density lipoprotein

Table 1: Oxidative stress markers in NAFLD/NASH

OxS marker	NAFLD/NASH	Animals\Humans	Localization	Clinico-pathological features	Mechanisms	Ref
↑HNE, 8-OHdG	NAFLD+NASH	Human	Liver biopsies	Necro-inflammation, fibrosis	↑ Grade of necro-inflammation	(186)
↓ CoQ9/CoQ10 ratio **↑ CoQ9**	HFD-induced NAFLD	Rat	Plasma	↑ ALT, AST/ALT ↑ IR ↓ Plasma protein thiols	Adaptive response to OxS	(23)
↑ CoQ9	HFD-induced/ HFHF-induced NASH	Rat	Liver tissue, Plasma	↑ ALT, ↑ Hepatic TG, ↑Liver weight	↑ Hepatic fibrosis	(96)
↑ oxLDL **↑ TBARS**	NASH	Human	Serum	↓ Vit E ↑ IR	↑ CVD	(32)
↑ MDA **↓ CoQ10** **↓ SOD** **↓ Catalase**	NAFLD/NASH	Human	Plasma, liver biopsy	↑ LDL ↑Necroinflammatory activity ↑ Fibrosis ↑ TG ↑ IR	↑ BMI and lipid metabolism = OxS NAFLD ↑ IR= ↓antioxidants in NAFLD	(243)
↑ TBARS **↑ PCC**	NAFLD	Human	Ultrasound of the liver	↓ GSH/GSSG	Adaptive response to OxS in NAFLD	(139)

					with or without T2D	
↑ **MDA-HSA** ↓ **Vit D**	Pediatric NAFLD	Human	Biopsy, Plasma	↑ Protein carbonyls ↑ Hepatic 8-OHG	↑Lobular inflammation ↑NAFLD activity score ↑ NASH	(147,148)
↑ **8-OHdG**	NASH	Human	Liver Biopsy, Serum	↑ GGT	Adaptive response to OxS	(86)
↑ **OSI**	NAFLD	Human	Plasma	↑ TOS/TAS ratio	↑ IR	(165)
↑ **sNOX2-dp** ↑ **Urinary 8-iso-PGF2*α***	NAFLD	Human	Plasma	↑ CK-18	↑ NAFLD Fibrosis score ↑ MetS score	(49,50)
↑ **CYP2E1 (Protein/activity)**	MCD	Rat	Liver	↑ ALT	↑ Macrovesicular steatosis ↑Inflammatory infiltration	(232)
↑ **CYP2E1**	Obese NASH	Human	Liver biopsy after, Gastroplasty	↑ Drug chlorzoxazone	↑ Severity of steatosis	(58)
↑ **GSH** ↑ **MDA**	Male Sprague-Dawley NASH	Rat	Serum, Liver tissue	↓ N-acetylcysteine	↑ Macrovesicular steatosis, ↑ Hepatocyte	(180)

					ballooning, and ↑ Necroinflammation	
↑ **Hydrogen peroxide**	Choline-deficient diet-induced NAFLD	Rat	Liver tissue, Liver mitochondria	↓ Mitochondrial complex I activity ↓ Respiratory control ratio ↓ Cardiolipin	Mitochondrial dysfunction ↑ Steatosis	(162)

CK-18, cytokeratin-18 tertiles; GGT, γ-glutamyltranspeptidase; GSH, glutathione; HAS, human serum albumin; IR, insulin resistance; MDA, malondialdehyde; PCC, protein carbonyl; oxLDL, oxidized LDL; OSI, oxidative stress index; sNOX-dp, NOX2-derived peptide; TAS, total antioxidant status; TBARS, thiobarbituric acid-reacting substances; TOS, total oxidant status; 8OHDG, 8-hydroxy-2-deoxyguanosine; 8-iso-PGF2α, 8-iso-prostaglandin F2α

Table 2: Genetic variants influencing redox status and the stress response in NAFLD/NASH

Oxidative stress					
Gene	**Name**	**Cytogenetic location**	**Mutations**	**Susceptibility**	**REF**
SOD2	Superoxide dismutase 2, mitochondrial	6q25.3	C47T, rs4880	Advanced fibrosis Type 2 diabetes mellitus Histological severity of NASH Glutathione and catalase could impair hepatocytes function.	(4) (151)
			MnSOD T/T	NASH progression	(137)
UCP3	Uncoupling protein 3	11q13.4	−55C/T	Insulin resistance NASH progression.	(7)
			rs11235972 GG	Prevalence of NAFLD	(238)
GCLC	Glutamate-cysteine ligase, catalytic subunit	6p12.1	−129C/T	NASH progression	(152)
GST	Glutathione-S-transferase		GSTM1 GSTP1	NAFLD progression	(83)
UGT1A1	Uridine-5′-diphosphoglucuronosyltransferase 1A1		UGT1A1*6	Risk factor for pediatric NAFLD.	(110)

Hepatic iron accumulation

HFE	Haemochromatosis	6p22.2	C282Y	Increased steatosis grade in NAFLD	(142,218)
			C282Y	NAFLD progression and insulin deficiency NASH progression	(141,217)
			H63D	Increase total cholesterol Increase low-density lipoprotein	(167)
				Increased steatosis grade in NAFLD	(142,218)
			A736V	Hepatic iron accumulation in patients with NAFLD	(219)

Table 3: Preclinical and clinical intervention studies targeting NAFLD

Compound	Dose	Duration	Animals or humans	Species/number	Hepatic outcomes	Mechanisms	Ref
Vitamin E	150 IU/kg	10 W	HFD induced NAFLD	Rat 10/GR	↓ Steatosis score ↓ Grading	↓ Plasma cholesterol	(215)
Polyphenols Resveratrol	500 mg/d	12 W	Randomized double-blind placebo-controlled clinical trial	Human 25/GR	↓ Liver steatosis ↓ ALT ↓ Hepatocellular apoptosis	↓ Inflammation ↓ hs-CRP ↓ Serum TNFA ↓ Serum IL-6 ↓ Cytokeratin-18 ↓ NF-KB in mononuclear cells	(63)
Polyphenols Resveratrol	30 mg/kg/d	60 d	HFD-induced NAFLD	Mice 6/GR	↓ Liver weight ↓ ALT, AST	↓ ACC ↓ SREBP1c ↓ PPARγ ↓ Inflammation (hepatic TNFA	(9)

						mRNA, IL-6 mRNA, NF-KB) ↑ SIRT1	
Polyphenols Green tea extract	1-2% of diet	8 W	HFD-induced NASH	Rats 15-16/GR	↓ Hepatic steatosis ↓ ALT, AST	↓ Inflammation (mRNA and protein of TNFA, MCP-1) ↓ NF-KB ↑ GSH	(157)
Polyphenols Quercetin	150 mg/kg	2-4 W	MCD-deficient diet-induced NASH	Mice 16/GR	↓ Steatosis ↓ Ballooning	↓ Inflammation (liver TLR4, HMGB1, protein of TNFA, IL-6) ↓ TBARS ↓ α-SMA and collagen deposition ↓ SMAD7 ↓ NF-KB	(125)

Polyphenols Proanthocyanidin	100 mg/kg/BW/d	15 d	HFRD-induced NAFLD	Mice 6/GR	↓ Steatosis ↓ Inflammatory infiltrate	↓ SREBP1c mRNA ↓ HMG-COAR mRNA ↑ PPARα ↓ Perilipin ↓ Adipophilin ↓ TIP47 ↓ FSP27	(245)
Herbal medicine Tamarindus Indica Linn	45, 90, 180 mg/kg/BW	6 W	HFD-induced NAFLD	Rats 6/GR	↓ ALT ↓ Micro-and macrovesicular steatosis ↓ Ballooning ↓ Inflammatory infiltrate ↓ Hepatomegaly	↓ OxS	(183)
Herbal medicine Osthol	20 mg/kg 5 times/W	2 W	HFD-induced NAFLD	Rats 10/GR	↓ Steatosis	↓ OxS (HNE) ↓ FAS mRNA ↓ SCD1 mRNA	(136)

(cnidium monnieri extract)						↓ SREB1c mRNA ↓ PPARγ ↑ Carnitine octanoyltransferase	
Silibin	20 mg/kg/day	4 W	Db/db MCD	Mice 8/GR	↓ Serum TG, serum ALT, IR ↓ Liver ALT ↓ Liver TBARS. iNOS, ROS ↑ L-FABP mRNA	↓ NAFLD activity score, NASH ↓ NF-KB ↓ OxS	(178,179)
Silibin Realsil ®	200 mg/kd/day	5 W	HFD induced NASH	Rats 6/GR	↓ MDA ↓ IR	↓ Macrovesicular steatosis ↓ Inflammatory cell infiltration ↓ TNF-a ↓ Hepatic PPARa	(75)
Sylimarin	140 mg/day	4 W	Randomized double-blind placebo-	Human 22/GR	↓ Lipid profile,	↓ Steatosis	(76)

			controlled clinical trial (NAFLD)		↓ ALT, AST ↓ IR		
Silibin Realsil ® + Vitamin E	94 mgx4/day	26-52 W	Retrospective study (NAFLD)	Human 20-39/GR	↓ ALT, GGT ↓ IR ↓ TNF-a	↓ Liver damage ↓ Inflammation	(117)
PUFA	5% EPA-diet	40 W	Hepatocyte *Pten* deficient mice	Mice 16/GR	↓ Liver weight ↓ Hepatic TG, ALT ↓SREBP-1c	↓ Plasma ROS level ↓ Inflammatory cell infiltration ↓ Steatohepatitis	(88)
PUFA	98% EPA-diet	20 W	MCD diet-induced NASH	Rats 10/GR	↓ Liver weight ↓ AST, ALT ↓ Hepatic TGF-β1	↓ Hepatic ROS ↓ Serum oxidative markers	
PUFA	1.0 g/kg/ day of 46% DHA p.o.	6 W	choline-deficient HFD-induced NASH	Rats 3/GR	↓ Liver n-6/n-3 ratio ↓ Liver weight	↑ Antioxidants (SOD)	(204)

						↓ Liver fibrosis ↓ NASH progression	
PUFA	DHA (250 mg/day) DHA (500 mg/day)	24 W	Randomized double-blind placebo-controlled clinical trial (NAFLD)	Children 20/GR	↑ Insulin sensitivity index ↓ Serum TG	↓ Liver fat content. (by ultrasonography)	(145,146)
PUFA	520 mg n-3 PUFA (EPA 175 mg, DPA 235 mg, DHA 110 mg)	24 W	Randomized double-blind placebo-controlled clinical trial (NAFLD)	Human 72/GR	↓ ALT, AST, GGT	↓ Hepatic steatosis ↓ NAFLD symptom score,	(256)
PUFA	1 g fish oil × 2/day (orally)=(EPA +DHA: >85% of PUFAs, ratio: 0.9–1.5)	24 W	Randomized single-blind-controlled clinical trial (NAFLD)	Human 18/GR	↓ ALT, AST, GGT ↓ TNF-a ↓ IR ↑ HDL	↓ Hepatic steatosis ↓ Liver fat content. (by ultrasonography)	(196)

PUFA	0.83 g n-3 PUFAs (0.47 g EPA + 0.24 g DHA)	52 W	Randomized, placebo-controlled, dietary intervention trial.	Human 6/GR	↓ AST, ALT and GGT ↑ Serum adiponectin, HDL	↓ Liver fat content. (by ultrasonography)	(195)
Prebiotics Synbiotic capsule (Protexin) with 7 strains & fructooligosaccharides	2 capsules/day (containing 200 millions of strains)	4 W	Randomized double-blind placebo-controlled clinical trial (NAFLD)	Human 52/GR	↓ fibrosis score ↓ ALT, AST, GGT	↓ Inflammation ↓ hs-CRP ↓ Serum TNFA ↓ NF-KB in mononuclear cells	(61)
Probiotics VSL#3	1.5x109 colonies/mouse /d	4 W	Ob/ob	Mice/16/GR	↓ Fatty liver ↓ Inflammation (histology) ↓ Serum ALT ↓ SCD1 ↓ UCP2	↓ JNK ↓ NF-KB	(109)

Probiotics VSL#3	1.5x109 colonies/mouse /d	4 W	HFD induced steatosis	Mice/8/GR	↑ NKT ↓ IR ↓ Steatosis	↓ Hepatic inflammation ↓ Hepatic NF-KB	(119)
Probiotics Lactobacillus rhamnosus GG	5x107 Cfu/BW/d	8 W	HFRD-induced NAFLD	Mice/6/GR	↓ Steatosis ↓ Hepatocellular ballooning cells ↓ Inflammation (TNFA, IL-1B)	↓ ACC1 mRNA ↓ ChREB mRNA ↓ FAS ↑ Markers of intestine barrier ↓ Plasma LPS ↓ SREBP1c mRNA ↓ NF-KB	(172)
Probiotics Lactobacillus casei strain shirota	109 colony-forming units/mouse/d	6 W	MCD-induced NASH	Mice/12/GR	↓ Steatosis ↓ Inflammatory cell infiltration ↓ Hepatocelular ballooning cells ↓ Serum ALT	↓ FAS mRNA ↓ SREBP1c mRNA ↓ NF-KB ↓ Plasma LPS	(150)

		↓ Hepatic inflammation of stellate cells involved in collagen deposition and fibrosis

ACC1, acetyl-CoA carboxylase 1 ; ALT, alanine-aminotransferase; AST, aspartate aminotransferase ; BD, body weight; day; ChREBP, carbohydrate-responsive element-binding protein ; CRP, C-reactive protein ; HFD, high fat diet; FAS, fatty acid synthase ; FSP27, fat-specific protein 27; GGT, gamma-glutamyltransferase ; GR, group; HFRD, high fructose diet; HMGB1, high-mobility group box 1; IR, insulin resistance; JNK, Jun N-terminal kinase; LPS, Lipopolysaccharide ; MCD, methionine-choline-deficient; MCP-1, monocyte chemoattractant protein-1; NF-KB, nuclear factor kappaB; NKT, Natural Killer T cells; SREBP1c, sterol regulatory element binding protein 1c ; TGF-β1, transforming growth

REFERENCES

1. Aharoni-Simon M, Hann-Obercyger M, Pen S, Madar Z, Tirosh O. Fatty liver is associated with impaired activity of PPARgamma-coactivator 1alpha (PGC1alpha) and mitochondrial biogenesis in mice. *Lab Invest* 91: 1018-28, 2011.
2. Ahmed T, Gilani AU, Abdollahi M, Daglia M, Nabavi SF, Nabavi SM. Berberine and neurodegeneration: A review of literature. *Pharmacol Rep* 67: 970-9, 2015.
3. Akcam M, Boyaci A, Pirgon O, Kaya S, Uysal S, Dundar BN. Therapeutic effect of metformin and vitamin E versus prescriptive diet in obese adolescents with fatty liver. *Int J Vitam Nutr Res* 81: 398-406, 2011.
4. Al-Serri A, Anstee QM, Valenti L, Nobili V, Leathart JB, Dongiovanni P, Patch J, Fracanzani A, Fargion S, Day CP, Daly AK. The SOD2 C47T polymorphism influences NAFLD fibrosis severity: evidence from case-control and intra-familial allele association studies. *J Hepatol* 56: 448-54, 2012.
5. Albanes D, Till C, Klein EA, Goodman PJ, Mondul AM, Weinstein SJ, Taylor PR, Parnes HL, Gaziano JM, Song X, Fleshner NE, Brown PH, Meyskens FL, Jr., Thompson IM. Plasma tocopherols and risk of prostate cancer in the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *Cancer Prev Res (Phila)* 7: 886-95, 2014.
6. Alkhouri N, Carter-Kent C, Feldstein AE. Apoptosis in nonalcoholic fatty liver disease: diagnostic and therapeutic implications. *Expert Rev Gastroenterol Hepatol* 5: 201-12, 2011.
7. Aller R, De Luis DA, Izaola O, Gonzalez Sagrado M, Conde R, Alvarez T, Pacheco D, Velasco MC. Role of -55CT polymorphism of UCP3 gene on non alcoholic fatty liver disease and insulin resistance in patients with obesity. *Nutr Hosp* 25: 572-6, 2010.
8. Altinbas A, Sowa JP, Hasenberg T, Canbay A. The diagnosis and treatment of non-alcoholic fatty liver disease. *Minerva Gastroenterol Dietol* 61: 159-69, 2015.
9. Andrade JM, Paraiso AF, de Oliveira MV, Martins AM, Neto JF, Guimaraes AL, de Paula AM, Qureshi M, Santos SH. Resveratrol attenuates hepatic steatosis in high-fat fed mice by decreasing lipogenesis and inflammation. *Nutrition* 30: 915-9, 2014.
10. Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 346: 1221-31, 2002.
11. Aranda JF, Madrigal-Matute J, Rotllan N, Fernandez-Hernando C. MicroRNA modulation of lipid metabolism and oxidative stress in cardiometabolic diseases. *Free Radic Biol Med* 64: 31-9, 2013.

12. Ashraf NU, Sheikh TA. Endoplasmic reticulum stress and Oxidative stress in the pathogenesis of Non-alcoholic fatty liver disease. *Free Radic Res* 49: 1405-18, 2015.
13. Aubert J, Begriche K, Knockaert L, Robin MA, Fromenty B. Increased expression of cytochrome P450 2E1 in nonalcoholic fatty liver disease: mechanisms and pathophysiological role. *Clin Res Hepatol Gastroenterol* 35: 630-7, 2011.
14. Auger C, Teissedre PL, Gerain P, Lequeux N, Bornet A, Serisier S, Besancon P, Caporiccio B, Cristol JP, Rouanet JM. Dietary wine phenolics catechin, quercetin, and resveratrol efficiently protect hypercholesterolemic hamsters against aortic fatty streak accumulation. *J Agric Food Chem* 53: 2015-21, 2005.
15. Barchetta I, Carotti S, Labbadia G, Gentilucci UV, Muda AO, Angelico F, Silecchia G, Leonetti F, Fraioli A, Picardi A, Morini S, Cavallo MG. Liver vitamin D receptor, CYP2R1, and CYP27A1 expression: relationship with liver histology and vitamin D3 levels in patients with nonalcoholic steatohepatitis or hepatitis C virus. *Hepatology* 56: 2180-7, 2012.
16. Baur JA, Sinclair DA. Therapeutic potential of resveratrol: the in vivo evidence. *Nat Rev Drug Discov* 5: 493-506, 2006.
17. Beal MF, Matthews RT. Coenzyme Q10 in the central nervous system and its potential usefulness in the treatment of neurodegenerative diseases. *Mol Aspects Med* 18 Suppl: S169-79, 1997.
18. Bellentani S, Scaglioni F, Marino M, Bedogni G. Epidemiology of non-alcoholic fatty liver disease. *Dig Dis* 28: 155-61, 2010.
19. Berson A, De Beco V, Letteron P, Robin MA, Moreau C, El Kahwaji J, Verthier N, Feldmann G, Fromenty B, Pessayre D. Steatohepatitis-inducing drugs cause mitochondrial dysfunction and lipid peroxidation in rat hepatocytes. *Gastroenterology* 114: 764-74, 1998.
20. Bieghs V, van Gorp PJ, Walenbergh SM, Gijbels MJ, Verheyen F, Buurman WA, Briles DE, Hofker MH, Binder CJ, Shiri-Sverdlov R. Specific immunization strategies against oxidized low-density lipoprotein: a novel way to reduce nonalcoholic steatohepatitis in mice. *Hepatology* 56: 894-903, 2012.
21. Bieghs V, Wouters K, van Gorp PJ, Gijbels MJ, de Winther MP, Binder CJ, Lutjohann D, Febbraio M, Moore KJ, van Bilsen M, Hofker MH, Shiri-Sverdlov R. Role of scavenger receptor A and CD36 in diet-induced nonalcoholic steatohepatitis in hyperlipidemic mice. *Gastroenterology* 138: 2477-86, 2486 e1-3, 2010.
22. Bouzianas DG, Bouziana SD, Hatzitolios AI. Potential treatment of human nonalcoholic fatty liver disease with long-chain omega-3 polyunsaturated fatty acids. *Nutr Rev* 71: 753-71, 2013.

23. Bravo E, Palleschi S, Rossi B, Napolitano M, Tiano L, D'Amore E, Botham KM. Coenzyme Q metabolism is disturbed in high fat diet-induced non-alcoholic fatty liver disease in rats. *Int J Mol Sci* 13: 1644-57, 2012.

24. Bril F, Sninsky JJ, Baca AM, Robert Superko H, Portillo Sanchez P, Biernacki D, Maximos M, Lomonaco R, Orsak B, Suman A, Weber MH, McPhaul MJ, Cusi K. Hepatic Steatosis and Insulin Resistance, but not Steatohepatitis, Promote Atherogenic Dyslipidemia in NAFLD. *J Clin Endocrinol Metab*: jc20153111, 2015.

25. Browning JD, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, Grundy SM, Hobbs HH. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 40: 1387-95, 2004.

26. Brusq JM, Ancellin N, Grondin P, Guillard R, Martin S, Saintillan Y, Issandou M. Inhibition of lipid synthesis through activation of AMP kinase: an additional mechanism for the hypolipidemic effects of berberine. *J Lipid Res* 47: 1281-8, 2006.

27. Bujanda L, Hijona E, Larzabal M, Beraza M, Aldazabal P, Garcia-Urkia N, Sarasqueta C, Cosme A, Irastorza B, Gonzalez A, Arenas JI, Jr. Resveratrol inhibits nonalcoholic fatty liver disease in rats. *BMC Gastroenterol* 8: 40, 2008.

28. Butt MS, Sultan MT. Green tea: nature's defense against malignancies. *Crit Rev Food Sci Nutr* 49: 463-73, 2009.

29. Caldwell SH, Swerdlow RH, Khan EM, Iezzoni JC, Hespenheide EE, Parks JK, Parker WD, Jr. Mitochondrial abnormalities in non-alcoholic steatohepatitis. *J Hepatol* 31: 430-4, 1999.

30. Cano A, Ciaffoni F, Safwat GM, Aspichueta P, Ochoa B, Bravo E, Botham KM. Hepatic VLDL assembly is disturbed in a rat model of nonalcoholic fatty liver disease: is there a role for dietary coenzyme Q? *J Appl Physiol (1985)* 107: 707-17, 2009.

31. Cao SS, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease. *Antioxid Redox Signal* 21: 396-413, 2014.

32. Chalasani N, Deeg MA, Crabb DW. Systemic levels of lipid peroxidation and its metabolic and dietary correlates in patients with nonalcoholic steatohepatitis. *Am J Gastroenterol* 99: 1497-502, 2004.

33. Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, Charlton M, Sanyal AJ, American Gastroenterological A, American Association for the Study of Liver D, American College of G. The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Gastroenterological Association, American Association for the Study

of Liver Diseases, and American College of Gastroenterology. *Gastroenterology* 142: 1592-609, 2012.

34. Chang CJ, Tzeng TF, Liou SS, Chang YS, Liu IM. Kaempferol regulates the lipid-profile in high-fat diet-fed rats through an increase in hepatic PPARalpha levels. *Planta Med* 77: 1876-82, 2011.
35. Chen S, Zhao X, Ran L, Wan J, Wang X, Qin Y, Shu F, Gao Y, Yuan L, Zhang Q, Mi M. Resveratrol improves insulin resistance, glucose and lipid metabolism in patients with non-alcoholic fatty liver disease: a randomized controlled trial. *Dig Liver Dis* 47: 226-32, 2015.
36. Chitranjali T, Anoop Chandran P, Muraleedhara Kurup G. Omega-3 fatty acid concentrate from Dunaliella salina possesses anti-inflammatory properties including blockade of NF-kappaB nuclear translocation. *Immunopharmacol Immunotoxicol* 37: 81-9, 2015.
37. Chiu KC, Chu A, Go VL, Saad MF. Hypovitaminosis D is associated with insulin resistance and beta cell dysfunction. *Am J Clin Nutr* 79: 820-5, 2004.
38. Chowdhry S, Nazmy MH, Meakin PJ, Dinkova-Kostova AT, Walsh SV, Tsujita T, Dillon JF, Ashford ML, Hayes JD. Loss of Nrf2 markedly exacerbates nonalcoholic steatohepatitis. *Free Radic Biol Med* 48: 357-71, 2010.
39. Cicero AF, Rosticci M, Parini A, Morbini M, Urso R, Grandi E, Borghi C. Short-term effects of a combined nutraceutical of insulin-sensitivity, lipid level and indexes of liver steatosis: a double-blind, randomized, cross-over clinical trial. *Nutr J* 14: 30, 2015.
40. Corey KE, Vuppalanchi R, Vos M, Kohli R, Molleston JP, Wilson L, Unalp-Arida A, Cummings OW, Lavine JE, Chalasani N, Nonalcoholic Steatohepatitis Clinical Research N. Improvement in liver histology is associated with reduction in dyslipidemia in children with nonalcoholic fatty liver disease. *J Pediatr Gastroenterol Nutr* 60: 360-7, 2015.
41. Cortez-Pinto H, Chatham J, Chacko VP, Arnold C, Rashid A, Diehl AM. Alterations in liver ATP homeostasis in human nonalcoholic steatohepatitis: a pilot study. *JAMA* 282: 1659-64, 1999.
42. Cullinan SB, Diehl JA. PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. *J Biol Chem* 279: 20108-17, 2004.
43. Day CP. Non-alcoholic steatohepatitis (NASH): where are we now and where are we going? *Gut* 50: 585-8, 2002.
44. Day CP, James OF. Hepatic steatosis: innocent bystander or guilty party? *Hepatology* 27: 1463-6, 1998.
45. Day CP, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology* 114: 842-5, 1998.

46. de Wit NJ, Afman LA, Mensink M, Muller M. Phenotyping the effect of diet on non-alcoholic fatty liver disease. *J Hepatol* 57: 1370-3, 2012.

47. Deckelbaum RJ, Worgall TS, Seo T. n-3 fatty acids and gene expression. *Am J Clin Nutr* 83: 1520S-1525S, 2006.

48. Del Ben M, Baratta F, Polimeni L, Angelico F. Non-alcoholic fatty liver disease and cardiovascular disease: epidemiological, clinical and pathophysiological evidences. *Intern Emerg Med* 7 Suppl 3: S291-6, 2012.

49. Del Ben M, Polimeni L, Baratta F, Bartimoccia S, Carnevale R, Loffredo L, Pignatelli P, Violi F, Angelico F. Serum Cytokeratin-18 Is Associated with NOX2-Generated Oxidative Stress in Patients with Nonalcoholic Fatty Liver. *Int J Hepatol* 2014: 784985, 2014.

50. Del Ben M, Polimeni L, Carnevale R, Bartimoccia S, Nocella C, Baratta F, Loffredo L, Pignatelli P, Violi F, Angelico F. NOX2-generated oxidative stress is associated with severity of ultrasound liver steatosis in patients with non-alcoholic fatty liver disease. *BMC Gastroenterol* 14: 81, 2014.

51. Deltour L, Foglio MH, Duester G. Metabolic deficiencies in alcohol dehydrogenase Adh1, Adh3, and Adh4 null mutant mice. Overlapping roles of Adh1 and Adh4 in ethanol clearance and metabolism of retinol to retinoic acid. *J Biol Chem* 274: 16796-801, 1999.

52. Dentin R, Benhamed F, Hainault I, Fauveau V, Foufelle F, Dyck JR, Girard J, Postic C. Liver-specific inhibition of ChREBP improves hepatic steatosis and insulin resistance in ob/ob mice. *Diabetes* 55: 2159-70, 2006.

53. Derdak Z, Villegas KA, Harb R, Wu AM, Sousa A, Wands JR. Inhibition of p53 attenuates steatosis and liver injury in a mouse model of non-alcoholic fatty liver disease. *J Hepatol* 58: 785-91, 2013.

54. Di Minno MN, Russolillo A, Lupoli R, Ambrosino P, Di Minno A, Tarantino G. Omega-3 fatty acids for the treatment of non-alcoholic fatty liver disease. *World J Gastroenterol* 18: 5839-47, 2012.

55. Diehl AM. Lessons from animal models of NASH. *Hepatol Res* 33: 138-44, 2005.

56. Dong SF, Yasui N, Negishb H, Kishimoto A, Sun JN, Ikeda K. Increased Oxidative Stress in Cultured 3T3-L1 Cells was Attenuated by Berberine Treatment. *Nat Prod Commun* 10: 895-7, 2015.

57. Dowman JK, Armstrong MJ, Tomlinson JW, Newsome PN. Current therapeutic strategies in non-alcoholic fatty liver disease. *Diabetes Obes Metab* 13: 692-702, 2011.

58. Emery MG, Fisher JM, Chien JY, Kharasch ED, Dellinger EP, Kowdley KV, Thummel KE. CYP2E1 activity before and after weight loss in morbidly obese subjects with nonalcoholic fatty liver disease. *Hepatology* 38: 428-35, 2003.

59. Eraslan S, Kizilgul M, Uzunlulu M, Colak Y, Ozturk O, Tuncer I. Frequency of metabolic syndrome and 25-hydroxyvitamin D3 levels in patients with non-alcoholic fatty liver disease. *Minerva Med* 104: 447-53, 2013.

60. Erhardt A, Stahl W, Sies H, Lirussi F, Donner A, Haussinger D. Plasma levels of vitamin E and carotenoids are decreased in patients with Nonalcoholic Steatohepatitis (NASH). *Eur J Med Res* 16: 76-8, 2011.

61. Eslamparast T, Poustchi H, Zamani F, Sharafkhah M, Malekzadeh R, Hekmatdoost A. Synbiotic supplementation in nonalcoholic fatty liver disease: a randomized, double-blind, placebo-controlled pilot study. *Am J Clin Nutr* 99: 535-42, 2014.

62. Esposito E, Iacono A, Bianco G, Autore G, Cuzzocrea S, Vajro P, Canani RB, Calignano A, Raso GM, Meli R. Probiotics reduce the inflammatory response induced by a high-fat diet in the liver of young rats. *J Nutr* 139: 905-11, 2009.

63. Faghihzadeh F, Adibi P, Rafiei R, Hekmatdoost A. Resveratrol supplementation improves inflammatory biomarkers in patients with nonalcoholic fatty liver disease. *Nutr Res* 34: 837-43, 2014.

64. Farhangi MA, Alipour B, Jafarvand E, Khoshbaten M. Oral coenzyme Q10 supplementation in patients with nonalcoholic fatty liver disease: effects on serum vaspin, chemerin, pentraxin 3, insulin resistance and oxidative stress. *Arch Med Res* 45: 589-95, 2014.

65. Farsi F, Mohammadshahi M, Alavinejad P, Rezazadeh A, Zarei M, Engali KA. Functions of Coenzyme Q10 Supplementation on Liver Enzymes, Markers of Systemic Inflammation, and Adipokines in Patients Affected by Nonalcoholic Fatty Liver Disease: A Double-Blind, Placebo-Controlled, Randomized Clinical Trial. *J Am Coll Nutr*: 1-8, 2015.

66. Feillet-Coudray C, Aoun M, Fouret G, Bonafos B, Ramos J, Casas F, Cristol JP, Coudray C. Effects of long-term administration of saturated and n-3 fatty acid-rich diets on lipid utilisation and oxidative stress in rat liver and muscle tissues. *Br J Nutr* 110: 1789-802, 2013.

67. Feldstein AE, Lopez R, Tamimi TA, Yerian L, Chung YM, Berk M, Zhang R, McIntyre TM, Hazen SL. Mass spectrometric profiling of oxidized lipid products in human nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *J Lipid Res* 51: 3046-54, 2010.

68. Fujita N, Miyachi H, Tanaka H, Takeo M, Nakagawa N, Kobayashi Y, Iwasa M, Watanabe S, Takei Y. Iron overload is associated with hepatic oxidative damage to DNA in nonalcoholic steatohepatitis. *Cancer Epidemiol Biomarkers Prev* 18: 424-32, 2009.

69. Gambino R, Musso G, Cassader M. Redox balance in the pathogenesis of nonalcoholic fatty liver disease: mechanisms and therapeutic opportunities. *Antioxid Redox Signal* 15: 1325-65, 2011.

70. Gazak R, Walterova D, Kren V. Silybin and silymarin--new and emerging applications in medicine. *Curr Med Chem* 14: 315-38, 2007.

71. Gerber PA, Gouni-Berthold I, Berneis K. Omega-3 fatty acids: role in metabolism and cardiovascular disease. *Curr Pharm Des* 19: 3074-93, 2013.

72. Gnoni GV, Paglialonga G. Resveratrol inhibits fatty acid and triacylglycerol synthesis in rat hepatocytes. *Eur J Clin Invest* 39: 211-8, 2009.

73. Goto M, Kitamura H, Alam MM, Ota N, Haseba T, Akimoto T, Shimizu A, Takano-Yamamoto T, Yamamoto M, Motohashi H. Alcohol dehydrogenase 3 contributes to the protection of liver from nonalcoholic steatohepatitis. *Genes Cells* 20: 464-80, 2015.

74. Grattagliano I, Palmieri VO, Portincasa P, Moschetta A, Palasciano G. Oxidative stress-induced risk factors associated with the metabolic syndrome: a unifying hypothesis. *J Nutr Biochem* 19: 491-504, 2008.

75. Haddad Y, Vallerand D, Brault A, Haddad PS. Antioxidant and hepatoprotective effects of silibinin in a rat model of nonalcoholic steatohepatitis. *Evid Based Complement Alternat Med* 2011: nep164, 2011.

76. Hajiaghamohammadi AA, Ziaee A, Oveisi S, Masroor H. Effects of metformin, pioglitazone, and silymarin treatment on non-alcoholic Fatty liver disease: a randomized controlled pilot study. *Hepat Mon* 12: e6099, 2012.

77. Han YP, Kong M, Zheng S, Ren Y, Zhu L, Shi H, Duan Z. Vitamin D in liver diseases: from mechanisms to clinical trials. *J Gastroenterol Hepatol* 28 Suppl 1: 49-55, 2013.

78. Haque JA, McMahan RS, Campbell JS, Shimizu-Albergine M, Wilson AM, Botta D, Bammler TK, Beyer RP, Montine TJ, Yeh MM, Kavanagh TJ, Fausto N. Attenuated progression of diet-induced steatohepatitis in glutathione-deficient mice. *Lab Invest* 90: 1704-17, 2010.

79. Hardwick RN, Fisher CD, Canet MJ, Lake AD, Cherrington NJ. Diversity in antioxidant response enzymes in progressive stages of human nonalcoholic fatty liver disease. *Drug Metab Dispos* 38: 2293-301, 2010.

80. Hargreaves IP. Coenzyme Q10 as a therapy for mitochondrial disease. *Int J Biochem Cell Biol* 49: 105-11, 2014.

81. Harrison SA, Torgerson S, Hayashi P, Ward J, Schenker S. Vitamin E and vitamin C treatment improves fibrosis in patients with nonalcoholic steatohepatitis. *Am J Gastroenterol* 98: 2485-90, 2003.

82. Hasegawa T, Yoneda M, Nakamura K, Makino I, Terano A. Plasma transforming growth factor-beta1 level and efficacy of alpha-tocopherol in patients with non-alcoholic steatohepatitis: a pilot study. *Aliment Pharmacol Ther* 15: 1667-72, 2001.

83. Hashemi M, Eskandari-Nasab E, Fazaeli A, Bahari A, Hashemzehi NA, Shafieipour S, Taheri M, Moazeni-Roodi A, Zakeri Z, Bakhshipour A, Ghavami S. Association of genetic polymorphisms of glutathione-S-transferase genes (GSTT1, GSTM1, and GSTP1) and susceptibility to nonalcoholic fatty liver disease in Zahedan, Southeast Iran. *DNA Cell Biol* 31: 672-7, 2012.

84. Hashemian Z, Hashemi A, Fateminasab M. The Benefits of vitamin E on liver function and the hemopoietic System in thalassemia Patients. *Iran J Ped Hematol Oncol* 2: 153-8, 2012.

85. Ikura Y, Ohsawa M, Suekane T, Fukushima H, Itabe H, Jomura H, Nishiguchi S, Inoue T, Naruko T, Ehara S, Kawada N, Arakawa T, Ueda M. Localization of oxidized phosphatidylcholine in nonalcoholic fatty liver disease: impact on disease progression. *Hepatology* 43: 506-14, 2006.

86. Irie M, Sohda T, Iwata K, Kunimoto H, Fukunaga A, Kuno S, Yotsumoto K, Sakurai K, Iwashita H, Hirano G, Ueda SI, Yokoyama K, Morihara D, Nishizawa S, Anan A, Takeyama Y, Sakamoto M, Shakado S, Sakisaka S. Levels of the oxidative stress marker gamma-glutamyltranspeptidase at different stages of nonalcoholic fatty liver disease. *J Int Med Res* 40: 924-33, 2012.

87. Ishigami A, Kondo Y, Nanba R, Ohsawa T, Handa S, Kubo S, Akita M, Maruyama N. SMP30 deficiency in mice causes an accumulation of neutral lipids and phospholipids in the liver and shortens the life span. *Biochem Biophys Res Commun* 315: 575-80, 2004.

88. Ishii H, Horie Y, Ohshima S, Anezaki Y, Kinoshita N, Dohmen T, Kataoka E, Sato W, Goto T, Sasaki J, Sasaki T, Watanabe S, Suzuki A, Ohnishi H. Eicosapentaenoic acid ameliorates steatohepatitis and hepatocellular carcinoma in hepatocyte-specific Pten-deficient mice. *J Hepatol* 50: 562-71, 2009.

89. Jayaprakasam B, Olson LK, Schutzki RE, Tai MH, Nair MG. Amelioration of obesity and glucose intolerance in high-fat-fed C57BL/6 mice by anthocyanins and ursolic acid in Cornelian cherry (Cornus mas). *J Agric Food Chem* 54: 243-8, 2006.

90. Jou J, Choi SS, Diehl AM. Mechanisms of disease progression in nonalcoholic fatty liver disease. *Semin Liver Dis* 28: 370-9, 2008.

91. Kasparova S, Sumbalova Z, Bystricky P, Kucharska J, Liptaj T, Mlynarik V, Gvozdjakova A. Effect of coenzyme Q10 and vitamin E on brain energy metabolism in the animal model of Huntington's disease. *Neurochem Int* 48: 93-9, 2006.

92. Kim WS, Lee YS, Cha SH, Jeong HW, Choe SS, Lee MR, Oh GT, Park HS, Lee KU, Lane MD, Kim JB. Berberine improves lipid dysregulation in obesity by controlling central and peripheral AMPK activity. *Am J Physiol Endocrinol Metab* 296: E812-9, 2009.

93. Kitson MT, Roberts SK. D-livering the message: the importance of vitamin D status in chronic liver disease. *J Hepatol* 57: 897-909, 2012.

94. Kitteringham NR, Abdullah A, Walsh J, Randle L, Jenkins RE, Sison R, Goldring CE, Powell H, Sanderson C, Williams S, Higgins L, Yamamoto M, Hayes J, Park BK. Proteomic analysis of Nrf2 deficient transgenic mice reveals cellular defence and lipid metabolism as primary Nrf2-dependent pathways in the liver. *J Proteomics* 73: 1612-31, 2010.

95. Klebanoff SJ. Myeloperoxidase: friend and foe. *J Leukoc Biol* 77: 598-625, 2005.

96. Kohli R, Kirby M, Xanthakos SA, Softic S, Feldstein AE, Saxena V, Tang PH, Miles L, Miles MV, Balistreri WF, Woods SC, Seeley RJ. High-fructose, medium chain trans fat diet induces liver fibrosis and elevates plasma coenzyme Q9 in a novel murine model of obesity and nonalcoholic steatohepatitis. *Hepatology* 52: 934-44, 2010.

97. Kohli R, Pan X, Malladi P, Wainwright MS, Whitington PF. Mitochondrial reactive oxygen species signal hepatocyte steatosis by regulating the phosphatidylinositol 3-kinase cell survival pathway. *J Biol Chem* 282: 21327-36, 2007.

98. Kondo Y, Inai Y, Sato Y, Handa S, Kubo S, Shimokado K, Goto S, Nishikimi M, Maruyama N, Ishigami A. Senescence marker protein 30 functions as gluconolactonase in L-ascorbic acid biosynthesis, and its knockout mice are prone to scurvy. *Proc Natl Acad Sci U S A* 103: 5723-8, 2006.

99. Kondo Y, Ishigami A. Involvement of senescence marker protein-30 in glucose metabolism disorder and non-alcoholic fatty liver disease. *Geriatr Gerontol Int* 16 Suppl 1: 4-16, 2016.

100. Kong WJ, Zhang H, Song DQ, Xue R, Zhao W, Wei J, Wang YM, Shan N, Zhou ZX, Yang P, You XF, Li ZR, Si SY, Zhao LX, Pan HN, Jiang JD. Berberine reduces insulin resistance through protein kinase C-dependent up-regulation of insulin receptor expression. *Metabolism* 58: 109-19, 2009.

101. Kucukazman M, Ata N, Dal K, Yeniova AO, Kefeli A, Basyigit S, Aktas B, Akin KO, Agladioglu K, Ure OS, Topal F, Nazligul Y, Beyan E, Ertugrul DT. The association of vitamin D deficiency with non-alcoholic fatty liver disease. *Clinics (Sao Paulo)* 69: 542-6, 2014.

102. Kushnareva Y, Murphy AN, Andreyev A. Complex I-mediated reactive oxygen species generation: modulation by cytochrome c and NAD(P)+ oxidation-reduction state. *Biochem J* 368: 545-53, 2002.

103. Lavine JE. Vitamin E treatment of nonalcoholic steatohepatitis in children: a pilot study. *J Pediatr* 136: 734-8, 2000.

104. Le TH, Caldwell SH, Redick JA, Sheppard BL, Davis CA, Arseneau KO, Iezzoni JC, Hespenheide EE, Al-Osaimi A, Peterson TC. The zonal distribution of megamitochondria with crystalline inclusions in nonalcoholic steatohepatitis. *Hepatology* 39: 1423-9, 2004.

105. Lee JW, Choe SS, Jang H, Kim J, Jeong HW, Jo H, Jeong KH, Tadi S, Park MG, Kwak TH, Man Kim J, Hyun DH, Kim JB. AMPK activation with glabridin ameliorates adiposity and lipid dysregulation in obesity. *J Lipid Res* 53: 1277-86, 2012.

106. Lee YE, Kim JW, Lee EM, Ahn YB, Song KH, Yoon KH, Kim HW, Park CW, Li G, Liu Z, Ko SH. Chronic resveratrol treatment protects pancreatic islets against oxidative stress in db/db mice. *PLoS One* 7: e50412, 2012.

107. Leite NC, Salles GF, Araujo AL, Villela-Nogueira CA, Cardoso CR. Prevalence and associated factors of non-alcoholic fatty liver disease in patients with type-2 diabetes mellitus. *Liver Int* 29: 113-9, 2009.

108. Li AN, Li S, Zhang YJ, Xu XR, Chen YM, Li HB. Resources and biological activities of natural polyphenols. *Nutrients* 6: 6020-47, 2014.

109. Li Z, Yang S, Lin H, Huang J, Watkins PA, Moser AB, Desimone C, Song XY, Diehl AM. Probiotics and antibodies to TNF inhibit inflammatory activity and improve nonalcoholic fatty liver disease. *Hepatology* 37: 343-50, 2003.

110. Lin YC, Chang PF, Hu FC, Chang MH, Ni YH. Variants in the UGT1A1 gene and the risk of pediatric nonalcoholic fatty liver disease. *Pediatrics* 124: e1221-7, 2009.

111. Liu S, Shi W, Li G, Jin B, Chen Y, Hu H, Liu L, Xie F, Chen K, Yin D. Plasma reactive carbonyl species levels and risk of non-alcoholic fatty liver disease. *J Gastroenterol Hepatol* 26: 1010-5, 2011.

112. Liu Y, Song X, Han Y, Zhou F, Zhang D, Ji B, Hu J, Lv Y, Cai S, Wei Y, Gao F, Jia X. Identification of anthocyanin components of wild Chinese blueberries and amelioration of light-

induced retinal damage in pigmented rabbit using whole berries. *J Agric Food Chem* 59: 356-63, 2011.

113. Liu Y, Wang D, Zhang D, Lv Y, Wei Y, Wu W, Zhou F, Tang M, Mao T, Li M, Ji B. Inhibitory effect of blueberry polyphenolic compounds on oleic acid-induced hepatic steatosis in vitro. *J Agric Food Chem* 59: 12254-63, 2011.

114. Liu Z, Jiang C, Zhang J, Liu B, Du Q. Resveratrol inhibits inflammation and ameliorates insulin resistant endothelial dysfunction via regulation of AMP-activated protein kinase and sirtuin 1 activities. *J Diabetes* 8: 324-335, 2016.

115. Loguercio C, Andreone P, Brisc C, Brisc MC, Bugianesi E, Chiaramonte M, Cursaro C, Danila M, de Sio I, Floreani A, Freni MA, Grieco A, Groppo M, Lazzari R, Lobello S, Lorefice E, Margotti M, Miele L, Milani S, Okolicsanyi L, Palasciano G, Portincasa P, Saltarelli P, Smedile A, Somalvico F, Spadaro A, Sporea I, Sorrentino P, Vecchione R, Tuccillo C, Del Vecchio Blanco C, Federico A. Silybin combined with phosphatidylcholine and vitamin E in patients with nonalcoholic fatty liver disease: a randomized controlled trial. *Free Radic Biol Med* 52: 1658-65, 2012.

116. Loguercio C, De Simone T, D'Auria MV, de Sio I, Federico A, Tuccillo C, Abbatecola AM, Del Vecchio Blanco C, Italian ACG. Non-alcoholic fatty liver disease: a multicentre clinical study by the Italian Association for the Study of the Liver. *Dig Liver Dis* 36: 398-405, 2004.

117. Loguercio C, Federico A, Trappoliere M, Tuccillo C, de Sio I, Di Leva A, Niosi M, D'Auria MV, Capasso R, Del Vecchio Blanco C, Real Sud G. The effect of a silybin-vitamin e-phospholipid complex on nonalcoholic fatty liver disease: a pilot study. *Dig Dis Sci* 52: 2387-95, 2007.

118. Loguercio C, Federico A, Tuccillo C, Terracciano F, D'Auria MV, De Simone C, Del Vecchio Blanco C. Beneficial effects of a probiotic VSL#3 on parameters of liver dysfunction in chronic liver diseases. *J Clin Gastroenterol* 39: 540-3, 2005.

119. Ma X, Hua J, Li Z. Probiotics improve high fat diet-induced hepatic steatosis and insulin resistance by increasing hepatic NKT cells. *J Hepatol* 49: 821-30, 2008.

120. Malaguarnera L, Madeddu R, Palio E, Arena N, Malaguarnera M. Heme oxygenase-1 levels and oxidative stress-related parameters in non-alcoholic fatty liver disease patients. *J Hepatol* 42: 585-91, 2005.

121. Malhotra JD, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? *Antioxid Redox Signal* 9: 2277-93, 2007.

122. Manco M, Calvani M, Nanni G, Greco AV, Iaconelli A, Gasbarrini G, Castagneto M, Mingrone G. Low 25-hydroxyvitamin D does not affect insulin sensitivity in obesity after bariatric surgery. *Obes Res* 13: 1692-700, 2005.

123. Mangge H, Becker K, Fuchs D, Gostner JM. Antioxidants, inflammation and cardiovascular disease. *World J Cardiol* 6: 462-77, 2014.

124. Mann JP, De Vito R, Mosca A, Alisi A, Armstrong MJ, Raponi M, Baumann U, Nobili V. Portal inflammation is independelty associated with fibrosis and metabolic syndrome in paediatric non-alcoholic fatty liver disease. *Hepatology*, 2015.

125. Marcolin E, San-Miguel B, Vallejo D, Tieppo J, Marroni N, Gonzalez-Gallego J, Tunon MJ. Quercetin treatment ameliorates inflammation and fibrosis in mice with nonalcoholic steatohepatitis. *J Nutr* 142: 1821-8, 2012.

126. Masterton GS, Plevris JN, Hayes PC. Review article: omega-3 fatty acids - a promising novel therapy for non-alcoholic fatty liver disease. *Aliment Pharmacol Ther* 31: 679-92, 2010.

127. Meakin PJ, Chowdhry S, Sharma RS, Ashford FB, Walsh SV, McCrimmon RJ, Dinkova-Kostova AT, Dillon JF, Hayes JD, Ashford ML. Susceptibility of Nrf2-null mice to steatohepatitis and cirrhosis upon consumption of a high-fat diet is associated with oxidative stress, perturbation of the unfolded protein response, and disturbance in the expression of metabolic enzymes but not with insulin resistance. *Mol Cell Biol* 34: 3305-20, 2014.

128. Medjakovic S, Mueller M, Jungbauer A. Potential health-modulating effects of isoflavones and metabolites via activation of PPAR and AhR. *Nutrients* 2: 241-79, 2010.

129. Miller ER, 3rd, Pastor-Barriuso R, Dalal D, Riemersma RA, Appel LJ, Guallar E. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. *Ann Intern Med* 142: 37-46, 2005.

130. Mohr D, Bowry VW, Stocker R. Dietary supplementation with coenzyme Q10 results in increased levels of ubiquinol-10 within circulating lipoproteins and increased resistance of human low-density lipoprotein to the initiation of lipid peroxidation. *Biochim Biophys Acta* 1126: 247-54, 1992.

131. Molendi-Coste O, Legry V, Leclercq IA. Why and How Meet n-3 PUFA Dietary Recommendations? *Gastroenterol Res Pract* 2011: 364040, 2011.

132. Musso G, Gambino R, De Michieli F, Durazzo M, Pagano G, Cassader M. Adiponectin gene polymorphisms modulate acute adiponectin response to dietary fat: Possible pathogenetic role in NASH. *Hepatology* 47: 1167-77, 2008.

133. Nadal-Casellas A, Amengual-Cladera E, Proenza AM, Llado I, Gianotti M. Long-term high-fat-diet feeding impairs mitochondrial biogenesis in liver of male and female rats. *Cell Physiol Biochem* 26: 291-302, 2010.

134. Nagasawa T, Inada Y, Nakano S, Tamura T, Takahashi T, Maruyama K, Yamazaki Y, Kuroda J, Shibata N. Effects of bezafibrate, PPAR pan-agonist, and GW501516, PPARdelta agonist, on development of steatohepatitis in mice fed a methionine- and choline-deficient diet. *Eur J Pharmacol* 536: 182-91, 2006.

135. Nakano T, Cheng YF, Lai CY, Hsu LW, Chang YC, Deng JY, Huang YZ, Honda H, Chen KD, Wang CC, Chiu KW, Jawan B, Eng HL, Goto S, Chen CL. Impact of artificial sunlight therapy on the progress of non-alcoholic fatty liver disease in rats. *J Hepatol* 55: 415-25, 2011.

136. Nam HH, Jun DW, Jeon HJ, Lee JS, Saeed WK, Kim EK. Osthol attenuates hepatic steatosis via decreased triglyceride synthesis not by insulin resistance. *World J Gastroenterol* 20: 11753-61, 2014.

137. Namikawa C, Shu-Ping Z, Vyselaar JR, Nozaki Y, Nemoto Y, Ono M, Akisawa N, Saibara T, Hiroi M, Enzan H, Onishi S. Polymorphisms of microsomal triglyceride transfer protein gene and manganese superoxide dismutase gene in non-alcoholic steatohepatitis. *J Hepatol* 40: 781-6, 2004.

138. Nan YM, Wu WJ, Fu N, Liang BL, Wang RQ, Li LX, Zhao SX, Zhao JM, Yu J. Antioxidants vitamin E and 1-aminobenzotriazole prevent experimental non-alcoholic steatohepatitis in mice. *Scand J Gastroenterol* 44: 1121-31, 2009.

139. Narasimhan S, Gokulakrishnan K, Sampathkumar R, Farooq S, Ravikumar R, Mohan V, Balasubramanyam M. Oxidative stress is independently associated with non-alcoholic fatty liver disease (NAFLD) in subjects with and without type 2 diabetes. *Clin Biochem* 43: 815-21, 2010.

140. Nassir F, Ibdah JA. Role of mitochondria in nonalcoholic fatty liver disease. *Int J Mol Sci* 15: 8713-42, 2014.

141. Nelson JE, Bhattacharya R, Lindor KD, Chalasani N, Raaka S, Heathcote EJ, Miskovsky E, Shaffer E, Rulyak SJ, Kowdley KV. HFE C282Y mutations are associated with advanced hepatic fibrosis in Caucasians with nonalcoholic steatohepatitis. *Hepatology* 46: 723-9, 2007.

142. Nelson JE, Brunt EM, Kowdley KV, Nonalcoholic Steatohepatitis Clinical Research N. Lower serum hepcidin and greater parenchymal iron in nonalcoholic fatty liver disease patients with C282Y HFE mutations. *Hepatology* 56: 1730-40, 2012.

143. Niki E. Role of vitamin E as a lipid-soluble peroxyl radical scavenger: in vitro and in vivo evidence. *Free Radic Biol Med* 66: 3-12, 2014.

144. Niu X, Zhang H, Li W, Wang Y, Mu Q, Wang X, He Z, Yao H. Protective effect of cavidine on acetic acid-induced murine colitis via regulating antioxidant, cytokine profile and NF-kappaB signal transduction pathways. *Chem Biol Interact* 239: 34-45, 2015.

145. Nobili V, Alisi A, Della Corte C, Rise P, Galli C, Agostoni C, Bedogni G. Docosahexaenoic acid for the treatment of fatty liver: randomised controlled trial in children. *Nutr Metab Cardiovasc Dis* 23: 1066-70, 2013.

146. Nobili V, Bedogni G, Alisi A, Pietrobattista A, Rise P, Galli C, Agostoni C. Docosahexaenoic acid supplementation decreases liver fat content in children with non-alcoholic fatty liver disease: double-blind randomised controlled clinical trial. *Arch Dis Child* 96: 350-3, 2011.

147. Nobili V, Giorgio V, Liccardo D, Bedogni G, Morino G, Alisi A, Cianfarani S. Vitamin D levels and liver histological alterations in children with nonalcoholic fatty liver disease. *Eur J Endocrinol* 170: 547-53, 2014.

148. Nobili V, Parola M, Alisi A, Marra F, Piemonte F, Mombello C, Sutti S, Povero D, Maina V, Novo E, Albano E. Oxidative stress parameters in paediatric non-alcoholic fatty liver disease. *Int J Mol Med* 26: 471-6, 2010.

149. Novo E, Busletta C, Bonzo LV, Povero D, Paternostro C, Mareschi K, Ferrero I, David E, Bertolani C, Caligiuri A, Cannito S, Tamagno E, Compagnone A, Colombatto S, Marra F, Fagioli F, Pinzani M, Parola M. Intracellular reactive oxygen species are required for directional migration of resident and bone marrow-derived hepatic pro-fibrogenic cells. *J Hepatol* 54: 964-74, 2011.

150. Okubo H, Sakoda H, Kushiyama A, Fujishiro M, Nakatsu Y, Fukushima T, Matsunaga Y, Kamata H, Asahara T, Yoshida Y, Chonan O, Iwashita M, Nishimura F, Asano T. Lactobacillus casei strain Shirota protects against nonalcoholic steatohepatitis development in a rodent model. *Am J Physiol Gastrointest Liver Physiol* 305: G911-8, 2013.

151. Oliveira CP, Stefano JT. Genetic polymorphisms and oxidative stress in non-alcoholic steatohepatitis (NASH): A mini review. *Clin Res Hepatol Gastroenterol* 39 Suppl 1: S35-40, 2015.

152. Oliveira CP, Stefano JT, Cavaleiro AM, Zanella Fortes MA, Vieira SM, Rodrigues Lima VM, Santos TE, Santos VN, de Azevedo Salgado AL, Parise ER, Ferreira Alves VA, Carrilho FJ, Correa-Giannella ML. Association of polymorphisms of glutamate-cystein ligase and microsomal triglyceride transfer protein genes in non-alcoholic fatty liver disease. *J Gastroenterol Hepatol* 25: 357-61, 2010.

153. Orellana M, Rodrigo R, Varela N, Araya J, Poniachik J, Csendes A, Smok G, Videla LA. Relationship between in vivo chlorzoxazone hydroxylation, hepatic cytochrome P450 2E1 content and liver injury in obese non-alcoholic fatty liver disease patients. *Hepatol Res* 34: 57-63, 2006.

154. Pagliaro B, Santolamazza C, Simonelli F, Rubattu S. Phytochemical Compounds and Protection from Cardiovascular Diseases: A State of the Art. *Biomed Res Int* 2015: 918069, 2015.

155. Panchal SK, Brown L. Cardioprotective and hepatoprotective effects of ellagitannins from European oak bark (Quercus petraea L.) extract in rats. *Eur J Nutr* 52: 397-408, 2013.

156. Paredes-Lopez O, Cervantes-Ceja ML, Vigna-Perez M, Hernandez-Perez T. Berries: improving human health and healthy aging, and promoting quality life--a review. *Plant Foods Hum Nutr* 65: 299-308, 2010.

157. Park HJ, Lee JY, Chung MY, Park YK, Bower AM, Koo SI, Giardina C, Bruno RS. Green tea extract suppresses NFkappaB activation and inflammatory responses in diet-induced obese rats with nonalcoholic steatohepatitis. *J Nutr* 142: 57-63, 2012.

158. Pektas MB, Koca HB, Sadi G, Akar F. Dietary Fructose Activates Insulin Signaling and Inflammation in Adipose Tissue: Modulatory Role of Resveratrol. *Biomed Res Int* 2016: 8014252, 2016.

159. Peng W, Ma YY, Zhang K, Zhou AY, Zhang Y, Wang H, Du Z, Zhao DG. Synthesis and Biological Evaluation of Novel Resveratrol-NSAID Derivatives as Anti-inflammatory Agents. *Chem Pharm Bull (Tokyo)* 64: 609-15, 2016.

160. Perez-Carreras M, Del Hoyo P, Martin MA, Rubio JC, Martin A, Castellano G, Colina F, Arenas J, Solis-Herruzo JA. Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. *Hepatology* 38: 999-1007, 2003.

161. Pessayre D. Role of mitochondria in non-alcoholic fatty liver disease. *J Gastroenterol Hepatol* 22 Suppl 1: S20-7, 2007.

162. Petrosillo G, Portincasa P, Grattagliano I, Casanova G, Matera M, Ruggiero FM, Ferri D, Paradies G. Mitochondrial dysfunction in rat with nonalcoholic fatty liver Involvement of complex I, reactive oxygen species and cardiolipin. *Biochim Biophys Acta* 1767: 1260-7, 2007.

163. Petta S, Grimaudo S, Tripodo C, Cabibi D, Calvaruso M, Di Cristina A, Guarnotta C, Macaluso FS, Minissale MG, Marchesini G, Craxi A. The hepatic expression of vitamin D receptor is inversely associated with the severity of liver damage in genotype 1 chronic hepatitis C patients. *J Clin Endocrinol Metab* 100: 193-200, 2015.

164. Phung N, Pera N, Farrell G, Leclercq I, Hou JY, George J. Pro-oxidant-mediated hepatic fibrosis and effects of antioxidant intervention in murine dietary steatohepatitis. *Int J Mol Med* 24: 171-80, 2009.

165. Pirgon O, Bilgin H, Cekmez F, Kurku H, Dundar BN. Association between insulin resistance and oxidative stress parameters in obese adolescents with non-alcoholic fatty liver disease. *J Clin Res Pediatr Endocrinol* 5: 33-9, 2013.

166. Polyak SJ, Oberlies NH, Pecheur EI, Dahari H, Ferenci P, Pawlotsky JM. Silymarin for HCV infection. *Antivir Ther* 18: 141-7, 2013.

167. Raszeja-Wyszomirska J, Kurzawski G, Lawniczak M, Miezynska-Kurtycz J, Lubinski J. Nonalcoholic fatty liver disease and HFE gene mutations: a Polish study. *World J Gastroenterol* 16: 2531-6, 2010.

168. Reddy NM, Potteti HR, Mariani TJ, Biswal S, Reddy SP. Conditional deletion of Nrf2 in airway epithelium exacerbates acute lung injury and impairs the resolution of inflammation. *Am J Respir Cell Mol Biol* 45: 1161-8, 2011.

169. Rensen SS, Bieghs V, Xanthoulea S, Arfianti E, Bakker JA, Shiri-Sverdlov R, Hofker MH, Greve JW, Buurman WA. Neutrophil-derived myeloperoxidase aggravates non-alcoholic steatohepatitis in low-density lipoprotein receptor-deficient mice. *PLoS One* 7: e52411, 2012.

170. Rensen SS, Slaats Y, Nijhuis J, Jans A, Bieghs V, Driessen A, Malle E, Greve JW, Buurman WA. Increased hepatic myeloperoxidase activity in obese subjects with nonalcoholic steatohepatitis. *Am J Pathol* 175: 1473-82, 2009.

171. Ricci C, Pastukh V, Leonard J, Turrens J, Wilson G, Schaffer D, Schaffer SW. Mitochondrial DNA damage triggers mitochondrial-superoxide generation and apoptosis. *Am J Physiol Cell Physiol* 294: C413-22, 2008.

172. Ritze Y, Bardos G, Claus A, Ehrmann V, Bergheim I, Schwiertz A, Bischoff SC. Lactobacillus rhamnosus GG protects against non-alcoholic fatty liver disease in mice. *PLoS One* 9: e80169, 2014.

173. Rodriguez-Ramiro I, Vauzour D, Minihane AM. Polyphenols and non-alcoholic fatty liver disease: impact and mechanisms. *Proc Nutr Soc*: 1-14, 2015.

174. Rolo AP, Teodoro JS, Palmeira CM. Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. *Free Radic Biol Med* 52: 59-69, 2012.

175. Rosado IV, Langevin F, Crossan GP, Takata M, Patel KJ. Formaldehyde catabolism is essential in cells deficient for the Fanconi anemia DNA-repair pathway. *Nat Struct Mol Biol* 18: 1432-4, 2011.

176. Roth CL, Elfers CT, Figlewicz DP, Melhorn SJ, Morton GJ, Hoofnagle A, Yeh MM, Nelson JE, Kowdley KV. Vitamin D deficiency in obese rats exacerbates nonalcoholic fatty liver disease and increases hepatic resistin and Toll-like receptor activation. *Hepatology* 55: 1103-11, 2012.

177. Sakiyama H, Fujiwara N, Yoneoka Y, Yoshihara D, Eguchi H, Suzuki K. Cu,Zn-SOD deficiency induces the accumulation of hepatic collagen. *Free Radic Res*: 1-35, 2016.

178. Salamone F, Galvano F, Cappello F, Mangiameli A, Barbagallo I, Li Volti G. Silibinin modulates lipid homeostasis and inhibits nuclear factor kappa B activation in experimental nonalcoholic steatohepatitis. *Transl Res* 159: 477-86, 2012.

179. Salamone F, Galvano F, Marino Gammazza A, Paternostro C, Tibullo D, Bucchieri F, Mangiameli A, Parola M, Bugianesi E, Li Volti G. Silibinin improves hepatic and myocardial injury in mice with nonalcoholic steatohepatitis. *Dig Liver Dis* 44: 334-42, 2012.

180. Samuhasaneeto S, Thong-Ngam D, Kulaputana O, Patumraj S, Klaikeaw N. Effects of N-acetylcysteine on oxidative stress in rats with non-alcoholic steatohepatitis. *J Med Assoc Thai* 90: 788-97, 2007.

181. Santos CX, Tanaka LY, Wosniak J, Laurindo FR. Mechanisms and implications of reactive oxygen species generation during the unfolded protein response: roles of endoplasmic reticulum oxidoreductases, mitochondrial electron transport, and NADPH oxidase. *Antioxid Redox Signal* 11: 2409-27, 2009.

182. Sanyal AJ, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, Neuschwander-Tetri BA, Lavine JE, Tonascia J, Unalp A, Van Natta M, Clark J, Brunt EM, Kleiner DE, Hoofnagle JH, Robuck PR, Nash CRN. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med* 362: 1675-85, 2010.

183. Sasidharan SR, Joseph JA, Anandakumar S, Venkatesan V, Madhavan CN, Agarwal A. Ameliorative potential of Tamarindus indica on high fat diet induced nonalcoholic fatty liver disease in rats. *ScientificWorldJournal* 2014: 507197, 2014.

184. Satapathy SK, Sanyal AJ. Novel treatment modalities for nonalcoholic steatohepatitis. *Trends Endocrinol Metab* 21: 668-75, 2010.

185. Satapati S, Kucejova B, Duarte JA, Fletcher JA, Reynolds L, Sunny NE, He T, Nair LA, Livingston K, Fu X, Merritt ME, Sherry AD, Malloy CR, Shelton JM, Lambert J, Parks EJ, Corbin I, Magnuson MA, Browning JD, Burgess SC. Mitochondrial metabolism mediates oxidative stress and inflammation in fatty liver. *J Clin Invest* 125: 4447-62, 2015.

186. Seki S, Kitada T, Yamada T, Sakaguchi H, Nakatani K, Wakasa K. In situ detection of lipid peroxidation and oxidative DNA damage in non-alcoholic fatty liver diseases. *J Hepatol* 37: 56-62, 2002.

187. Sentman ML, Granstrom M, Jakobson H, Reaume A, Basu S, Marklund SL. Phenotypes of mice lacking extracellular superoxide dismutase and copper- and zinc-containing superoxide dismutase. *J Biol Chem* 281: 6904-9, 2006.

188. Serviddio G, Bellanti F, Vendemiale G. Free radical biology for medicine: learning from nonalcoholic fatty liver disease. *Free Radic Biol Med* 65: 952-68, 2013.

189. Serviddio G, Bellanti F, Vendemiale G, Altomare E. Mitochondrial dysfunction in nonalcoholic steatohepatitis. *Expert Rev Gastroenterol Hepatol* 5: 233-44, 2011.

190. Seymour EM, Singer AA, Kirakosyan A, Urcuyo-Llanes DE, Kaufman PB, Bolling SF. Altered hyperlipidemia, hepatic steatosis, and hepatic peroxisome proliferator-activated receptors in rats with intake of tart cherry. *J Med Food* 11: 252-9, 2008.

191. Shang J, Chen LL, Xiao FX, Sun H, Ding HC, Xiao H. Resveratrol improves non-alcoholic fatty liver disease by activating AMP-activated protein kinase. *Acta Pharmacol Sin* 29: 698-706, 2008.

192. Sharifi N, Amani R, Hajiani E, Cheraghian B. Does vitamin D improve liver enzymes, oxidative stress, and inflammatory biomarkers in adults with non-alcoholic fatty liver disease? A randomized clinical trial. *Endocrine* 47: 70-80, 2014.

193. Singal AK, Jampana SC, Weinman SA. Antioxidants as therapeutic agents for liver disease. *Liver Int* 31: 1432-48, 2011.

194. Soden JS, Devereaux MW, Haas JE, Gumpricht E, Dahl R, Gralla J, Traber MG, Sokol RJ. Subcutaneous vitamin E ameliorates liver injury in an in vivo model of steatocholestasis. *Hepatology* 46: 485-95, 2007.

195. Sofi F, Giangrandi I, Cesari F, Corsani I, Abbate R, Gensini GF, Casini A. Effects of a 1-year dietary intervention with n-3 polyunsaturated fatty acid-enriched olive oil on non-alcoholic fatty liver disease patients: a preliminary study. *Int J Food Sci Nutr* 61: 792-802, 2010.

196. Spadaro L, Magliocco O, Spampinato D, Piro S, Oliveri C, Alagona C, Papa G, Rabuazzo AM, Purrello F. Effects of n-3 polyunsaturated fatty acids in subjects with nonalcoholic fatty liver disease. *Dig Liver Dis* 40: 194-9, 2008.

197. St-Pierre J, Drori S, Uldry M, Silvaggi JM, Rhee J, Jager S, Handschin C, Zheng K, Lin J, Yang W, Simon DK, Bachoo R, Spiegelman BM. Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell* 127: 397-408, 2006.

198. Starzynski RR, Lipinski P, Drapier JC, Diet A, Smuda E, Bartlomiejczyk T, Gralak MA, Kruszewski M. Down-regulation of iron regulatory protein 1 activities and expression in superoxide dismutase 1 knock-out mice is not associated with alterations in iron metabolism. *J Biol Chem* 280: 4207-12, 2005.

199. Strange RC, Shipman KE, Ramachandran S. Metabolic syndrome: A review of the role of vitamin D in mediating susceptibility and outcome. *World J Diabetes* 6: 896-911, 2015.

200. Sugimoto H, Okada K, Shoda J, Warabi E, Ishige K, Ueda T, Taguchi K, Yanagawa T, Nakahara A, Hyodo I, Ishii T, Yamamoto M. Deletion of nuclear factor-E2-related factor-2 leads to rapid onset and progression of nutritional steatohepatitis in mice. *Am J Physiol Gastrointest Liver Physiol* 298: G283-94, 2010.

201. Sumida Y, Niki E, Naito Y, Yoshikawa T. Involvement of free radicals and oxidative stress in NAFLD/NASH. *Free Radic Res* 47: 869-80, 2013.

202. Syn WK, Choi SS, Diehl AM. Apoptosis and cytokines in non-alcoholic steatohepatitis. *Clin Liver Dis* 13: 565-80, 2009.

203. Tailleux A, Wouters K, Staels B. Roles of PPARs in NAFLD: potential therapeutic targets. *Biochim Biophys Acta* 1821: 809-18, 2012.

204. Takayama F, Nakamoto K, Totani N, Yamanushi T, Kabuto H, Kaneyuki T, Mankura M. Effects of docosahexaenoic acid in an experimental rat model of nonalcoholic steatohepatitis. *J Oleo Sci* 59: 407-14, 2010.

205. Takeuchi Y, Yahagi N, Izumida Y, Nishi M, Kubota M, Teraoka Y, Yamamoto T, Matsuzaka T, Nakagawa Y, Sekiya M, Iizuka Y, Ohashi K, Osuga J, Gotoda T, Ishibashi S, Itaka K, Kataoka K, Nagai R, Yamada N, Kadowaki T, Shimano H. Polyunsaturated fatty acids selectively suppress sterol regulatory element-binding protein-1 through proteolytic processing and autoloop regulatory circuit. *J Biol Chem* 285: 11681-91, 2010.

206. Tanaka N, Sano K, Horiuchi A, Tanaka E, Kiyosawa K, Aoyama T. Highly purified eicosapentaenoic acid treatment improves nonalcoholic steatohepatitis. *J Clin Gastroenterol* 42: 413-8, 2008.

207. Targher G. Non-alcoholic fatty liver disease, the metabolic syndrome and the risk of cardiovascular disease: the plot thickens. *Diabet Med* 24: 1-6, 2007.

208. Targher G, Bertolini L, Scala L, Cigolini M, Zenari L, Falezza G, Arcaro G. Associations between serum 25-hydroxyvitamin D3 concentrations and liver histology in patients with non-alcoholic fatty liver disease. *Nutr Metab Cardiovasc Dis* 17: 517-24, 2007.

209. Tariq Z, Green CJ, Hodson L. Are oxidative stress mechanisms the common denominator in the progression from hepatic steatosis towards non-alcoholic steatohepatitis (NASH)? *Liver Int* 34: e180-90, 2014.

210. Teodoro JS, Duarte FV, Gomes AP, Varela AT, Peixoto FM, Rolo AP, Palmeira CM. Berberine reverts hepatic mitochondrial dysfunction in high-fat fed rats: a possible role for SirT3 activation. *Mitochondrion* 13: 637-46, 2013.

211. Tessari P, Coracina A, Cosma A, Tiengo A. Hepatic lipid metabolism and non-alcoholic fatty liver disease. *Nutr Metab Cardiovasc Dis* 19: 291-302, 2009.

212. Timmers S, Konings E, Bilet L, Houtkooper RH, van de Weijer T, Goossens GH, Hoeks J, van der Krieken S, Ryu D, Kersten S, Moonen-Kornips E, Hesselink MK, Kunz I, Schrauwen-Hinderling VB, Blaak EE, Auwerx J, Schrauwen P. Calorie restriction-like effects of 30 days of resveratrol supplementation on energy metabolism and metabolic profile in obese humans. *Cell Metab* 14: 612-22, 2011.

213. Tsuruta Y, Nagao K, Kai S, Tsuge K, Yoshimura T, Koganemaru K, Yanagita T. Polyphenolic extract of lotus root (edible rhizome of Nelumbo nucifera) alleviates hepatic steatosis in obese diabetic db/db mice. *Lipids Health Dis* 10: 202, 2011.

214. Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol* 552: 335-44, 2003.

215. Tzanetakou IP, Doulamis IP, Korou LM, Agrogiannis G, Vlachos IS, Pantopoulou A, Mikhailidis DP, Patsouris E, Vlachos I, Perrea DN. Water Soluble Vitamin E Administration in Wistar Rats with Non-alcoholic Fatty Liver Disease. *Open Cardiovasc Med J* 6: 88-97, 2012.

216. Uchiyama S, Shimizu T, Shirasawa T. CuZn-SOD deficiency causes ApoB degradation and induces hepatic lipid accumulation by impaired lipoprotein secretion in mice. *J Biol Chem* 281: 31713-9, 2006.

217. Valenti L, Dongiovanni P, Fracanzani AL, Santorelli G, Fatta E, Bertelli C, Taioli E, Fiorelli G, Fargion S. Increased susceptibility to nonalcoholic fatty liver disease in heterozygotes for the mutation responsible for hereditary hemochromatosis. *Dig Liver Dis* 35: 172-8, 2003.

218. Valenti L, Fracanzani AL, Bugianesi E, Dongiovanni P, Galmozzi E, Vanni E, Canavesi E, Lattuada E, Roviaro G, Marchesini G, Fargion S. HFE genotype, parenchymal iron accumulation, and liver fibrosis in patients with nonalcoholic fatty liver disease. *Gastroenterology* 138: 905-12, 2010.

219. Valenti L, Rametta R, Dongiovanni P, Motta BM, Canavesi E, Pelusi S, Pulixi EA, Fracanzani AL, Fargion S. The A736V TMPRSS6 polymorphism influences hepatic iron overload in nonalcoholic fatty liver disease. *PLoS One* 7: e48804, 2012.

220. Valenzuela R, Espinosa A, Gonzalez-Manan D, D'Espessailles A, Fernandez V, Videla LA, Tapia G. N-3 long-chain polyunsaturated fatty acid supplementation significantly reduces liver oxidative stress in high fat induced steatosis. *PLoS One* 7: e46400, 2012.

221. Velayudham A, Dolganiuc A, Ellis M, Petrasek J, Kodys K, Mandrekar P, Szabo G. VSL#3 probiotic treatment attenuates fibrosis without changes in steatohepatitis in a diet-induced nonalcoholic steatohepatitis model in mice. *Hepatology* 49: 989-97, 2009.

222. Videla LA, Rodrigo R, Araya J, Poniachik J. Insulin resistance and oxidative stress interdependency in non-alcoholic fatty liver disease. *Trends Mol Med* 12: 555-8, 2006.

223. Videla LA, Rodrigo R, Orellana M, Fernandez V, Tapia G, Quinones L, Varela N, Contreras J, Lazarte R, Csendes A, Rojas J, Maluenda F, Burdiles P, Diaz JC, Smok G, Thielemann L, Poniachik J. Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients. *Clin Sci (Lond)* 106: 261-8, 2004.

224. Vilar L, Oliveira CP, Faintuch J, Mello ES, Nogueira MA, Santos TE, Alves VA, Carrilho FJ. High-fat diet: a trigger of non-alcoholic steatohepatitis? Preliminary findings in obese subjects. *Nutrition* 24: 1097-102, 2008.

225. Walenbergh SM, Koek GH, Bieghs V, Shiri-Sverdlov R. Non-alcoholic steatohepatitis: the role of oxidized low-density lipoproteins. *J Hepatol* 58: 801-10, 2013.

226. Wang GL, Fu YC, Xu WC, Feng YQ, Fang SR, Zhou XH. Resveratrol inhibits the expression of SREBP1 in cell model of steatosis via Sirt1-FOXO1 signaling pathway. *Biochem Biophys Res Commun* 380: 644-9, 2009.

227. Wang L, Jiang Z, Lei XG. Knockout of SOD1 alters murine hepatic glycolysis, gluconeogenesis, and lipogenesis. *Free Radic Biol Med* 53: 1689-96, 2012.

228. Wang N, Tan HY, Li L, Yuen MF, Feng Y. Berberine and Coptidis Rhizoma as potential anticancer agents: Recent updates and future perspectives. *J Ethnopharmacol* 176: 35-48, 2015.

229. Wang S, Kamat A, Pergola P, Swamy A, Tio F, Cusi K. Metabolic factors in the development of hepatic steatosis and altered mitochondrial gene expression in vivo. *Metabolism* 60: 1090-9, 2011.

230. Weber M, Baker MB, Moore JP, Searles CD. MiR-21 is induced in endothelial cells by shear stress and modulates apoptosis and eNOS activity. *Biochem Biophys Res Commun* 393: 643-8, 2010.

231. Wei Y, Clark SE, Morris EM, Thyfault JP, Uptergrove GM, Whaley-Connell AT, Ferrario CM, Sowers JR, Ibdah JA. Angiotensin II-induced non-alcoholic fatty liver disease is mediated by oxidative stress in transgenic TG(mRen2)27(Ren2) rats. *J Hepatol* 49: 417-28, 2008.

232. Weltman MD, Farrell GC, Liddle C. Increased hepatocyte CYP2E1 expression in a rat nutritional model of hepatic steatosis with inflammation. *Gastroenterology* 111: 1645-53, 1996.

233. Wiedemann M, Kontush A, Finckh B, Hellwege HH, Kohlschutter A. Neonatal blood plasma is less susceptible to oxidation than adult plasma owing to its higher content of bilirubin and lower content of oxidizable Fatty acids. *Pediatr Res* 53: 843-9, 2003.

234. Wu KC, Cui JY, Klaassen CD. Beneficial role of Nrf2 in regulating NADPH generation and consumption. *Toxicol Sci* 123: 590-600, 2011.

235. Wu W, Xiao H, Laguna-Fernandez A, Villarreal G, Jr., Wang KC, Geary GG, Zhang Y, Wang WC, Huang HD, Zhou J, Li YS, Chien S, Garcia-Cardena G, Shyy JY. Flow-Dependent Regulation of Kruppel-Like Factor 2 Is Mediated by MicroRNA-92a. *Circulation* 124: 633-41, 2011.

236. Xiao J, Ho CT, Liong EC, Nanji AA, Leung TM, Lau TY, Fung ML, Tipoe GL. Epigallocatechin gallate attenuates fibrosis, oxidative stress, and inflammation in non-alcoholic fatty liver disease rat model through TGF/SMAD, PI3 K/Akt/FoxO1, and NF-kappa B pathways. *Eur J Nutr* 53: 187-99, 2014.

237. Xu P, Wang H, Kayoumu A, Wang M, Huang W, Liu G. Diet rich in Docosahexaenoic Acid/Eicosapentaenoic Acid robustly ameliorates hepatic steatosis and insulin resistance in seipin deficient lipodystrophy mice. *Nutr Metab (Lond)* 12: 58, 2015.

238. Xu YP, Liang L, Wang CL, Fu JF, Liu PN, Lv LQ, Zhu YM. Association between UCP3 gene polymorphisms and nonalcoholic fatty liver disease in Chinese children. *World J Gastroenterol* 19: 5897-903, 2013.

239. Xu YZ, Zhang X, Wang L, Zhang F, Qiu Q, Liu ML, Zhang GR, Wu XL. An increased circulating angiotensin II concentration is associated with hypoadiponectinemia and postprandial hyperglycemia in men with nonalcoholic fatty liver disease. *Intern Med* 52: 855-61, 2013.

240. Yan HM, Xia MF, Wang Y, Chang XX, Yao XZ, Rao SX, Zeng MS, Tu YF, Feng R, Jia WP, Liu J, Deng W, Jiang JD, Gao X. Efficacy of Berberine in Patients with Non-Alcoholic Fatty Liver Disease. *PLoS One* 10: e0134172, 2015.

241. Yang J, Yin J, Gao H, Xu L, Wang Y, Xu L, Li M. Berberine improves insulin sensitivity by inhibiting fat store and adjusting adipokines profile in human preadipocytes and metabolic syndrome patients. *Evid Based Complement Alternat Med* 2012: 363845, 2012.

242. Yates MS, Tran QT, Dolan PM, Osburn WO, Shin S, McCulloch CC, Silkworth JB, Taguchi K, Yamamoto M, Williams CR, Liby KT, Sporn MB, Sutter TR, Kensler TW. Genetic versus

chemoprotective activation of Nrf2 signaling: overlapping yet distinct gene expression profiles between Keap1 knockout and triterpenoid-treated mice. *Carcinogenesis* 30: 1024-31, 2009.

243. Yesilova Z, Yaman H, Oktenli C, Ozcan A, Uygun A, Cakir E, Sanisoglu SY, Erdil A, Ates Y, Aslan M, Musabak U, Erbil MK, Karaeren N, Dagalp K. Systemic markers of lipid peroxidation and antioxidants in patients with nonalcoholic Fatty liver disease. *Am J Gastroenterol* 100: 850-5, 2005.

244. Yimin, Furumaki H, Matsuoka S, Sakurai T, Kohanawa M, Zhao S, Kuge Y, Tamaki N, Chiba H. A novel murine model for non-alcoholic steatohepatitis developed by combination of a high-fat diet and oxidized low-density lipoprotein. *Lab Invest* 92: 265-81, 2012.

245. Yogalakshmi B, Sreeja S, Geetha R, Radika MK, Anuradha CV. Grape seed proanthocyanidin rescues rats from steatosis: a comparative and combination study with metformin. *J Lipids* 2013: 153897, 2013.

246. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global Epidemiology of Non-Alcoholic Fatty Liver Disease-Meta-Analytic Assessment of Prevalence, Incidence and Outcomes. *Hepatology*, 2015.

247. Yu W, Fu YC, Wang W. Cellular and molecular effects of resveratrol in health and disease. *J Cell Biochem* 113: 752-9, 2012.

248. Zein CO, Yerian LM, Gogate P, Lopez R, Kirwan JP, Feldstein AE, McCullough AJ. Pentoxifylline improves nonalcoholic steatohepatitis: a randomized placebo-controlled trial. *Hepatology* 54: 1610-9, 2011.

249. Zhang BJ, Xu D, Guo Y, Ping J, Chen LB, Wang H. Protection by and anti-oxidant mechanism of berberine against rat liver fibrosis induced by multiple hepatotoxic factors. *Clin Exp Pharmacol Physiol* 35: 303-9, 2008.

250. Zhang P, Qiang X, Zhang M, Ma D, Zhao Z, Zhou C, Liu X, Li R, Chen H, Zhang Y. Demethyleneberberine, a natural mitochondria-targeted antioxidant, inhibits mitochondrial dysfunction, oxidative stress, and steatosis in alcoholic liver disease mouse model. *J Pharmacol Exp Ther* 352: 139-47, 2015.

251. Zhang Q, Liu J, Liu J, Huang W, Tian L, Quan J, Wang Y, Niu R. oxLDL induces injury and defenestration of human liver sinusoidal endothelial cells via LOX1. *J Mol Endocrinol* 53: 281-93, 2014.

252. Zhang X, Zhao Y, Zhang M, Pang X, Xu J, Kang C, Li M, Zhang C, Zhang Z, Zhang Y, Li X, Ning G, Zhao L. Structural changes of gut microbiota during berberine-mediated prevention of obesity and insulin resistance in high-fat diet-fed rats. *PLoS One* 7: e42529, 2012.

253. Zhang YK, Yeager RL, Tanaka Y, Klaassen CD. Enhanced expression of Nrf2 in mice attenuates the fatty liver produced by a methionine- and choline-deficient diet. *Toxicol Appl Pharmacol* 245: 326-34, 2010.

254. Zhao HL, Sui Y, Qiao CF, Yip KY, Leung RK, Tsui SK, Lee HM, Wong HK, Zhu X, Siu JJ, He L, Guan J, Liu LZ, Xu HX, Tong PC, Chan JC. Sustained antidiabetic effects of a berberine-containing Chinese herbal medicine through regulation of hepatic gene expression. *Diabetes* 61: 933-43, 2012.

255. Zhou JY, Zhou SW, Zhang KB, Tang JL, Guang LX, Ying Y, Xu Y, Zhang L, Li DD. Chronic effects of berberine on blood, liver glucolipid metabolism and liver PPARs expression in diabetic hyperlipidemic rats. *Biol Pharm Bull* 31: 1169-76, 2008.

256. Zhu FS, Liu S, Chen XM, Huang ZG, Zhang DW. Effects of n-3 polyunsaturated fatty acids from seal oils on nonalcoholic fatty liver disease associated with hyperlipidemia. *World J Gastroenterol* 14: 6395-400, 2008.

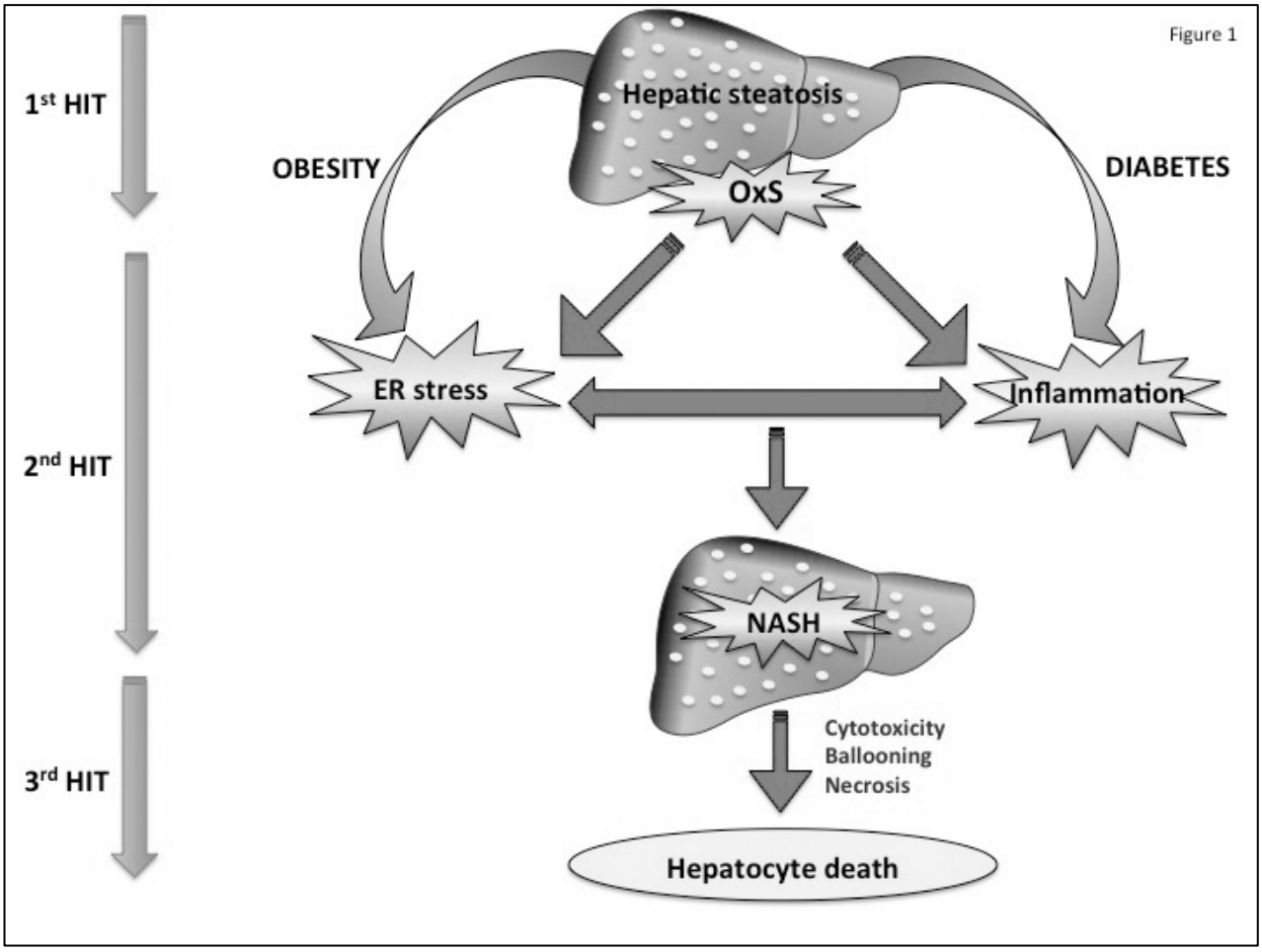

Figure 1. Progression of NAFLD to NASH.

Abundant triglyceride accumulation in the liver in response to fatty acid mobilization from the adipose tissue causes reactive oxygen species (ROS) production (1st Hit). Hepatocytes, overwhelmed by ROS, develop oxidative stress (OxS) that induces endoplasmic reticulum (ER) stress and inflammation (2nd Hit). Such aggravation leads to NASH, which may in turn progress to hepatic fibrosis and cirrhosis due to marked cytoxicity, ballooning, apoptosis and necrosis (3rd Hit).

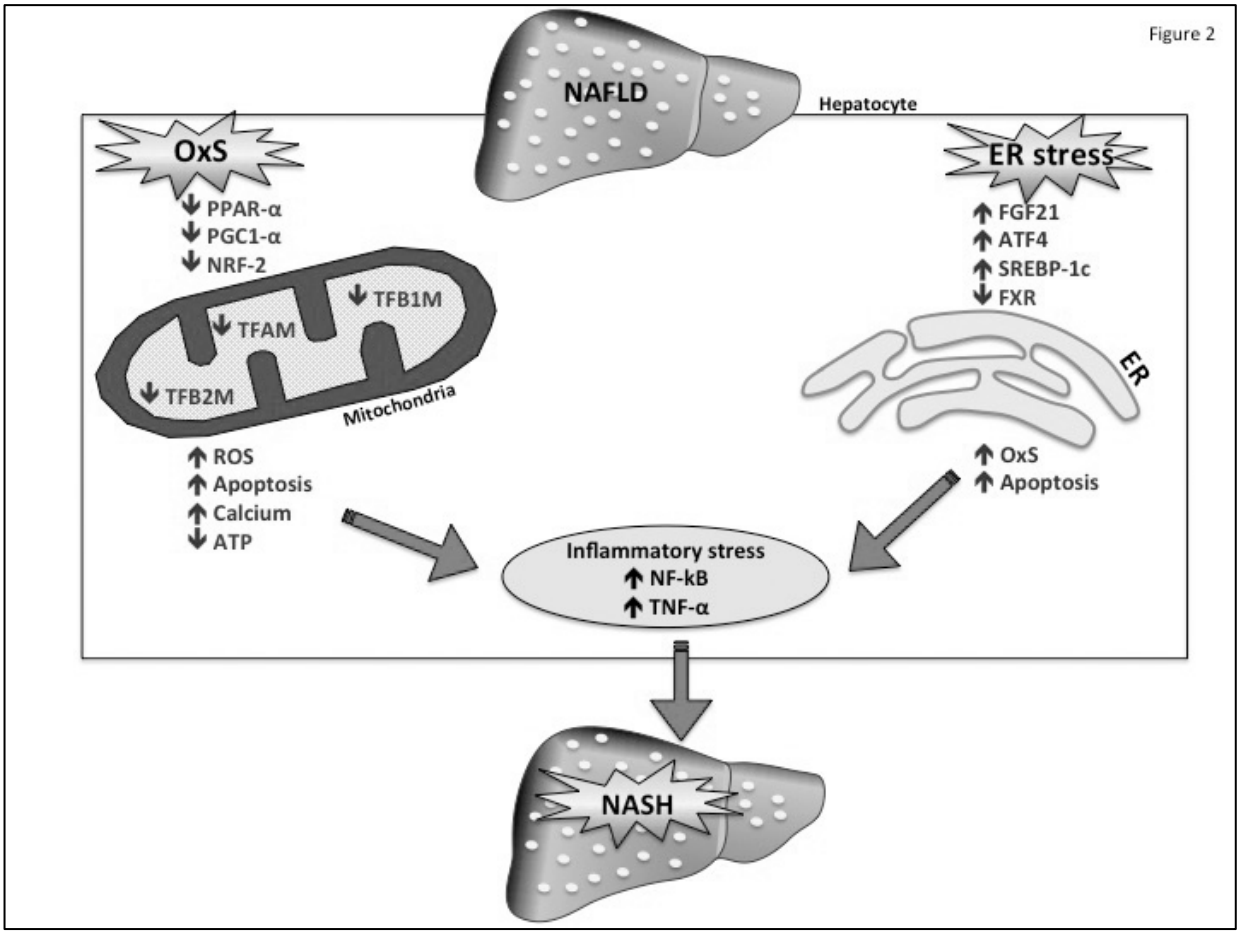

Figure 2. Combined action of mitochondria and endoplamsic reticulum on the progression of NAFLD to NASH.

Several intracellular processes related to NAFLD, including mitochondrial oxidative stress (OxS) and dysfunctions as well as endoplasmic reticulum (ER) stress, trigger inflammation via activation of NF-KB. Additional transcription factors are implicated in the molecular mechanisms resulting in the development of steatohepatitis (NASH).

Revue 3

«The Epigenetic Machinery in Vascular Dysfunction and Hypertension»

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CONTRIBUTION DES AUTEURS

Spahis S a effectué la revue critique de la littérature, a compilé les données et crée les Figures et Tables qui accompagnent la revue. Elle a contribué à la rédaction, soumission et révision de la revue, sous la supervision du Directeur de recherche, Dr Levy E.

Bigras JL, Delvin E et Borys JM ont participé à la révision du manuscrit.

Levy E a contribué à la rédaction et la révision du manuscrit.

THE EPIGENETIC MACHINERY IN VASCULAR DYSFUNCTION AND HYPERTENSION

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Abstract

Hypertension (HT) is among the major components of the metabolic syndrome, i.e., obesity, dyslipidemia and hyperglycemia/insulin resistance. It represents a significant health problem with foremost risks for chronic cardiovascular disease and a significant cause of morbidity and mortality worldwide. Therefore, it is not surprising that this disorder constitutes a serious public health concern. Although multiple studies have stressed the multifactorial nature of HT, the pathogenesis remains largely unknown. However, if we want to reduce the global prevalence of HT, restrain the number of deaths (currently 9.4 million/year in the world), and alleviate the socio-economic burden, a deeper insight into the mechanisms is urgently needed in order to define new meaningful therapeutic targets. Recently, the role of epigenetics in the development of various complex diseases has attracted much attention. In the present review, we provide a critical update on the available literature and ongoing research regarding the epigenetic modifications of genes involved in several pathways of elevated blood pressure, especially those linked to the vascular epithelium. This review also focuses on the role of microRNA (miRNA) in the regulation of gene expression associated with HT and of fetal programming mediating susceptibility to HT in adulthood.

ABBREVIATION LIST

ACE;	angiotensin-converting enzyme
AGTR1;	angiotensin II receptor type 1
Ang;	angiotensin
CMV;	cytomegalovirus
CNS;	central nervous system
Dot-1;	Disruptor of telomere silencing 1
ENaC;	epithelial of sodium channel
eNOS;	endothelial nitric oxide synthase
H3K4;	histone H3 lysine 4
HT;	hypertension
miRNA;	microRNA
NET;	noradrenaline transporter
OxS;	oxidative stress
RAAS;	Renin-Angiotensin-Aldosterone System
ROS;	reactive oxygen species
SMC;	smooth muscle cells
SNP;	single nucleotide polymorphism
SOD;	superoxide dismutase

I. INTRODUCTION

Metabolic syndrome is a clinical entity characterized by the co-presence of multiple dysmetabolic processes such as obesity, insulin-resistance, hypertension (HT) and dyslipidemia. One of its key components, arterial

HT, impacts a large proportion of the population and represents a major public health concern. It is estimated that approximately one third of adult individuals will be hypertensive in the world by 2025, with an increased risk of developing stroke, myocardial infarction, heart failure, and end-stage renal disease (1). Many mechanisms have been proposed to explain HT pathogenesis, but the dynamic interactions between genetics and environmental factors constitute the primary triggers (2). The increasing prevalence worldwide is accompanied by disability and premature death, therefore requiring the elucidation of the precise etiology in order to develop appropriate strategies for prevention and treatment. Except for rare familial forms, HT is a polygenic trait and single nucleotide polymorphisms (SNPs) of the Renin-Angiotensin-Aldosterone System (RAAS) genes [e.g. angiotensinogen, renin, angiotensin II type I receptor (*AGTR1)*, angiotensin (Ang) converting enzyme and aldosterone synthase] display the ability to dysregulate RAAS and influence HT pathophysiology (3). Additional genetic variants related to lipid metabolism, sodium regulating system, and sympathetic nervous system may play a role in the development of HT by altering the function and/or the expression of the encoded proteins, thereby creating phenotypes that predispose to HT (4). Importantly, despite the weighty progress in discovering genetic biomarkers, this can only inform a small part of the phenotypic variance of blood pressure. On the other hand, due to the role of development and environmental stressors that influence blood pressure, there is a need to examine other mechanisms. Therefore, this review will critically discuss the associative relationship between endothelial dysfunction, HT, and epigenetic modifications.

II. EPIGENETICS

Multiple pathways are regulated by epigenetic phenomena like DNA methylation, histone modification, chromatin remodelling, and microRNA (miRNA) mediated targeting of various genes. Epigenetics delivers a valuable stratum of information on top of the DNA sequence and is critical for establishing gene expression profiles. Indeed, hypo- and hyper-methylation of bases in DNA, as well as loss of imprinting, dictate the outcome of cell fate without modifications of the DNA sequences. Methylation is site-specific and is performed by DNA

methyltransferases that transfer a methyl group from S-adenosyl methionine to cytosine bases of CpG dinucleotides (concentrating in short CpG-rich regions known commonly as "CpG islands") at gene promoters and regulatory regions (**FIG. 1**). Although transcriptional silencing results from DNA methylation, it remains uncertain whether this reflects repression or a lack of activation (5), but what is sure is the induction of pathological processes by atypical patterns of DNA methylation.

Histone modification is another powerful epigenetic mechanism that controls gene expression via transcription, replication, and repair (6). As histones are complexed to DNA, which leads to its compaction and assembly into the basic unit of chromatin the nucleosome, modifications (acetylation and methylation) in different histone proteins (H2A, H2B, H3, and H4) cause the DNA to be more easily freed from the nucleosome and accessed by proteins involved in transcription, repair, and other genetic functions (**FIG. 2**). For example, hyperacetylated histones lead to uncompressed chromatin with increased DNA accessibility to the transcription machinery resulting in gene activation, whereas histone deacetylation leads to condensed chromatin and gene inactivation.

miRNAs are a class of small, noncoding RNA transcripts (~22 nucleotides). Mature miRNAs are generally located in intergenic regions or in antisense orientation to protein-coding genes and negatively control gene expression through either repression of translation or by increasing mRNA degradation (7). They elicit their functions by binding primarily to the 3' untranslated region of target mRNAs through complementary base pairing mechanisms and subsequently preventing the translation of the bound mRNA transcript. Individual miRNA families can target many different mRNAs and, in this way, fine-tune gene expression post-transcriptionally to modulate crucial cellular and molecular activity in response to external and internal stressors. Expressed in all cell types, miRNAs are essential master regulators of many processes (8).

III. EPIGENETICS AND ETIOLOGICAL FACTORS OF HYPERTENSION: VASCULAR ENDOTHELIUM

a) Smooth muscle cell plasticity and epigenetics

The main function of smooth muscle cells (SMC), the major cell type in blood vessels, is to finely modulate blood flow and blood pressure through vessel wall contraction and relaxation. With their extraordinary plasticity, SMC can promptly switch between two phenotypes, i.e. a differentiated and quiescent "contractile" condition and a vastly proliferative and migratory

"synthetic" status. This efficient remodelling is deployed to cope with various cues such as growth factors and signals (9). Dysfunction of SMC results in atherosclerosis, HT, aneurysm, asthma and cancer (10). Many groups have addressed the issue relating to SMC regulation to better target specific molecules/pathways facilitating the beneficial prevention or efficient treatment of these disorders. Their efforts have been rewarded by the discovery of epigenome modification as the critical regulator of SMC (11). The pioneering work of Manabe et al. points out that the critical serum response factor for SMC-specific transcription is unable to connect to the target sites of endogenous marker genes in chromatin in undifferentiated SMC cells, but it has this potential in differentiated cells following its association with hyperacetylation of histones H3 and H4 (12). Clearly, these studies reveal the involvement of chromatin-remodelling along with selective recruitment of serum response factor to CArG (CC[A/T]TATA[A/T]GG) box elements in the induction of cell-selective marker genes during SMC differentiation. Subsequently, miRNAs have been recognized as important modulators of SMC phenotype via the modulation of the smooth muscle- and cardiomyocyte-specific transcriptional co-activator myocardin (13). DNA methylation is the third mechanism that controls SMC phenotype and the development of vascular disease. In view of the relationship between vascular wall remodelling and human essential HT or genetic HT of the rat (14), epigenetic alterations may not only affect SMC gene expression and phenotype, but also blood pressure. Therefore, regulatory epigenetic mechanisms warrant careful examination in the future.

b) Endothelial dysregulation and blood flow

Endothelial cells of the tunica intima, the innermost layer of blood vessels, are in close contact with blood flow. They experience mechanical stresses able to impact their morphology and function. For example, the surface of endothelial cells is continuously exposed to various hemodynamic forces related to blood flow, which include shear stress (friction of the blood flow with the endothelial layer), tensile stress (due to pulsatile nature of blood pumped from the heart), and normal stress (the pressure differential across the vessel wall). Therefore, it is not surprising that arteries may alter shape under blood fluid dynamics, which in turn can alter blood flow. Growing evidence suggests that endothelial responses to blood flow are modulated through epigenetic mechanisms such as histone modification, chromatin remodelling, and miRNA. In particular, miRNA mediates the adaptation of endothelial cells

subjected to shear stress, thereby transforming the mechanical stimuli into intracellular signals. The importance of this concept may be illustrated through miR-19a that, in response to shear stress, induces an anti-proliferative effect through inhibition of cyclin D1, thereby preventing excessive cellular proliferation, loose intercellular junctions, and raised permeability to lipoproteins (15). Similarly, in response to shear stress, miR-143/145 increases and contributes to the lowering of Ang-converting enzyme that generates Ang II (16).

DNA methylation represents another route by which disturbed local arterial flow modifies gene expression in the arterial endothelium (17). For instance, hyper- and hypo-methylation can result in suppression and stimulation of transcription in human arterial endothelial cells in response to disturbed flow (17). Thus, it is essential to understand epigenetic regulation of vascular endothelium given the reciprocal influences among arterial biomechanics, blood flow and HT.

c) Obesity

Although many epidemiological investigations have stressed the relationship between weight gain and HT, the precise pathogenic mechanisms remain complex and inadequately defined. At the moment, this association is mainly attributable to multifactorial mechanisms, including genetics, insulin resistance, inflammation, oxidative stress (OxS), and environmental stressors (**Fig. 3**). Growing evidence suggests that epigenetic modifications also play a role in this complex relationship. For example, *ADRB3* DNA hypermethylation is detected in human visceral adipose tissue in conjunction with obesity and HT (18). In fact, these epigenetic alterations might be involved in the development of obesity and its related metabolic complications. Similarly, epigenetic events in the adipose tissue have the potential to influence the rise of blood pressure. Cortisol-induced recruitment of corticosteroid receptors can trigger DNA demethylation at two putative corticosteroid response elements and activate the expression of the angiotensinogen gene in visceral adipose tissue via interactions between corticosteroid receptors and CCAAT/enhancer binding protein, which contribute to the development of HT (19). Obesity-associated adipose tissue expansion is accompanied by a proinflammatory state and an increase in adipokines such as leptin and tumor necrosis factor-α, and these signaling molecules are regulated by promoter DNA methylation (20). Further studies are warranted to define the mechanisms by which obesity influences HT.

d) Oxidative stress

Elevated levels of OxS have been reported in hypertensive rats and patients. In these conditions, reactive oxygen species (ROS)-mediated OxS contribute significantly to organ damage in patients with HT (21). The OxS in HT vascular remodelling has been associated with endothelial dysfunction, inflammation, cell migration, apoptosis, and angiogenesis (**Fig. 3**). Similarly, the activity of the epithelial Na(+) channel (ENaC), which plays a critical role in the regulation of Na(+) reabsorption and contributes to HT, is enhanced by ROS (22).

There is a strong link between OxS and epigenetic changes via chemical DNA modifications. In fact, ROS directly modify cytosine with oxidative conversion of 5-methylcytosine to 5-hydroxymethylcytosine, which blocks the binding of Dnmt1 to DNA (23). Furthermore, peroxides cause nucleobase modification and induce improper Dnmt1 methylation within CpG sequences, prompting gene silencing. Therefore, OxS may be strongly implicated in HT pathogenesis via changes in methylation pattern. A good example is superoxide dismutase (SOD) deficiency in pulmonary arterial HT, which is caused by hypermethylation and silencing of the gene with resultant increase in H_2O_2 production. Treatment with 5-azacytidine, a methyltransferase inhibitor, reverses the hypermethylation and raises SOD expression. Such an increase in SOD in the animal model reduces pulmonary arterial pressure and thickness of the arterial media (24). Histone deacetylation (specifically via class I HDAC3) is another epigenetic mechanism that lowers extracellular SOD3 expression, whereas HDAC inhibitors protect against human idiopathic pulmonary arterial HT in part by increasing SOD3 expression (25).

e) Central nervous system control of hypertension

Ample evidence supports the role of the central nervous system (CNS) in the pathogenesis and maintenance of HT (**Fig. 3**). In particular, the hypothalamus plays a crucial role in co-ordinating and integrating the activity of neural networks that control central blood pressure (26). The mechanisms include: (i) the baroreceptors that sense acute variations in blood vessel pressure and lower or raise CNS activity; (ii) the renin–Ang system; (iii) adrenergic receptors (or adrenoceptors) that bind catecholamines to increase heart rate, myocardial contractility, and to regulate vascular tone; (iv) factors produced by endothelial cells, which lead to vasodilation (e.g. nitric oxide) or vasoconstriction (e.g. endothelin); (v) natriuretic peptides that are secreted in response to increased pressure; and (vi) the kinin–kallikrein system, which influences vascular tone and renal salt handling (26). Epigenetic regulation of these systems may influence the development or sustainment of HT. For example, the noradrenaline transporter (NET) gene,

rich in CpG dinucleotides and potentially rendering it susceptible to methylation and silencing (27), displays increased methylation in its promoter region in patients with HT (27). Similarly, the inhibitory transcription factor, MeCP2, which causes gene silencing primarily by binding to heavily methylated promoter region DNA, is clearly enriched on the promoter region of the NET gene in essential HT (28). Additionally, miR-155 targets mRNA belonging to the renin-Ang system by binding to AGTR1 mRNA, which may impact blood pressure. Renin mRNA is overexpressed in response to miR-181a and miR-663 in kidneys of patients with HT (29). Thus, epigenetic modifications may represent the mechanisms of neurogenic HT.

f) Renal disease-related hypertension

Renal disease represents both a cause and consequence of HT, forming a vicious circle whereby HT causes kidney damage, which then exacerbates the high blood pressure (**Fig. 3**). Some animal models of high blood pressure (spontaneously hypertensive and salt-loaded stroke-prone rats) mimic humans in terms of pathological progression of hypertensive renal injury (30). These models have been very instrumental for the identification of multiple genes implicated in HT and kidney damage. For example, increase in the levels of renin, a key component of the renin-Ang aldosterone system, results in raised plasma concentrations of Ang II and aldosterone, provoking systemic vasoconstriction and Na(+) resorption in the kidney to favor blood pressure elevation (31). Disruptor of telomere silencing 1 (Dot-1) is a member of the histone methyltransferase family, which methylates Lys-79 in histones and suppresses gene transcription. In addition, Dot1α-AF9 complex facilitates histone hypermethylation. Under basal circumstances, epithelial Na (+) channel (ENAC) gene transcription is constrained. It can be induced by aldosterone and serum glucocorticoid regulated kinase 1 (SGK1). Aldosterone diminishes Dot1α and AF9 expression and leads to histone hypomethylation and increased ENAC gene transcription. SGK1 phosphorylates AF9 at Ser435 and disrupts Dot1α-AF9 complex, thus increasing ENac synthesis (32). β_2-adrenergic stimulation in response to enhanced renal sympathetic activity in salt-induced HT suppresses HDAC8 activity (via cAMP/PKA signaling), which favors the accessibility of glucocorticoid receptors to the negative glucocorticoid receptor response element in the *WNK4 (*the β_2-adrenergic stimulant-glucocorticoid receptor-with-no-lysine kinase) promoter, thereby resulting in the suppression of *WNK4* transcription, activation of NaCL cotransporters in the nephron and elevated Na(+) retention and blood pressure upon salt loading (33). Moreover, through interaction with miRNA,

the expression of the human antigen R, which links and modulates the expression of soluble guanylate cyclase transcripts α_1 and β_1, is reduced in older hypertensive rats, thereby promoting the decline of soluble guanylate cyclase levels and worsening HT. Overall, these findings show that epigenetic markers may offer valuable new insight into the relationship between renal pathophysiology and HT.

g) Inflammation

Accumulating evidence suggests that inflammation contributes to HT (**Fig. 3**). Consistent with this point, phosphoinositide 3-kinase γ is implicated in HT (34) by modulating vascular function and particularly the recruitment of immune cells to inflammatory sites, including kidney and bone marrow (35). In the last decade, hypothalamic inflammation was emphasized as a potential driver of pathogenic HT. In addition, obesity-associated HT is associated with the activation of pro-inflammatory signalling pathways. The augmented expression of Ang II can lead to endothelial damage, while promoting inflammation, mesangial cell proliferation, and vasoconstriction (36). Recently, downregulation of miR-129 and DNMT3a in mice, as well as IL-17A upregulation mediated by changes in methylation in kidneys has been related to inflammation and HT (37). Furthermore, the adherence to Mediterranean diet by subjects with cardiovascular risk factors, including HT, has been reported to be associated with the methylation of genes related to inflammation (*EEF2, COL18A1, IL4I1, LEPR, PLAGL1, IFRD1, MAPKAPK2, PPARGC1B*) (38). Therefore, the compliance to Mediterranean may uphold a healthful impact via anti-inflammatory effects mediated by epigenetic modifications.

h) Angiogenesis

Angiogenesis represents the formation of new blood vessels from existing vasculature and involves endothelial cell proliferation, migration, tube formation, and extracellular matrix degradation (39). Angiogenesis is both an essential adaptive response to physiological stress and an endogenous repair mechanism after ischemic injury. This is a highly regulated process, mainly through synchronised expression of chemokines and growth factors together with their receptors (40). In fact, the neovessel growth process is critical for vascular homeostasis after injury, and its impairment results in the development of various disorders such as HT (**Fig. 3**). In this context, hypertensive patients and animals displayed loss of pre-capillary arterioles and capillaries, known as microvascular/capillary rarefaction, and are characterized by imbalance of pro- and anti-angiogenic factors (41).

The importance of epigenetic control has been highlighted with the induction of Let-7 and miR-103/107 in endothelial cells and angiogenesis stimulation (**Table 1**). In link with HT, blood flow patterns play a significant role in sprouting angiogenesis. MiR-126 facilitates integration of a physiological stimulus with growth factor signaling in endothelial cells to guide angiogenesis (42). In addition, miR-18a and miR-19 reduce the secreted angiogenesis inhibitors connective tissue growth factor and thrombospondin-1, thereby influencing angiogenesis, which is critically dependent on the balance of pro- versus anti-angiogenic cytokines. Similarly, the combination of miR-17 and let-7b is able to rescue the limited angiogenesis in the corpus luteum of Dicer deficient mice (43). Conversely, overexpression of miR-92a impairs *in vitro* angiogenesis in zebra fish (44). In summary, epigenetics play a role in angiogenesis homeostasis, which may directly impact blood pressure.

IV. METHYLATION PATTERN AND HYPERTENSION

The overall outcome of the genome-wide association and replication study carried out by Kato et al is largely supportive of blood pressure regulation by methylation modification (45). In fact, the strong correlation between the genetic polymorphisms and blood pressure in 320,251 individuals identifies DNA methylation as the modulatory process linking sequence variation and blood pressure. Interestingly, the 12 newly identified SNP loci include those involved with vascular smooth muscle (*IGFBP3*, *KCNK3*, *PDE3A*, *PRDM6*) and renal function (*ARHGAP24*, *OSR1*, *SLC22A7*, *TBX2*). Previous observations had already emphasized the relationship between DNA methylation extent and essential HT characteristics, e.g. onset, timing, and severity. There are also alterations in the methylation of gene sequences influencing arterial pressure such as hydroxysteroid dehydrogenase-11β2, the enzyme converting cortisol to cortisone, that has a large role in Na(+) reabsorption by the kidneys and consequently plays a significant role in fluid-related arterial pressure (46). Subsequent to the hypermethylation of the hydroxysteroid dehydrogenase-11β2 promoter, the conversion of cortisol to cortisone is inhibited, thereby favouring essential HT (47).

Methylation changes in genes of the renin-Ang system, which highly influence the profile of blood pressure, Na(+) retention, and vasoconstriction, can trigger the development of HT (48). Importantly, the renin-Ang system displays CpG islands in the promoter region of some enzyme components such as ATPase, H^+ transporting, lysosomal accessory protein 2 (*ATP6AP2*) encoding (P)RR), angiotensin-converting enzyme *(ACE)* and *AT1R*. In spontaneously

hypertensive rats, hypomethylation of AT1aR and Na-K-Cl cotransporter 1 promoters leads to raised expression of AT1aR expression with augmented blood pressure (49). The administration of ACE inhibitors concomitantly lowers global DNA methylation and blood pressure (50). Similarly, exposure of mice to intermittent hypoxia causes hypomethylation of CpG islands associated with the transcription start site of the *ACE1* and *AGT* genes in mesenteric artery cells, which may explain higher systolic blood pressure, impaired baroreflex responses, and decreased heart rate (51). The *in vitro* and animal studies are also valid in humans given the reduced 5-methylcytosine levels in peripheral blood mononuclear cells from patients affected by essential HT (52). The hypoxia process, in particular, suggests that major epigenetic modifications in gene pathways are involved in endothelial cell biology and function, which may lead to long-lasting changes in renin–angiotensin system modulation and endothelial function, and ultimately promoting hypertension. All together, these observations indicate that epigenetics is involved in blood pressure regulation. However, additional efforts are clearly necessary to increase causal and direct evidence, especially in humans, whereby methylation modification induces HT. It is basically the transgenerational inheritance that has mostly benefited from various groups' insight to highlight the role of DNA methylation patterns in the development of HT.

V. HISTONE MODIFICATIONS AND HYPERTENSION

Only limited data are available concerning the contribution of post-translational histone modifications to chromatin modifications and to arterial HT. Studies have demonstrated so far that the activation of histone deacetylase 8 lowers the binding of the acetylated histones 3 and 4 to the promoter of the beta-adrenergic receptor–GR-WNK4 pathway, which decreases the transcriptional activity of *WNK4* and salt-sensitive HT (53). Furthermore, feeding mice with a high-salt diet leads to deficiency of lysine-specific demethylase-1, which induces demethylation of histone H3 lysine 4 (H3K4) or H3K9, alters gene transcription of endothelial nitric oxide synthase (eNOS), guanylate cyclase, and raises arterial HT (54).

VI. miRNA AND HYPERTENSION

As mentioned before, miRNA deregulation has the ability to lead to impaired cellular function and disease development. miRNAs may be important in the pathogenesis of endothelial dysfunction. In this context, miR-126 seems indispensable for the integrity of vascular endothelium, which generally contributes to HT in conditions of homeostasis perturbation (55).

The basic importance of miR-126 has been confirmed with the observed demonstration that suppression of miR-126 results in leaky vessels and haemorrhage (55). On another side, miR-217 causes endothelial senescence by down-regulating silent information regulator 1 and subsequently to its targets Forkhead Box Protein O1 and endothelial eNOS (56). Furthermore, miR-21 induces cellular dysfunction in coronary artery disease (57). As kidney function is closely associated with blood pressure, particularly with regard to the control of sodium homeostasis, blood volume, and arteriolar resistance via the renin-Ang aldosterone system, several investigators attempted to determine miRNA profile in renal diseases. Indeed, the deletion of Dicer, the enzyme processing pre-miRNAs into mature miRNAs, results in total suppression of miRNAs combined with mouse embryonic lethality. The Dicer ablation in the renin-secreting juxtaglomerular cells causes a significant decline in plasma renin and blood pressure (58). In other words, the development of kidney abnormalities provokes changes in miRNA targets in link with HT pathogenesis.

Different miRNAs seem to contribute to the pathogenesis of HT in view of their capacity to regulate the expression of key molecules related to vascular tone. To mention just a few examples: (i) miR-125a-5p and miR-125b-5p down-regulate endothelin-1 expression in endothelial cells (59) in line with their lessened level in hypertensive rats; (ii) miR-155 alters the expression of endothelial eNOS and *AGTR1,* the two main players in vascular homeostasis (60); and (iii) miR-Let-7g displays the capacity to maintain endothelial function and suppress inflammation (61).

Intriguingly, no alterations were reported in the expression of 118 miRNAs in the Dahl salt-sensitive animal model of HT, yet other animal studies have succeeded in delivering functional insights on the role of miRNAs (62). Supression of miR-143/145 leads to a lessening of blood pressure in mice (62). On the contrary, reduced levels of miR-181 have been associated with high renin mRNA and HT in the Schlager BHP/2J mouse in the active circadian period (63), which is in line with the down-regulation of miR-181 in in kidneys of hypertensive patients (64). Further investigation on Dahl salt-sensitive hypertensive animals shows that five miRNAs are differentially expressed when compared to consomic SS-13BN controls (65). In particular, miR-29b is down-regulated in the hypertensive animals.

In humans, a direct association has been reported between various miRNAs and HT as shown in **Table 1**. Additionally, using Omics tools, a group of investigators could evaluate the

expression of miRNAs on a large scale. Relying on a microarray analysis, miR-210, miR-425 and miR-505 are upregulated in a Chinese population, which is consistent with its inhibitory effects on epithelial cell migration and tube formation through the modulation of fibroblast growth factor 18 (66). Genome-wide assay, carried out to document the differential miRNA expression in hypertensive patients and healthy controls, points out the up-regulation of 9 miRNAs and down-regulation of 18 miRNAs in subjects with HT (67). Interestingly, in keeping with the high titres of cytomegalovirus (CMV) in hypertensive patients, this investigation has detected hcmv-miR-UL112, a human CMV–encoded miRNA, which is able to interact with interferon regulatory factor-1 involved in infectious and inflammatory responses. The question that begs asking is whether CMV could contribute to HT by modulating miRNA pathways. Another interesting issue is the high levels of miR-122 and miR-637 in the plasma of subjects with white coat HT (higher blood pressure in a medical setting than at home), whereas hypertensive subjects exhibit up-regulation of miR-21, miR-122, miR-637, and MiR-let-7e compared to healthy controls (68). The most noticeable point is the antagonism between the low levels of miR-296-5p in hypertensive patients and the high levels in white-coat hypertensives, which allows the distinction between white-coat and non-white-coat hypertensive individuals.

Comparative studies between healthy subjects and patients with various disorders (metabolic syndrome, type 2 diabetes, hypercholesterolemia or HT) disclose the down-regulation of plasma miR-150, miR-192 and miR-27a in subjects with hypercholesterolemia or HT, and up-regulation of miR-130a, miR-195 and miR-92a in subjects with HT and metabolic syndrome (69). The raised concentrations of plasma miR-130a, miR-195 and miR-92a are positively correlated with blood pressure values (69). In this context, it is relevant to mention that the dysregulation of miR-27a in subjects with HT is in line with the low levels of miR-27a in aortas of spontaneously hypertensive rat (70).

VII. FETAL PROGRAMMING AND HYPERTENSION

The developmental origin of HT has been related to maternal and perinatal conditions, as is the case for various cardiovascular disorders (71). In fact, the pioneering observations were proposed by Barker and colleagues who put forward the idea that persons born with a low birth weight are likely to exhibit raised cardiometabolic and cardiovascular mortality in adulthood (72). The central hypothesis is that changes occurring during critical time windows can become permanent, thereby allowing a variety of phenotypes to be generated. This developmental

plasticity has provided an organism with the ability to modify structure and functions because of environmental cues, which may explain how adult-onset hypertension results from nutritional insults in early life. Renal programming, characterized by a reduced number of nephrons due to undernutrition, has been associated with essential HT (73). Furthermore, the relationship between maternal undernutrition, placental insufficiency, reduced nephron number, and HT is confirmed in experimental animal models with an emphasis on nitric oxide-ROS imbalance (74). Apparently, epigenomic modifications play a significant role in the development of HT. For example, glucocorticoid-driven effects on blood pressure were mediated by an aberrant increase in methylation of glucocorticoid receptor promoter region (75). Moreover, the consumption of an imbalanced diet (high protein and very low carbohydrate foods) led to elevated methylation of 11β-HSD2 promoter that promoted blood pressure (76). Further studies evidenced the various epigenetic changes, including decreased CpG methylation of the p53 promoter in the kidney following uteroplacental insufficiency, which was associated to a reduced glomerular number (77).

VIII. THERAPEUTIC POTENTIAL OF miRNAs TO TREAT HYPERTENSION

miRNAs target various genes, control signaling pathways, and regulate numerous biologic processes, and their deregulation affects cellular functions that promote multiple disorders. Thus, it is reasonable to put forward that miRNAs may represent efficient tools in a therapeutic arsenal. miRNA-related therapeutic strategies have been designed to produce chemically modified oligonucleotides known as antagomirs, or anti-miRs, in order to silence specific endogenous miRNA. Quite recently, miR-155 restoration in mice lacking mineralocorticoid receptors in SMC rescues the aging phenotype, resulting in improved Ang II-mediated vasoconstriction, vascular OxS and blood pressure (78). As low levels of miR-21 are strongly associated with an improvement in arterial stiffness in patients with well-controlled essential HT (79), miR-21 may become a potential diagnostic/prognostic marker and a future therapeutic target not only in hypertensive subjects, but also in heart failure since its overexpression in animal models induces fibrosis and cardiac dysfunction. However, these data should be confirmed by further work, since, paradoxically, the administration of exogenous miR-21 by recombinant adeno-associated virus is sufficient to lower blood pressure in the spontaneously hypertensive rat model (80). In rats with other forms of HT, including pulmonary artery HT, epigenetic treatment has been applied. When miR-204 is supplied by nebulization, there is a

lowering of the pressure in the pulmonary artery along with a decline in thickness of the medium and small pulmonary arteries (81). Similarly, the administration of miR-140-5p mimic prevents the development of experimental pulmonary artery HT in rat models (82). Noteworthy, not all miRNA treatments with antagomirs or specific miRNA provide beneficial effects, as miR-223 is not able to attenuate pulmonary artery HT after administration in the monocrotaline rat model (83). So far, only a limited number of miRNAs have undergone clinical trials, as many miRNAs have pleiotropic effects. Therefore, inhibition or overexpression of these small non-coding RNA molecules could have off-target effects. For now, only the miRNA's with organ or tissue specificity have been employed in clinical trials. Moreover, issues related to delivery, pharmacokinetics, and targeting the specific organ and gene specificity still need to be addressed.

IX. CONCLUSIONS

In this review, we could note that changes in the epigenetic regulation of genes contribute to the induction the metabolic syndrome components as exemplified by artery hypertension. We have particularly attempted to break down the pathophysiology of arterial hypertension into a number of its etiological factors, each in conjunction with specific epigenetic modifications. Additional studies are evidently needed to look more closely at this subject in order to clarify the regulatory mechanisms involving DNA methylation, histone modification, chromatin remodelling and miRNA mediated targeting. Furthermore, it is mandatory to address arterial hypertension as a whole clinical entity with the combination of all the epigenetic mechanisms. Only then can we gain a better overview of the interactions among the etiological factors of hypertension, which dictate the initiation and evolution of the disease and allow the possibility to develop a unified theory for this multifactorial disease. In meeting this challenge, we will open new avenues for successful, targeted anti-hypertensive drug therapy.

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Table 1. Regulation of miRNAs in humans during hypertension

miRNAs downregulated in hypertention		
	Systemic circulation	miR-9 (84), miR-18b (67), miR-26b (85), miR-133 (86), miR-133a (85), miR-143/145 (86), miR-150 (87), miR-296–5p (67)
	Kidney	miR-181 (29), miR-663 (29)
	Vascular smooth muscle cell	miR-204 (81)
	In vitro (HUVEC,	miR-221/222 (88)
	artery	miR-132 (89), miR-212 (89)
	Endothelial cells	miR-31 (90), miR-126 (91), miR-146 (92)
	Heart	miR-155-5p (93)
miRNAs upregulated in hypertention		
	Systemic circulation	Hcmv-miR-UL122 (67), let-7e (67), miR-1(86), miR-17 (69), miR-21 (86), miR-23a (69), miR-23b (94), miR-27a (69), miR-65 (69), miR-92a (69), miR-103 (69), miR-122 (68), miR-126 (95), miR-130a (94), miR-155 (95), miR-181a (96), miR-183 (69), miR-191 (94), miR-192 (69), miR-195 (69), miR-197 (69), miR-208b (85), miR-210 (95), miR-221/222 (97), miR-320a (69), miR-425 (66), miR-499 (85), miR-505 (66), miR-509-5p (69), miR-516b (67), miR-584 (69), miR-600 (67), miR-605 (67), miR-623 (67), miR-637 (68)
	In vitro (HUVEC,	miR-24 (98), miR-24 (99), miR-27 (98), miR-155 (100), miR-200 (101)
	Endothelial cells	miR-1 (102), miR-29b (89), miR-129-3p (89), miR-143/145 (16), miR-212 (89), miR-217 (103), miR-221/222 (104)
	Artery	miR-145 (105)
	Heart	miR-182 (106)

REFERENCES

Papers of particular interest have been highlighted as:

*** of special interest**

**** of outstanding interest**

1. Carretero OA, Oparil S. Essential hypertension. Circulation. 2000;101(3):329-35.
2. Simon PH, Sylvestre M-P, Tremblay J, Hamet P. Key Considerations and Methods in the Study of Gene–Environment Interactions. American journal of hypertension. 2016:hpw021.
3. Cat AND, Montezano AC, Touyz RM. Renin–angiotensin–aldosterone system: new concepts. Hypertension. 2013:84-100.
4. Wei LK, Au A, Teh LK, Lye HS. Recent Advances in the Genetics of Hypertension. 2016.
5. Baubec T, Schübeler D. Genomic patterns and context specific interpretation of DNA methylation. Current opinion in genetics & development. 2014;25:85-92.
6. Arnsdorf EJ, Tummala P, Castillo AB, Zhang F, Jacobs CR. The epigenetic mechanism of mechanically induced osteogenic differentiation. Journal of biomechanics. 2010;43(15):2881-6.
7. Lee RC, Ambros V. An extensive class of small RNAs in Caenorhabditis elegans. Science. 2001;294(5543):862-4.
8. Yoo AS, Staahl BT, Chen L, Crabtree GR. MicroRNA-mediated switching of chromatin-remodelling complexes in neural development. Nature. 2009;460(7255):642-6.
9. Floren M, Bonani W, Dharmarajan A, Motta A, Migliaresi C, Tan W. Human mesenchymal stem cells cultured on silk hydrogels with variable stiffness and growth factor differentiate into mature smooth muscle cell phenotype. Acta Biomater. 2016;31:156-66.
10. Byon CH, Heath JM, Chen Y. Redox signaling in cardiovascular pathophysiology: A focus on hydrogen peroxide and vascular smooth muscle cells. Redox Biol. 2016;9:244-53.
11. Liu R, Leslie KL, Martin KA. Epigenetic regulation of smooth muscle cell plasticity. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms. 2015;1849(4):448-53.

12. Manabe I, Owens GK. Recruitment of serum response factor and hyperacetylation of histones at smooth muscle–specific regulatory regions during differentiation of a novel p19-derived in vitro smooth muscle differentiation system. Circulation research. 2001;88(11):1127-34.
13. Cao D, Wang Z, Zhang C-L, Oh J, Xing W, Li S, et al. Modulation of smooth muscle gene expression by association of histone acetyltransferases and deacetylases with myocardin. Molecular and cellular biology. 2005;25(1):364-76.
14. Misárková E, Behuliak M, Bencze M, Zicha J. Excitation-contraction coupling and excitation-transcription coupling in blood vessels: their possible interactions in hypertensive vascular remodeling. Physiological Research. 2016;65(2):173.
15. Qin X, Wang X, Wang Y, Tang Z, Cui Q, Xi J, et al. MicroRNA-19a mediates the suppressive effect of laminar flow on cyclin D1 expression in human umbilical vein endothelial cells. Proc Natl Acad Sci U S A. 2010;107(7):3240-4.
16. Kohlstedt K, Trouvain C, Boettger T, Shi L, Fisslthaler B, Fleming I. AMP-activated protein kinase regulates endothelial cell angiotensin-converting enzyme expression via p53 and the post-transcriptional regulation of microRNA-143/145. Circ Res. 2013;112(8):1150-8.
17. Davies PF, Manduchi E, Stoeckert CJ, Jiménez JM, Jiang Y-Z. Emerging topic: flow-related epigenetic regulation of endothelial phenotype through DNA methylation. Vascular pharmacology. 2014;62(2):88-93.
18. Guay SP, Brisson D, Lamarche B, Biron S, Lescelleur O, Biertho L, et al. ADRB3 gene promoter DNA methylation in blood and visceral adipose tissue is associated with metabolic disturbances in men. Epigenomics. 2014;6(1):33-43.
19. Wang F, Demura M, Cheng Y, Zhu A, Karashima S, Yoneda T, et al. Dynamic CCAAT/enhancer binding protein-associated changes of DNA methylation in the angiotensinogen gene. Hypertension. 2014;63(2):281-8.
20. Campion J, Milagro FI, Martinez JA. Individuality and epigenetics in obesity. Obes Rev. 2009;10(4):383-92.
21. Coats A, Jain S. Protective effects of nebivolol from oxidative stress to prevent hypertension-related target organ damage. J Hum Hypertens. 2017.

22. Ilatovskaya DV, Pavlov TS, Levchenko V, Staruschenko A. ROS production as a common mechanism of ENaC regulation by EGF, insulin, and IGF-1. Am J Physiol Cell Physiol. 2013;304(1):C102-11.
23. Chia N, Wang L, Lu X, Senut MC, Brenner C, Ruden DM. Hypothesis: environmental regulation of 5-hydroxymethylcytosine by oxidative stress. Epigenetics. 2011;6(7):853-6.
24. Archer SL. Acquired Mitochondrial Abnormalities, Including Epigenetic Inhibition of Superoxide Dismutase 2, in Pulmonary Hypertension and Cancer: Therapeutic Implications. Adv Exp Med Biol. 2016;903:29-53.
25. Nozik-Grayck E, Woods C, Stearman RS, Venkataraman S, Ferguson BS, Swain K, et al. Histone deacetylation contributes to low extracellular superoxide dismutase expression in human idiopathic pulmonary arterial hypertension. Am J Physiol Lung Cell Mol Physiol. 2016;311(1):L124-34.
26. Hirooka Y, Kishi T, Ito K, Sunagawa K. Potential clinical application of recently discovered brain mechanisms involved in hypertension. Hypertension. 2013;62(6):995-1002.
27. Esler M, Alvarenga M, Pier C, Richards J, El-Osta A, Barton D, et al. The neuronal noradrenaline transporter, anxiety and cardiovascular disease. J Psychopharmacol. 2006;20(4 Suppl):60-6.
28. Esler M, Eikelis N, Schlaich M, Lambert G, Alvarenga M, Kaye D, et al. Human sympathetic nerve biology: parallel influences of stress and epigenetics in essential hypertension and panic disorder. Ann N Y Acad Sci. 2008;1148:338-48.
29. Marques FZ, Campain AE, Tomaszewski M, Zukowska-Szczechowska E, Yang YH, Charchar FJ, et al. Gene expression profiling reveals renin mRNA overexpression in human hypertensive kidneys and a role for microRNAs. Hypertension. 2011;58(6):1093-8.
30. Hultstrom M. Development of structural kidney damage in spontaneously hypertensive rats. J Hypertens. 2012;30(6):1087-91.
31. Dobrian AD, Schriver SD, Prewitt RL. Role of angiotensin II and free radicals in blood pressure regulation in a rat model of renal hypertension. Hypertension. 2001;38(3):361-6.
32. Zhang D, Yu ZY, Cruz P, Kong Q, Li S, Kone BC. Epigenetics and the control of epithelial sodium channel expression in collecting duct. Kidney Int. 2009;75(3):260-7.

33. Nishimoto M, Fujita T. Renal mechanisms of salt-sensitive hypertension: contribution of two steroid receptor-associated pathways. Am J Physiol Renal Physiol. 2015;308(5):F377-87.
34. Oudit GY, Penninger JM. Cardiac regulation by phosphoinositide 3-kinases and PTEN. Cardiovasc Res. 2009;82(2):250-60.
35. Kang H, Chang W, Hurley M, Vignery A, Wu D. Important roles of PI3Kgamma in osteoclastogenesis and bone homeostasis. Proc Natl Acad Sci U S A. 2010;107(29):12901-6.
36. Benigni A, Cassis P, Remuzzi G. Angiotensin II revisited: new roles in inflammation, immunology and aging. EMBO Mol Med. 2010;2(7):247-57.
37. Weber G, Pushpakumar S, Sen U. Hydrogen Sulfide Alleviates Hypertensive Kidney Dysfunction through an Epigenetic Mechanism. Am J Physiol Heart Circ Physiol. 2017:ajpheart 00637 2016.
38. Arpon A, Riezu-Boj JI, Milagro FI, Razquin C, Martinez-Gonzalez MA, Corella D, et al. Adherence to Mediterranean diet is associated with methylation changes in inflammation-related genes in peripheral blood cells. J Physiol Biochem. 2017.
39. Chung AS, Lee J, Ferrara N. Targeting the tumour vasculature: insights from physiological angiogenesis. Nat Rev Cancer. 2010;10(7):505-14.
40. Henning RJ. Therapeutic angiogenesis: angiogenic growth factors for ischemic heart disease. Future Cardiol. 2016;12(5):585-99.
41. Marek-Trzonkowska N, Kwieczynska A, Reiwer-Gostomska M, Kolinski T, Molisz A, Siebert J. Arterial Hypertension Is Characterized by Imbalance of Pro-Angiogenic versus Anti-Angiogenic Factors. PLoS One. 2015;10(5):e0126190.
42. Nicoli S, Standley C, Walker P, Hurlstone A, Fogarty KE, Lawson ND. MicroRNA-mediated integration of haemodynamics and Vegf signalling during angiogenesis. Nature. 2010;464(7292):1196-200.
43. Otsuka M, Zheng M, Hayashi M, Lee JD, Yoshino O, Lin S, et al. Impaired microRNA processing causes corpus luteum insufficiency and infertility in mice. J Clin Invest. 2008;118(5):1944-54.

44. Bonauer A, Carmona G, Iwasaki M, Mione M, Koyanagi M, Fischer A, et al. MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice. Science. 2009;324(5935):1710-3.
45. Kato N, Loh M, Takeuchi F, Verweij N, Wang X, Zhang W, et al. Trans-ancestry genome-wide association study identifies 12 genetic loci influencing blood pressure and implicates a role for DNA methylation. Nature genetics. 2015;47(11):1282-93.
46. Raftopoulos L, Katsi V, Makris T, Tousoulis D, Stefanadis C, Kallikazaros I. Epigenetics, the missing link in hypertension. Life sciences. 2015;129:22-6.
47. Udali S, Guarini P, Moruzzi S, Choi SW, Friso S. Cardiovascular epigenetics: from DNA methylation to microRNAs. Mol Aspects Med. 2013;34(4):883-901.
48. Friso S, Carvajal CA, Fardella CE, Olivieri O. Epigenetics and arterial hypertension: the challenge of emerging evidence. Transl Res. 2015;165(1):154-65.
49. Pei F, Wang X, Yue R, Chen C, Huang J, Huang J, et al. Differential expression and DNA methylation of angiotensin type 1A receptors in vascular tissues during genetic hypertension development. Mol Cell Biochem. 2015;402(1-2):1-8.
50. De Vries N, Prestes P, Rana I, Harrap SB, Charchar FJ. YIA 03-04 EPIGENETIC CHANGES AFTER ACUTE TREATMENT WITH ACUTE ANGIOTENSIN CONVERTING ENZYME INHIBITORS (ACEi). J Hypertens. 2016;34 Suppl 1 - ISH 2016 Abstract Book:e204-e5.
51. Chu A, Gozal D, Cortese R, Wang Y. Cardiovascular dysfunction in adult mice following postnatal intermittent hypoxia. Pediatr Res. 2015;77(3):425-33.
52. Ast J, Jablecka A, Bogdanski P, Smolarek I, Krauss H, Chmara E. Evaluation of the antihypertensive effect of L-arginine supplementation in patients with mild hypertension assessed with ambulatory blood pressure monitoring. Med Sci Monit. 2010;16(5):CR266-71.
53. Seckl JR, Meaney MJ. Glucocorticoid programming. Ann N Y Acad Sci. 2004;1032:63-84.
54. Pojoga LH, Williams JS, Yao TM, Kumar A, Raffetto JD, do Nascimento GR, et al. Histone demethylase LSD1 deficiency during high-salt diet is associated with enhanced vascular contraction, altered NO-cGMP relaxation pathway, and hypertension. Am J Physiol Heart Circ Physiol. 2011;301(5):H1862-71.

55. Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, Hill JA, et al. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. Dev Cell. 2008;15(2):261-71.
56. Menghini R, Casagrande V, Cardellini M, Martelli E, Terrinoni A, Amati F, et al. MicroRNA 217 modulates endothelial cell senescence via silent information regulator 1. Circulation. 2009;120(15):1524-32.
57. Sabatel C, Malvaux L, Bovy N, Deroanne C, Lambert V, Gonzalez ML, et al. MicroRNA-21 exhibits antiangiogenic function by targeting RhoB expression in endothelial cells. PLoS One. 2011;6(2):e16979.
58. Sequeira-Lopez ML, Weatherford ET, Borges GR, Monteagudo MC, Pentz ES, Harfe BD, et al. The microRNA-processing enzyme dicer maintains juxtaglomerular cells. J Am Soc Nephrol. 2010;21(3):460-7.
59. Li D, Yang P, Xiong Q, Song X, Yang X, Liu L, et al. MicroRNA-125a/b-5p inhibits endothelin-1 expression in vascular endothelial cells. J Hypertens. 2010;28(8):1646-54.
60. Pankratz F, Bemtgen X, Zeiser R, Leonhardt F, Kreuzaler S, Hilgendorf I, et al. MicroRNA-155 Exerts Cell-Specific Antiangiogenic but Proarteriogenic Effects During Adaptive Neovascularization. Circulation. 2015;131(18):1575-89.
61. Liao YC, Wang YS, Guo YC, Lin WL, Chang MH, Juo SH. Let-7g improves multiple endothelial functions through targeting transforming growth factor-beta and SIRT-1 signaling. J Am Coll Cardiol. 2014;63(16):1685-94.
62. Xin M, Small EM, Sutherland LB, Qi X, McAnally J, Plato CF, et al. MicroRNAs miR-143 and miR-145 modulate cytoskeletal dynamics and responsiveness of smooth muscle cells to injury. Genes Dev. 2009;23(18):2166-78.
63. Head GA, Gueguen C, Marques FZ, Jackson KL, Eikelis N, Stevenson ER, et al. 6b.01: Effect of Renal Denervation on Blood Pressure and Microrna 181a in Hypertensive Schlager Mice. J Hypertens. 2015;33 Suppl 1:e76.
64. Marques FZ, Booth SA, Charchar FJ. The emerging role of non-coding RNA in essential hypertension and blood pressure regulation. J Hum Hypertens. 2015;29(8):459-67.
65. Liu Y, Taylor NE, Lu L, Usa K, Cowley AW, Jr., Ferreri NR, et al. Renal medullary microRNAs in Dahl salt-sensitive rats: miR-29b regulates several collagens and related genes. Hypertension. 2010;55(4):974-82.

66. Yang Q, Jia C, Wang P, Xiong M, Cui J, Li L, et al. MicroRNA-505 identified from patients with essential hypertension impairs endothelial cell migration and tube formation. Int J Cardiol. 2014;177(3):925-34.
67. Li S, Zhu J, Zhang W, Chen Y, Zhang K, Popescu LM, et al. Signature microRNA expression profile of essential hypertension and its novel link to human cytomegalovirus infection. Circulation. 2011;124(2):175-84.
68. Cengiz M, Karatas OF, Koparir E, Yavuzer S, Ali C, Yavuzer H, et al. Differential expression of hypertension-associated microRNAs in the plasma of patients with white coat hypertension. Medicine (Baltimore). 2015;94(13):e693.
69. Karolina DS, Tavintharan S, Armugam A, Sepramaniam S, Pek SL, Wong MT, et al. Circulating miRNA profiles in patients with metabolic syndrome. J Clin Endocrinol Metab. 2012;97(12):E2271-6.
70. Gu Q, Wang B, Zhang XF, Ma YP, Liu JD, Wang XZ. Contribution of renin-angiotensin system to exercise-induced attenuation of aortic remodeling and improvement of endothelial function in spontaneously hypertensive rats. Cardiovasc Pathol. 2014;23(5):298-305.
71. Haugen AC, Schug TT, Collman G, Heindel JJ. Evolution of DOHaD: the impact of environmental health sciences. J Dev Orig Health Dis. 2015;6(2):55-64.
72. Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet. 1986;1(8489):1077-81.
73. Hoy WE, Hughson MD, Bertram JF, Douglas-Denton R, Amann K. Nephron number, hypertension, renal disease, and renal failure. J Am Soc Nephrol. 2005;16(9):2557-64.
74. Paixao AD, Alexander BT. How the kidney is impacted by the perinatal maternal environment to develop hypertension. Biol Reprod. 2013;89(6):144.
75. Hogg K, Blair JD, McFadden DE, von Dadelszen P, Robinson WP. Early onset pre-eclampsia is associated with altered DNA methylation of cortisol-signalling and steroidogenic genes in the placenta. PLoS One. 2013;8(5):e62969.
76. Drake AJ, McPherson RC, Godfrey KM, Cooper C, Lillycrop KA, Hanson MA, et al. An unbalanced maternal diet in pregnancy associates with offspring epigenetic changes in genes controlling glucocorticoid action and foetal growth. Clin Endocrinol (Oxf). 2012;77(6):808-15.

77. Pham TD, MacLennan NK, Chiu CT, Laksana GS, Hsu JL, Lane RH. Uteroplacental insufficiency increases apoptosis and alters p53 gene methylation in the full-term IUGR rat kidney. Am J Physiol Regul Integr Comp Physiol. 2003;285(5):R962-70.
78. DuPont JJ, McCurley A, Davel AP, McCarthy J, Bender SB, Hong K, et al. Vascular mineralocorticoid receptor regulates microRNA-155 to promote vasoconstriction and rising blood pressure with aging. JCI Insight. 2016;1(14):e88942.
79. Parthenakis F, Marketou M, Kontaraki J, Patrianakos A, Nakou H, Touloupaki M, et al. Low Levels of MicroRNA-21 Are a Marker of Reduced Arterial Stiffness in Well-Controlled Hypertension. J Clin Hypertens (Greenwich). 2016.
80. Li H, Zhang X, Wang F, Zhou L, Yin Z, Fan J, et al. MicroRNA-21 Lowers Blood Pressure in Spontaneous Hypertensive Rats by Upregulating Mitochondrial Translation. Circulation. 2016;134(10):734-51.
81. Courboulin A, Paulin R, Giguere NJ, Saksouk N, Perreault T, Meloche J, et al. Role for miR-204 in human pulmonary arterial hypertension. J Exp Med. 2011;208(3):535-48.
82. Rothman AM, Arnold ND, Pickworth JA, Iremonger J, Ciuclan L, Allen RM, et al. MicroRNA-140-5p and SMURF1 regulate pulmonary arterial hypertension. J Clin Invest. 2016;126(7):2495-508.
83. Gubrij IB, Pangle AK, Pang L, Johnson LG. Reversal of MicroRNA Dysregulation in an Animal Model of Pulmonary Hypertension. PLoS One. 2016;11(1):e0147827.
84. Kontaraki JE, Marketou ME, Zacharis EA, Parthenakis FI, Vardas PE. MicroRNA-9 and microRNA-126 expression levels in patients with essential hypertension: potential markers of target-organ damage. J Am Soc Hypertens. 2014;8(6):368-75.
85. Kontaraki JE, Marketou ME, Parthenakis FI, Maragkoudakis S, Zacharis EA, Petousis S, et al. Hypertrophic and antihypertrophic microRNA levels in peripheral blood mononuclear cells and their relationship to left ventricular hypertrophy in patients with essential hypertension. J Am Soc Hypertens. 2015;9(10):802-10.
86. Kontaraki JE, Marketou ME, Zacharis EA, Parthenakis FI, Vardas PE. Differential expression of vascular smooth muscle-modulating microRNAs in human peripheral blood mononuclear cells: novel targets in essential hypertension. J Hum Hypertens. 2014;28(8):510-6.

87. Rhodes CJ, Wharton J, Boon RA, Roexe T, Tsang H, Wojciak-Stothard B, et al. Reduced microRNA-150 is associated with poor survival in pulmonary arterial hypertension. Am J Respir Crit Care Med. 2013;187(3):294-302.
88. Zhu N, Zhang D, Chen S, Liu X, Lin L, Huang X, et al. Endothelial enriched microRNAs regulate angiotensin II-induced endothelial inflammation and migration. Atherosclerosis. 2011;215(2):286-93.
89. Eskildsen TV, Jeppesen PL, Schneider M, Nossent AY, Sandberg MB, Hansen PB, et al. Angiotensin II regulates microRNA-132/-212 in hypertensive rats and humans. Int J Mol Sci. 2013;14(6):11190-207.
90. Kumar S, Kim CW, Simmons RD, Jo H. Role of flow-sensitive microRNAs in endothelial dysfunction and atherosclerosis: mechanosensitive athero-miRs. Arterioscler Thromb Vasc Biol. 2014;34(10):2206-16.
91. Harris TA, Yamakuchi M, Kondo M, Oettgen P, Lowenstein CJ. Ets-1 and Ets-2 regulate the expression of microRNA-126 in endothelial cells. Arterioscler Thromb Vasc Biol. 2010;30(10):1990-7.
92. Cheng HS, Sivachandran N, Lau A, Boudreau E, Zhao JL, Baltimore D, et al. MicroRNA-146 represses endothelial activation by inhibiting pro-inflammatory pathways. EMBO Mol Med. 2013;5(7):1017-34.
93. Yang Y, Zhou Y, Cao Z, Tong XZ, Xie HQ, Luo T, et al. miR-155 functions downstream of angiotensin II receptor subtype 1 and calcineurin to regulate cardiac hypertrophy. Exp Ther Med. 2016;12(3):1556-62.
94. Wei C, Henderson H, Spradley C, Li L, Kim IK, Kumar S, et al. Circulating miRNAs as potential marker for pulmonary hypertension. PLoS One. 2013;8(5):e64396.
95. Park MY, Herrmann SM, Saad A, Widmer RJ, Tang H, Zhu XY, et al. Circulating and renal vein levels of microRNAs in patients with renal artery stenosis. Nephrol Dial Transplant. 2015;30(3):480-90.
96. Marques FZ, Romaine SP, Denniff M, Eales J, Dormer J, Garrelds IM, et al. Signatures of miR-181a on renal transcriptome and blood pressure. Mol Med. 2015.
97. Mandraffino G, Imbalzano E, Sardo MA, D'Ascola A, Mamone F, Lo Gullo A, et al. Circulating progenitor cells in hypertensive patients with different degrees of cardiovascular involvement. J Hum Hypertens. 2014;28(9):543-50.

98. Thulasingam S, Massilamany C, Gangaplara A, Dai H, Yarbaeva S, Subramaniam S, et al. miR-27b*, an oxidative stress-responsive microRNA modulates nuclear factor-kB pathway in RAW 264.7 cells. Mol Cell Biochem. 2011;352(1-2):181-8.
99. Zhang W, Yan L, Li Y, Chen W, Hu N, Wang H, et al. Roles of miRNA-24 in regulating endothelial nitric oxide synthase expression and vascular endothelial cell proliferation. Mol Cell Biochem. 2015;405(1-2):281-9.
100. Sun HX, Zeng DY, Li RT, Pang RP, Yang H, Hu YL, et al. Essential role of microRNA-155 in regulating endothelium-dependent vasorelaxation by targeting endothelial nitric oxide synthase. Hypertension. 2012;60(6):1407-14.
101. Magenta A, Greco S, Gaetano C, Martelli F. Oxidative stress and microRNAs in vascular diseases. Int J Mol Sci. 2013;14(9):17319-46.
102. Wang L, Yuan Y, Li J, Ren H, Cai Q, Chen X, et al. MicroRNA-1 aggravates cardiac oxidative stress by post-transcriptional modification of the antioxidant network. Cell Stress Chaperones. 2015;20(3):411-20.
103. Hulsmans M, Holvoet P. The vicious circle between oxidative stress and inflammation in atherosclerosis. J Cell Mol Med. 2010;14(1-2):70-8.
104. Celic T, Metzinger-Le Meuth V, Six I, Massy ZA, Metzinger L. The mir-221/222 Cluster is a Key Player in Vascular Biology via the Fine-Tuning of Endothelial Cell Physiology. Curr Vasc Pharmacol. 2017;15(1):40-6.
105. Santovito D, Mandolini C, Marcantonio P, De Nardis V, Bucci M, Paganelli C, et al. Overexpression of microRNA-145 in atherosclerotic plaques from hypertensive patients. Expert Opin Ther Targets. 2013;17(3):217-23.
106. Li N, Hwangbo C, Jaba IM, Zhang J, Papangeli I, Han J, et al. miR-182 Modulates Myocardial Hypertrophic Response Induced by Angiogenesis in Heart. Sci Rep. 2016;6:21228.

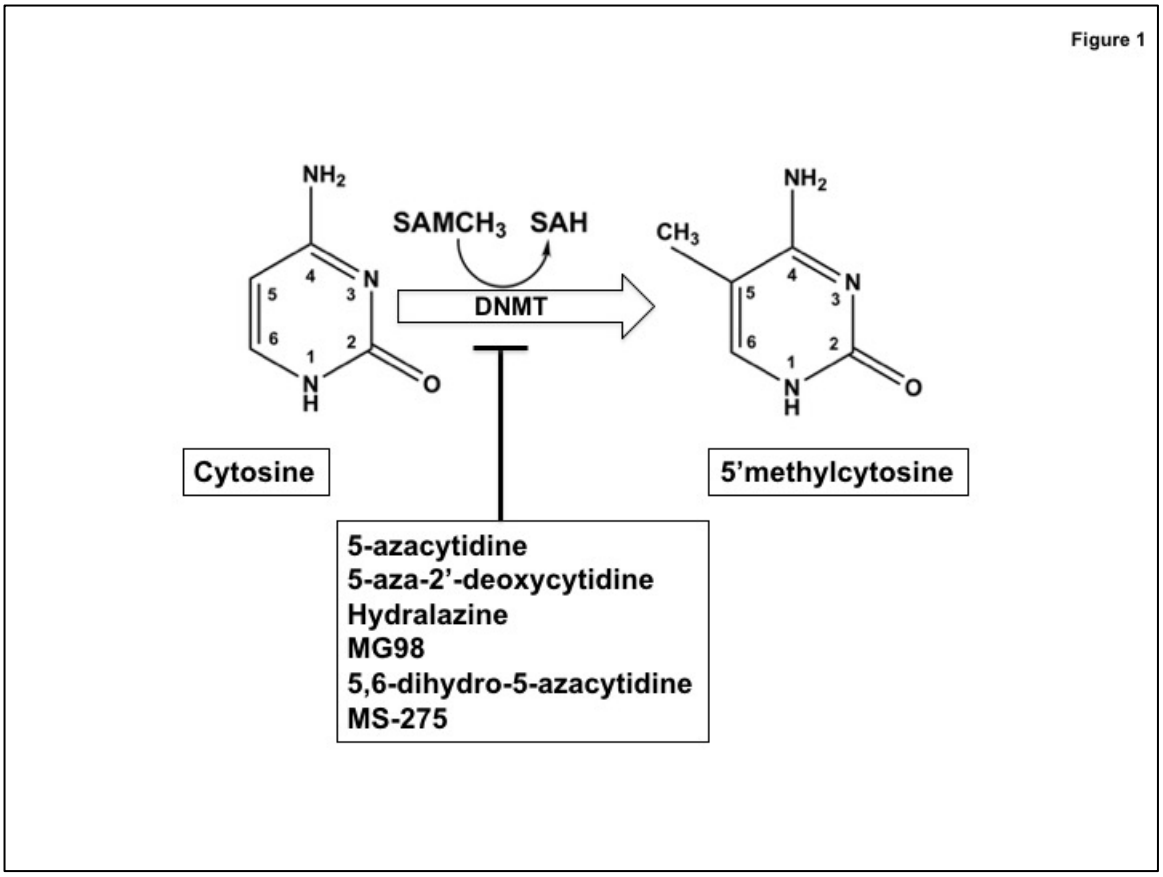

FIGURE 1. DNA methylation process for the regulation of gene expression.

DNA methylation predominantly occurs at the 5-carbon of cytosine base ring. The methyl deposition is catalyzed by DNA methyltransferase proteins (DNMTs) to produce 5-methylcytosine. DNMTs use S-adenosyl-methionine (SAM) as a primary methyl group donor and is converted to S-adenosine homocysteine (SAH) during the process. 5- methylcytosine is frequently located in clusters of CpG dinucleotides, called CpG islands, which are frequently found in gene promoter regions. 5- methylcytosine is generally a repressive mark since it acts by blocking gene transcription. Three conserved DNMT enzymes are responsible for the methyl mark: DNMT3A and DNMT3B catalyse *de novo* DNA methylation, whereas DNMT1 represents the maintenance enzyme, which restores the fully methylated state of DNA after replication. DNMT inhibitors can modulate aberrant DNA methylation pattern in a reversible way, which may be efficiently exploited for epigenetic therapy.

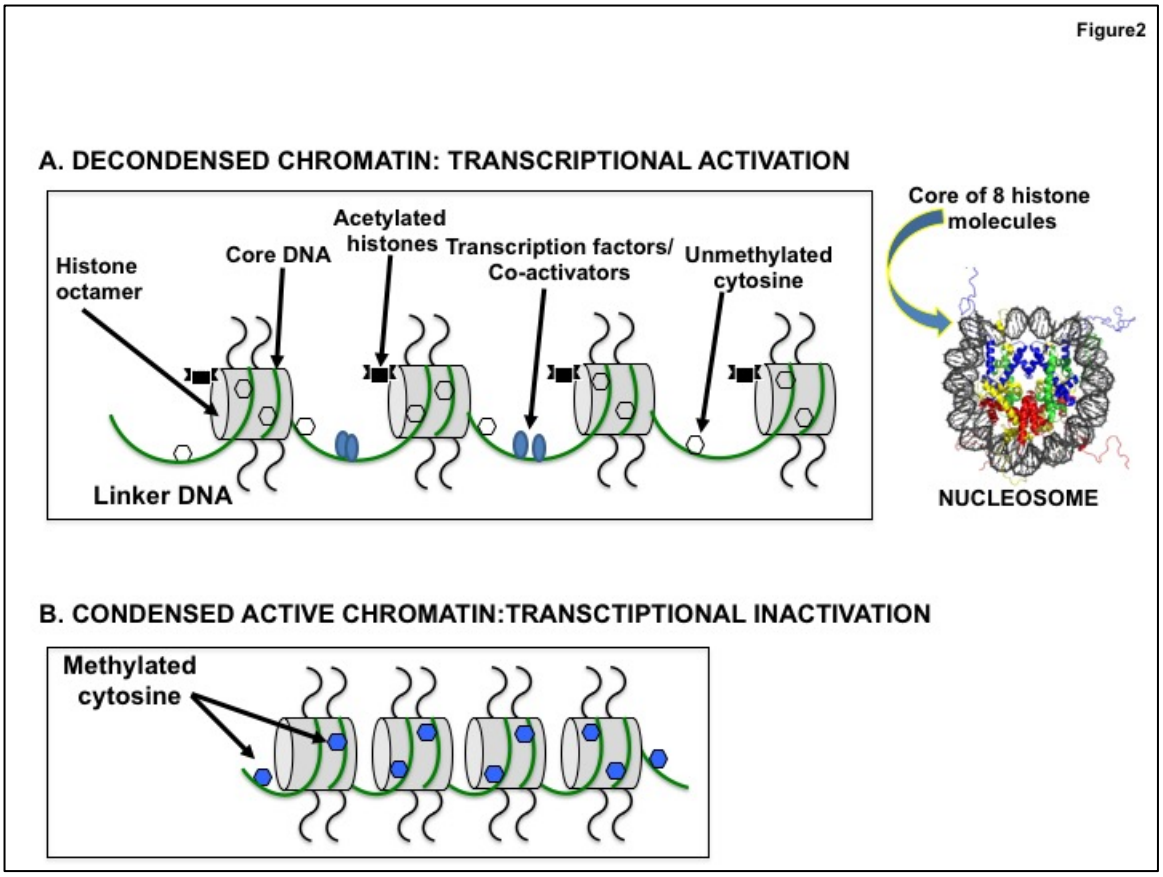

FIGURE 2. Chromatin remodelling as a function of histone modification.

The basic unit of chromatin is the nucleosome, which comprises DNA wrapped around a core of histone proteins. Changes in histones through methylation or acetylation, occurring at N-terminal histone tails, impact gene expression through chromatin remodelling. Such modifications disturb the binding affinity between histones and DNA depending on their degree of loosening or lightening. Histone acetylation allows the access of transcription factors to DNA via the conversion of condensed chromatin (heterochromatin) into a more relaxed structure (euchromatin), which favours gene expression. In contrast, methylation of specific lysine residues in H3 and H4 mediates gene silencing and is able to directly interfere with the binding of transcription factors.

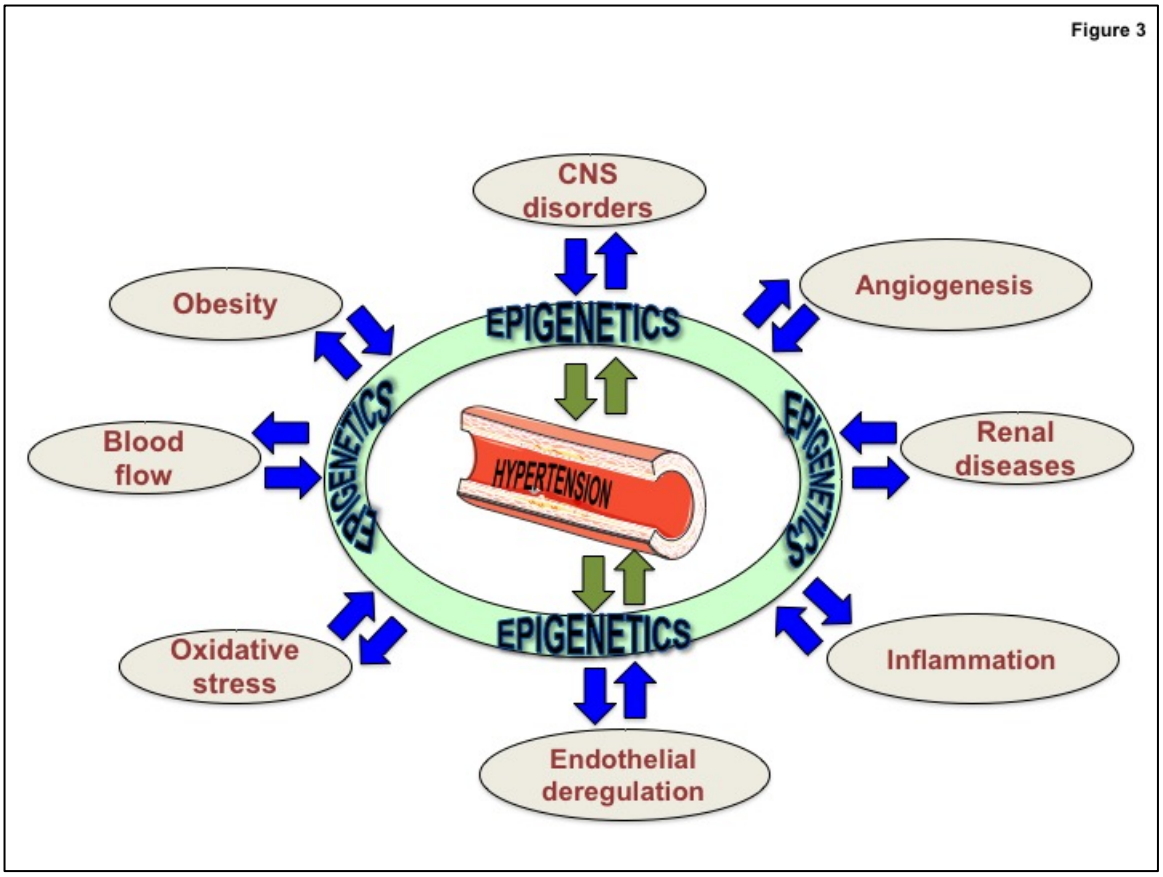

FIGURE 3. Epigenetic modifications as the potential link between hypertension and its related disorders.

Hypertension is recognized as a major risk factor for the development of various disorders, which in turn can contribute to its development, progression and damaging effects. Oxidative stress, inflammation, angiogenesis, blood flow, endothelial deregulation, obesity, central nervous system (CSN) and renal diseases are among the different mechanisms influencing blood pressure via epigenetic modifications. Conversely, epigenetic alterations elicited by hypertension may predispose or exacerbate the outlined disorders.

DISCUSSION/CONCLUSION

Plusieurs travaux de recherche ont été réalisés en préparation de cette thèse de doctorat. L'ensemble des projets sont reliés entre eux et ont un lien commun avec les désordres cardiométaboliques, notamment l'obésité, l'insulinorésistance et autres composantes culminant avec le développement du SyM. Avant de discuter minutieusement l'ensemble de nos résultats, une préface de notre programme scientifique a été élaborée pour faciliter la compréhension aux différents lecteurs.

Des protéines clés jouent un rôle essentiel dans le transport des graisses et formation des CM. Leurs défauts génétiques engendrent la malabsorption intestinale des lipides, mais leur surexpression comme c'est le cas de la protéine Sar1b GTPase, en réponse à des polymorphismes, à une alimentation sous optimale et à un style de vie inadéquat (sédentaire) transforment l'intestin en un organe insulino-résistant, ce qui déclenche une « hyperabsorption » des lipides via la formation exagérée des CM, qui finit par produire une dyslipidémie postprandiale (**Articles 1-2**).

Les conséquences de la dyslipidémie favorisent également une insulinorésistance systémique qui, avec la formation des résidus de CM et des AG, va affecter le foie, entre autres pour accentuer le dépôt lipidique et contribuer au développement de la NAFLD (**Article 1-2**).

À cette fin, plusieurs mécanismes pathophysiologiques sont alors initiés au niveau hépatique et incluent le SOx, l'inflammation, les dysfonctions mitochondriales, la lipogenèse, la gluconéogenèse et la dérégulation de plusieurs facteurs de transcription (**Article 3**).

Des aliments exhibant des propriétés hypolipidémiantes, antioxydantes et anti-inflammatoires, tels que les AGPI n-3, peuvent tendre à normaliser les conditions cardiométaboliques et pathophysiologiques, en freinant par exemple, la progression de la NAFLD vers le NASH (**Article 4-5**).

En support de ce programme de recherche, des revues de littérature critiques sont venues étayer et élargir ces concepts (**Revues 1-3**).

La compréhension des fonctions assumées par l'intestin et particulièrement, l'axe foie-intestin a marqué un tournant extraordinaire durant la dernière décade. Non seulement, l'intestin est actif dans la digestion et l'absorption des aliments, permettant ainsi leur passage de la lumière de l'intestin vers le milieu intérieur, mais il est équipé de systèmes coriaces à la fois nerveux «deuxième cerveau», immunitaire (rempart de défense) et métagénomique (microbiote) (662, 663). Évidemment, les différentes actions de ces systèmes agissent en symbiose. Notre laboratoire a contribué substantiellement au dévoilement de la majorité de ces rôles en soulignant également les désordres sous-jacents (664-670). En particulier, notre laboratoire a mis en évidence la double nature des protéines clés engendrant des troubles intestinaux relatifs au transport des lipides. Si d'un côté, des défauts génétiques dans les étapes clés produisent une malabsorption sévère des graisses, l'hyperabsorption, causée par exemple par un environnement nutritionnel riche en lipides, résulte en une surproduction de CM, une dyslipidémie postprandiale et l'athérosclérose.

Un des objectifs de mon travail doctoral était de participer activement à la consolidation de ce concept et de démontrer en outre l'existence d'un axe « intestin-foie ». En prenant comme exemple le Sar1b GTPase, une protéine essentielle au transport des CM du réticulum endoplasmique à l'appareil de Golgi (179, 667, 671-676), nous devons confronter les questions cardinales suivantes : *(i) Si la déficience de Sar1b empêche les vésicules du complexe COPII à se former et à transporter le cargo de CMs, résultant en une malabsorption lipidique sévère, est-ce qu'à l'opposé la surexpression de Sar1b engendre une surproduction des CM édifiant une dyslipidémie?; (ii) Quelle est la répercussion de la surexpression de Sar1b sur les fonctions cardiométaboliques comme l'insulinorésistance?; (iii) Comment la surexpression de Sar1b influence la condition hépatique?; et (iv) quelle est la distribution tissulaire de la Sar1b suite à sa surexpression?*

Pour apporter les réponses à ces interrogations, le modèle de souris transgénique $Sar1b^{+/+}$ a été créé. L'exposition des animaux à une diète riche en lipides a engendré: une augmentation

du poids corporel, de l'adiposité, de l'insulinorésistance, de la dyslipidémie et de la stéatose hépatique, ainsi qu'une altération les AGPI n-3 et une surproduction des CM suite à un gavage lipidique (172).

Donc, en plus de son rôle bien établi dans la mobilisation de COPII intracellulaire et le trafic des CM, le Sar1b GTPase peut réguler certaines fonctions liées à l'homéostasie du glucose et des lipides. Effectivement, même si la consommation de diète riche en lipides est généralement accompagnée de troubles cardiométaboliques (677-679), la présence de Sar1b GTPase en grande quantité stimulait encore davantage les désordres énoncés. Pour la première fois, nos expériences *in vivo* ont découvert l'implication directe de Sar1b GTPase dans l'accélération du transport des lipides intestinaux (172).

Alors que les mécanismes régulant son activité et son expression demeurent inconnues, nous proposons que, dans les situations où le flux CM-TG est stimulé par la charge graisseuse alimentaire, le Sar1b GTPase peut induire un tableau clinique plus préjudiciable. Cet effet nuisible est probablement occasionné par la disponibilité excessive des AG, produits par la lipolyse des CM, qui interfèrent avec la captation et la combustion du glucose, suscitant ainsi une insulinorésistance (680). D'ailleurs, en analysant la distribution de *SAR1B* dans les tissus périphériques, l'organe ayant l'expression génique la plus élevée est le muscle squelettique (suivi par le cœur et le foie) (178), muni de la plus grande capacité de captation des AG et de leur oxydation (681). En outre, la relation intestin-foie se trouve renforcée par la surexpression de Sar1b GTPase, qui a résulté en une stéatose hépatique, probablement à cause de la captation exagérée des CM en relation avec la dyslipidémie causée par l'intestin (172). Cependant, des efforts additionnels sont requis pour découvrir les mécanismes d'action décisifs.

La NAFLD est devenue la condition la plus fréquente parmi les pathologies affligeant le foie (584, 682). Cette accumulation de graisses, asymptomatique à son initiation mais lourde de conséquences par la suite, accompagne généralement l'obésité et le SyM. Sa progression vers le NASH, la fibrose, la cirrhose et le carcinome, constitue une menace redoutable (683). Même si la NAFLD devient un problème de santé publique majeur et inquiétant dans le monde entier, les causes sont assez méconnues. Des études pointent les interactions entre la prédisposition

génétique (371, 684) et les facteurs environnementaux (185, 584, 685-688) comme agents étiologiques, mais ce prédicat peut émaner essentiellement de l'obésité et du SyM escortant la NAFLD (185, 584). Les mécanismes d'action ne sont pas beaucoup plus élaborés étant donné les ambiguïtés apparentes dans la littérature scientifique proposant surtout l'abondance des AG mobilisés par le tissu adipeux et la RI (689), mais ces propositions peuvent être également attribuables aux désordres cardio-métaboliques convoyant la NAFLD (185, 584).

En outre, les travaux expérimentaux, bien qu'extrêmement profitables, sont généralement menés dans des modèles animaux et ne peuvent donc être extrapolés en toute quiétude à l'humain. De surcroît, l'investigation de la NAFLD a priorisé l'adulte quoique les enfants et adolescents en sont de plus en plus atteints puisque la stéatose hépatique touche près de 10% des enfants et 20 à 40% (1/4) des enfants obèses (690). Plus saisissant encore est le fait qu'il n'existe à ce jour aucun consensus de traitement officiel et aucun médicament éprouvé pour la prise en charge efficace des patients avec la NAFLD. Finalement, aucune attention n'a été donnée à la population Canadienne-Française, qui est principalement et historiquement connue pour ses risques excessifs athérosclérotiques.

Pour l'ensemble de ces raisons, une grande portion de cet ouvrage de doctorat a été consacrée à (*i) l'approfondissement des mécanismes étiologiques de la NAFLD en optant pour la stratégie d'œuvrer directement sur les foies humains obtenus suite à la chirurgie bariatrique; (ii) la compréhension de la physiopathologie de la NAFLD chez les enfants/adolescents; et (iii) la détermination de la réponse biochimique et clinique à une intervention nutritionnelle employant les AGPI n-3 auprès de sujets pédiatriques Canadiens-Français.*

De ce fait, la NAFLD est cliniquement difficile à diagnostiquer et la plupart des patients sont asymptomatiques, ce qui retarde la prise en charge rapide de la maladie. En outre, une pharmacothérapie efficace contre la NAFLD est limitée, en particulier chez les enfants et les adolescents (691). Par conséquent, il existe un besoin pressant d'identifier et de contrôler les voies moléculaires responsables qui peuvent donner de nouveaux outils thérapeutiques. À l'heure actuelle, même si des efforts intensifs ont produit des progrès considérables dans notre compréhension de la NAFLD (584), les mécanismes précis restent fragmentaires. De plus, très

peu d'études ont utilisé des spécimens hépatiques humains pour mettre en évidence les troubles métaboliques hépatiques et donc les mécanismes latents, mais ils se sont surtout concentrés sur des modèles animaux certes instructifs mais pouvant présenter des difficultés d'extrapolation à l'humain.

La possibilité d'obtenir des échantillons de foie de patients atteints d'obésité morbide pendant la chirurgie bariatrique nous a permis de traiter des questions suivantes : les individus en surpoids sains et en mauvaise santé/non-sains peuvent-ils être différenciés sur le plan métabolique en termes de stéatose et de complications hépatiques ? Quelles sont les anomalies lipidiques qui caractérisent les obèses métaboliquement anormaux? Existe-t-il une association entre les deux compartiments hépatique et systémique ? Quels sont les mécanismes dans le foie derrière la NAFLD ? Ces problématiques sont de grand intérêt car ce ne sont pas tous les individus présentant un excès de poids qui développent le SyM et progressent vers une complication hépatique, et les termes «obésité métaboliquement saine et non saine avec stéatose modérée ou sévère» ont été utilisés pour désigner ces individus. De plus, en ayant recours à l'analyse protéomique, est-il possible de découvrir une signature spécifique, qui mettra davantage l'accent sur les mécanismes de la NAFLD et pourrait identifier des cibles de traitement ou des marqueurs prédictifs?

Le présent travail montre d'abord que la NAFLD coexistait seulement chez les patients adultes obèses présentant des caractéristiques du SyM et une stéatose variant de minimale, modérée à sévère. 1) Nous les avons définis (Mi-NAFLD), comme des sujets insulinosensibles (sans RI) et présentant une stéatose minime de grade 1 (~8%); des patients avec une stéatose modérée (Mo-NAFLD) et sévère (Se-NAFLD), présentant une obésité morbide, une RI, un grade et un % de stéatose allant, respectivement, de (2, ~16%) à (>3, ~60%). 2). Leur tissu hépatique a révélé une teneur élevée en TG, ce qui confirme les résultats histologiques et les transaminases hépatiques élevées définies biochimiquement, mais les sujets Se-NAFLD avaient, en plus, une chute des PL, due essentiellement à une diminution de la PC, et un faible ratio PC/PE. 3) Leur tissu hépatique subissait le SOx et l'inflammation. 4) Leur composition en AG hépatiques a été perturbée par la diminution des AGPI, l'augmentation des AG saturés et l'accumulation des lipides dans le foie. 5) Cette observation a été validée par l'augmentation

des SP, l'index de lipogenèse (16:00/18:2n-6) et SCD1 (18:1n-9/18:0) hépatiques; des biomarqueurs clés dans la régulation de la lipogenèse hépatique et l'oxydation des lipides dans la NAFLD (692, 693). 6) Cette observation a aussi été validée dans leurs échantillons de foie qui ont montré une régulation à la hausse de la lipogenèse et une régulation négative de la ß-oxydation, comme le montrent les expressions protéiques des biomarqueurs spécifiques de ACC phosphorylé/FAS et de CPT-1/ACADL, respectivement. 7) Finalement, conformément à ces résultats, des altérations dans de puissants facteurs de transcription ont été notées (SREBP1c/PPARg).

Le foie est un organe cible du SyM, entraînant une NAFLD, au point que cette dernière est considérée comme une composante du SyM (694). À l'exception de l'hypertension, toutes les autres composantes du SyM (obésité, RI et dyslipidémie) étaient présentes dans le groupe Mo-NAFLD et Se-NAFLD. En particulier, l'insulinorésistance, qui représente le mécanisme pivot dans la NAFLD primaire, était significativement plus élevée dans le groupe de sujets avec stéatose sévère, comme le reflète l'indice HOMA élevé. Dans ces conditions, il semble raisonnable de s'attendre à ce que l'insulinorésistance induise SREBP-1c, qui peut conduire à une régulation positive des gènes impliqués dans la lipogenèse *de novo*, tels que FAS et ACC, favorisant ainsi la synthèse hépatique des AG et des TG (695). Cependant, seule l'expression de la protéine FAS a été stimulée alors que la protéine ACC, l'enzyme clé de la synthèse *de novo* d'AG, n'a pas répondu au facteur de transcription SREBP-1c. Par contre, l'activité de l'ACC a pu être stimulée par le peu de sa phosphorylation en Ser, probablement une conséquence du déficit en p-AMPK, connu pour phosphoryler et inactiver l'ACC (181). Ce stratagème explique comment la lipogenèse est régie par deux mécanismes à la fois, et dépend des facteurs de transcription SREBP-1c et de l'AMPK, respectivement.

Le foie est muni d'une capacité énorme pour la β-oxydation mitochondriale des AG. Comme la stéatose dans la NAFLD peut résulter non seulement de la lipogenèse, mais aussi de la diminution de la β-oxydation des AG, nous avons évalué les deux biomarqueurs importants de cette dernière soit la CPT-1 (considérée à la fois comme la passerelle mitochondriale dictant l'entrée des AG dans la matrice et comme le modulateur hépatique principal du flux oxydatif des AG (696)), et ACADL, l'enzyme qui catalyse la première étape de la β-oxydation

mitochondriale des esters d'acyl-CoA gras (697). Les deux enzymes de dégradation ont été régulées à la baisse dans le foie des patients Se-NAFLD. Puisque le PPARα est exprimé principalement dans les tissus riches en mitochondries, comme le foie, et qu'il est un facteur important régulant la β-oxydation des AG (698-700), nous avons également déterminé son expression protéique. L'analyse par immunobuvardage indiquait une chute de la masse protéique de PPARα. Il est possible que le déclin de PPARα affecte sa liaison avec le PPARγ response element (PPRE) situé sur le promoteur de CPT1 (701) ou via la surexpression de PPARγ qui stimule également l'activité de SCD1via PPRE sur le promoteur de SCD1 (702). D'autres mécanismes potentiels pouvant affecter la dégradation des AG dans le foie des patients Se-NAFLD incluent l'hypersinsulinémie/l'insulinorésistance (703) et la faible régulation de l'ACC par phosphorylation, conduisant à l'activation de l'ACC et à la surproduction du malonyl-CoA, un inhibiteur du CPT-1.

Chez la population pédiatrique d'origine Canadienne-Française, comparativement aux sujets sains, les patients diagnostiqués NAFLD sur la base de l'élévation de transaminases sanguines, de score d'activité NAFLD (NAS) et d'indice de foie gras (FLI), présentaient des anomalies évidentes d'IMC, de sensibilité à l'insuline, de lipides, d'adipokines et de facteurs inflammatoires. Leur composition d'AG dans les érythrocytes affichait des taux moindres de DHA et EPA, entrainant la diminution de l'indice oméga-3, constituant ainsi un risque élevé de maladies cardiovasculaires. Bien entendu, le diagnostic de NAFLD a été formulé en absence de consommation d'alcool, de traitement hépatotoxique et d'affection hépatique. À première vue, quatre éléments apparaissent évidents dans le tableau clinique de nos jeunes patients avec l'accumulation intrahépatique lipidique: (i) le lien direct avec l'IMC confirmant que l'obésité favorise généralement le développement de NAFLD; (ii) l'étroite association avec l'indice élevé de HOMA corroborant que l'insulinorésistance joue un rôle dominant dans la survenue de NAFLD; (iii) la coexistence avec plusieurs composantes du SyM validant l'allégation que ce syndrome prédispose au développement de la NAFLD; et (iv) la prédominance du genre mâle dans notre clinique consolidant la conception que le genre masculin est plus à risque de manifester la NAFLD, et expliquant le choix d'intégrer uniquement les mâles dans le présent travail. Dans ce dernier contexte, il est pertinent de souligner que la population du Québec, en

particulier les hommes, est plus apte à développer des troubles cardiométaboliques (704, 705) et qu'il y a une prédominance de garçons et d'hommes souffrant d'obésité, de SyM et de NAFLD selon diverses cliniques et études de méta-analyse, NHANES III et Statistiques Canada (301, 706-709).

La supplémentation en AGPI n-3 pendant 6 mois a entraîné une augmentation significative des concentrations de EPA et de DHA dans les globules rouges ainsi qu'une atténuation de la stéatose hépatique, reflétée par la réduction du rapport FLI, ALT et ALT/AST. De plus, la supplémentation a amélioré le profil lipidique et l'épaisseur de l'intima-média carotidienne, tout en augmentant l'adiponectine et le SOx. Nous concluons que dans l'absence actuelle de moyens thérapeutiques, les mesures diététiques et spécialement les AGPI n-3 demeurent l'avenue la plus appropriée pour traiter la NAFLD. Par ailleurs, comme la NAFLD survient fréquemment dans le contexte d'obésité, d'insulinorésistance et de DT2, une prise en charge agressive de tous les facteurs cardiométaboliques s'impose de façon primordiale afin d'éviter la progression de la NAFLD et les complications cardiovasculaires. Les résultats issus de cette intervention, nous ont permis de cibler des biomarqueurs biochimiques susceptibles de prédire l'avènement de la NAFLD chez les jeunes adolescents.

De plus, les interventions nutritionnelles sont encore peu nombreuses et demandent plus de conciliation avec les secteurs de l'agroalimentaire (agriculture, transformation et distribution des aliments, recherche et développement, « marketing » des produits alimentaires, etc.), de l'environnement bâti (aménagement du territoire, organisation du transport, planification urbaine, code du bâtiment, etc.), et du socioculturel (médias, publicités, mode, industries « du corps », conciliation travail-famille, politique familiale, lutte à la discrimination à l'égard du poids). La grande compréhension de ces modifications sociétales nous aidera à affaiblir l'escalade de l'obésité infantile dans toutes les parties du monde, qui en fait va de pair avec la multiplication des offres de restauration rapide, la consommation croissante de boissons sucrées, l'approche publicitaire intensive et agressive de l'industrie agroalimentaire ou encore la prolifération des jeux vidéo; ces dernières étant le plus souvent incriminées.

NAFLD et NASH sont des maladies multifactorielles impliquant une ramification de plusieurs événements intracellulaires. Étant donné que le SOx semble être parmi les processus les plus significatifs à transmettre des lésions hépatiques, nous avons effectué une analyse critique de la littérature pour mettre en relief les mécanismes conduisant à son développement. Notre revue a d'abord bien établi le lien de la NAFLD/NASH avec d'un côté le SOx amplifié et d'un autre côté la déficience de la défense antioxydante endogène (584). Parmi les mécanismes rapportés, on retrouve (i) les systèmes pro-oxydants tels que le cytochrome P450, la lipo-oxygénase, la cyclo-oxygénase et les produits radicalaires (347); (ii) la dysfonction de la mitochondrie, une source primaire des radicaux libres (710), culminant dans le développement et la progression de NAFLD (711-713); (iii) l'induction de la peroxydation des LDL dont les concentrations sanguines peuvent même prédire la sévérité de l'histologie hépatique chez les sujets avec NAFLD (714, 715); (iv) la libération de la myéloperoxydase par les neutrophiles, une enzyme dotée d'un potentiel oxydant puissant dans le foie (716); (v) les interactions entre le SOx et le stress du RE amplifiant considérablement le SOx (717); (vi) la dérégulation des miRNAs contribuant aux dérangements métaboliques (718), au SOx et par conséquent à la NAFLD (719-721); et (vii) les mutations génétiques et les polymorphismes provoquant des anomalies dans le système antioxydant (p. ex. la SOD2) (378, 722).

Si le SOx est actuellement reconnu comme un mécanisme central contribuant à la NAFLD et à ses complications (p. ex. NASH), plusieurs groupes de chercheurs ont raisonnablement entrepris des essais expérimentaux et cliniques avec des agents naturels antioxydants ou connus pour moduler le SOx (p. ex. la vitamine E, la vitamine D, le coenzyme Q, les polyphénols, les AGPI n-3, et les pré-probiotiques). Néanmoins, malgré les diverses données obtenues, il y a encore un besoin important d'essais robustes et bien contrôlés, faisant appel à de grandes cohortes, pour traduire les données probantes en contexte clinique et pour prouver l'efficacité de l'intervention, ce qui constitue une tâche ardue pour les chercheurs biomédicaux.

Comme le SOx est pivot dans la genèse du SyM qui, lui-même, promeut la NAFLD, nous avons également scruté la littérature afin d'en déduire les mécanismes sous-jacents (185). La compréhension détaillée des mécanismes du SyM est cruciale pour avancer à la fois le développement de stratégies de prévention efficaces et des outils d'intervention adéquats qui

pourraient freiner sa prévalence croissante et limiter ses comorbidités, en particulier la NAFLD, le DT2 et les MCV. En collectant les données de la littérature, il nous est apparu que le SOx est la cause première de l'initiation et l'évolution de SyM tout en constituant le mécanisme unificateur de ses composantes : l'obésité, la dyslipidémie, la RI, et l'hypertension. Très rapidement, la mitochondrie s'est dessinée comme la source du SOx et de ce fait la cible thérapeutique de choix. Les antioxydants, ciblant son SOx et son dysfonctionnement intrinsèque, peuvent non seulement éliminer les désordres mitochondriaux, mais ils ont également la capacité d'amoindrir l'accumulation de lipides ectopiques et les troubles du microbiote intestinal, améliorant ainsi le SyM et ses co-morbidités. Il est à noter que même si le SOx ne constitue pas le seul mécanisme suscitant les désordres cardiométaboliques, sa présence constitue un ''générateur'' d'inflammation, de troubles lipidiques, d'aberrations moléculaires et autres phénomènes qui finissent par l'amplifier et créer un cercle vicieux.

De plus, nous avions résumé et commenté sur les modifications intervenant dans la régulation épigénétique de gènes contrôlant les composantes du SyM, en particulier l'hypertension artérielle (718). Nous avons d'abord décomposé la physiopathologie de l'hypertension artérielle en ses facteurs étiologiques, et par la suite chacun en conjonction avec les changements épigénétiques qui lui sont propres. Nous avons relevé les lacunes et souligné le besoin d'effectuer des études supplémentaires afin de combler les déficiences et élucider de plus près les mécanismes de régulation impliquant la méthylation de l'ADN, les modifications d'histone, le remodelage de la chromatine et l'identité des miRNA. De plus, nous avons insisté sur la nécessité de considérer l'hypertension artérielle comme une entité clinique assez centrale regroupant une combinaison de mécanismes épigénétiques. Une meilleure vue de ses interactions pourrait nous aider à trouver des cibles thérapeutiques pour combattre les troubles cardiométaboliques et cardiovasculaires.

PERSPECTIVES ET LIMITES

L'atteinte des objectifs de cette présente thèse a permis : (i) de démontrer que l'élévation de protéines clés intra-entérocytaires peut être associée à un déclenchement de la dyslipidémie postprandiale et à la progression du SyM; (ii) d'identifier des biomarqueurs susceptibles d'améliorer la prise en charge de l'obésité; et (iii) d'identifier la population à risque de développer la NAFLD, et d'intervenir avec des approches thérapeutiques nutritionnelles.

Les perspectives découlant de notre travail sont tout à fait attrayantes et pertinentes aux trois aspects mentionnés ci-dessus :

(i) Il serait fascinant d'examiner si les polymorphismes de gain de fonction de la Sar1b GTPase chez l'humain sont associés à la dyslipidémie postprandiale et aux risques cardiométaboliques. Par ailleurs, nous avons mis l'emphase spécifiquement sur la Sar1b GTPase, mais d'autres facteurs cruciaux (ex. phospholipase D1) participant activement à la formation/sécrétion des CMs peuvent constituer des candidats extrêmement intéressants à investiguer.

(ii) La NAFLD survient fréquemment dans le contexte d'obésité, d'insulinorésistance et de DT2. Cependant, des études élégantes ont suggéré que la NAFLD peut être en amont des risques cardiométaboliques. Cette éventualité peut être testée dans un modèle animal approprié, ne présentant que la NAFLD afin de comprendre les mécanismes favorisant l'éclosion des risques cardiométaboliques. En outre, il serait captivant d'explorer si des nutriments fonctionnels peuvent inhiber le transit vers le SyM, le DT2 et les MCV.

(iii) En outre, nos travaux sur la NAFLD employant l'approche protéomique a révélé certains biomarqueurs métaboliques. Il serait fascinant de vérifier leur rôle dans des modèles cellulaires et animaux avec des modifications génétiques conduisant à leur surexpression ou à leur invalidation.

(iv) Finalement, comme on est à l'ère de la médecine de précision, il serait séduisant de déterminer si la nutrition personnalisée ciblant distincts biomarqueurs biochimiques de la NAFLD améliorait efficacement l'état métabolique. Cette perspective pourrait être examinée dans un modèle animal avant de la tester chez l'humain.

(v) Une autre voie à tester serait de dévoiler si le profil des dérivés des acides gras et les différents ratios et enzymes impliqués seraient des prédicteurs diagnostiques et thérapeutiques de la NAFLD.

Parmi les limites de nos études, notons le nombre restreint de patients suivis avec NAFLD, souvent le peu de disponibilité des échantillons biologiques, ce qui nous a empêché d'aller scruter à fond les mécanismes d'action, et finalement, le manque de l'opportunité de tester les résultats obtenus dans le modèle animal chez l'humain.

BIBLIOGRAPHIE

1. Anis AH, Zhang W, Bansback N, Guh DP, Amarsi Z, Birmingham CL. Obesity and overweight in Canada: an updated cost-of-illness study. Obesity reviews : an official journal of the International Association for the Study of Obesity. 2010;11(1):31-40.
2. Bouchard C. Current understanding of the etiology of obesity: genetic and nongenetic factors. The American journal of clinical nutrition. 1991;53(6 Suppl):1561S-5S.
3. Carmelli D, Cardon LR, Fabsitz R. Clustering of hypertension, diabetes, and obesity in adult male twins: same genes or same environments? American journal of human genetics. 1994;55(3):566-73.
4. Haslam DW, James WP. Obesity. Lancet. 2005;366(9492):1197-209.
5. Schwimmer JB, Deutsch R, Rauch JB, Behling C, Newbury R, Lavine JE. Obesity, insulin resistance, and other clinicopathological correlates of pediatric nonalcoholic fatty liver disease. The Journal of pediatrics. 2003;143(4):500-5.
6. Swinburn BA, Sacks G, Hall KD, McPherson K, Finegood DT, Moodie ML, et al. The global obesity pandemic: shaped by global drivers and local environments. Lancet. 2011;378(9793):804-14.
7. Szabo G, Bala S, Petrasek J, Gattu A. Gut-liver axis and sensing microbes. Digestive diseases. 2010;28(6):737-44.
8. O'Rahilly S, Farooqi IS. Human obesity: a heritable neurobehavioral disorder that is highly sensitive to environmental conditions. Diabetes. 2008;57(11):2905-10.
9. Rankinen T, Zuberi A, Chagnon YC, Weisnagel SJ, Argyropoulos G, Walts B, et al. The human obesity gene map: the 2005 update. Obesity. 2006;14(4):529-644.
10. Mellin LM, Slinkard LA, Irwin CE, Jr. Adolescent obesity intervention: validation of the SHAPEDOWN program. Journal of the American Dietetic Association. 1987;87(3):333-8.
11. Reilly JJ, Wilson ML, Summerbell CD, Wilson DC. Obesity: diagnosis, prevention, and treatment; evidence based answers to common questions. Archives of disease in childhood. 2002;86(6):392-4.

12. Whitaker RC, Wright JA, Pepe MS, Seidel KD, Dietz WH. Predicting obesity in young adulthood from childhood and parental obesity. The New England journal of medicine. 1997;337(13):869-73.
13. Larson NI, Wall MM, Story MT, Neumark-Sztainer DR. Home/family, peer, school, and neighborhood correlates of obesity in adolescents. Obesity. 2013;21(9):1858-69.
14. Dowd JB, Zajacova A. Long-term obesity and cardiovascular, inflammatory, and metabolic risk in U.S. adults. American journal of preventive medicine. 2014;46(6):578-84.
15. Kopelman PG. Obesity as a medical problem. Nature. 2000;404(6778):635-43.
16. Malik VS, Willett WC, Hu FB. Global obesity: trends, risk factors and policy implications. Nature reviews Endocrinology. 2013;9(1):13-27.
17. Popkin BM, Adair LS, Ng SW. Global nutrition transition and the pandemic of obesity in developing countries. Nutrition reviews. 2012;70(1):3-21.
18. Lobstein T, Jackson-Leach R, Moodie ML, Hall KD, Gortmaker SL, Swinburn BA, et al. Child and adolescent obesity: part of a bigger picture. Lancet. 2015.
19. Troiano RP, Flegal KM, Kuczmarski RJ, Campbell SM, Johnson CL. Overweight prevalence and trends for children and adolescents. The National Health and Nutrition Examination Surveys, 1963 to 1991. Archives of pediatrics & adolescent medicine. 1995;149(10):1085-91.
20. Krauss RM, Deckelbaum RJ, Ernst N, Fisher E, Howard BV, Knopp RH, et al. Dietary guidelines for healthy American adults. A statement for health professionals from the Nutrition Committee, American Heart Association. Circulation. 1996;94(7):1795-800.
21. Astrup A, Dyerberg J, Selleck M, Stender S. Nutrition transition and its relationship to the development of obesity and related chronic diseases. Obesity reviews : an official journal of the International Association for the Study of Obesity. 2008;9 Suppl 1:48-52.
22. Popkin BM. The nutrition transition in low-income countries: an emerging crisis. Nutrition reviews. 1994;52(9):285-98.
23. Moore JX, Chaudhary N, Akinyemiju T. Metabolic Syndrome Prevalence by Race/Ethnicity and Sex in the United States, National Health and Nutrition Examination Survey, 1988-2012. Prev Chronic Dis. 2017;14:E24.

24. Setayeshgar S, Whiting SJ, Vatanparast H. Metabolic syndrome in canadian adults and adolescents: prevalence and associated dietary intake. ISRN Obes. 2012;2012:816846.
25. Grundy SM, Brewer HB, Jr., Cleeman JI, Smith SC, Jr., Lenfant C, American Heart A, et al. Definition of metabolic syndrome: Report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. Circulation. 2004;109(3):433-8.
26. Isomaa B, Almgren P, Tuomi T, Forsen B, Lahti K, Nissen M, et al. Cardiovascular morbidity and mortality associated with the metabolic syndrome. Diabetes Care. 2001;24(4):683-9.
27. Lakka HM, Laaksonen DE, Lakka TA, Niskanen LK, Kumpusalo E, Tuomilehto J, et al. The metabolic syndrome and total and cardiovascular disease mortality in middle-aged men. JAMA. 2002;288(21):2709-16.
28. Klijs B, Angelini V, Mierau JO, Smidt N. The role of life-course socioeconomic and lifestyle factors in the intergenerational transmission of the metabolic syndrome: results from the LifeLines Cohort Study. Int J Epidemiol. 2016;45(4):1236-46.
29. Huang TT, Ball GD, Franks PW. Metabolic syndrome in youth: current issues and challenges. Appl Physiol Nutr Metab. 2007;32(1):13-22.
30. Reaven GM. Banting lecture 1988. Role of insulin resistance in human disease. Diabetes. 1988;37(12):1595-607.
31. Dandona P, Aljada A, Chaudhuri A, Mohanty P, Garg R. Metabolic syndrome: a comprehensive perspective based on interactions between obesity, diabetes, and inflammation. Circulation. 2005;111(11):1448-54.
32. Sandholt CH, Hansen T, Pedersen O. Beyond the fourth wave of genome-wide obesity association studies. Nutrition & diabetes. 2012;2:e37.
33. Silventoinen K, Rokholm B, Kaprio J, Sorensen TI. The genetic and environmental influences on childhood obesity: a systematic review of twin and adoption studies. International journal of obesity. 2010;34(1):29-40.
34. Mathes WF, Aylor DL, Miller DR, Churchill GA, Chesler EJ, de Villena FP, et al. Architecture of energy balance traits in emerging lines of the Collaborative Cross. American journal of physiology Endocrinology and metabolism. 2011;300(6):E1124-34.

35. Finucane MM, Stevens GA, Cowan MJ, Danaei G, Lin JK, Paciorek CJ, et al. National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9.1 million participants. Lancet. 2011;377(9765):557-67.
36. Ng M, Fleming T, Robinson M, Thomson B, Graetz N, Margono C, et al. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet. 2014;384(9945):766-81.
37. Xia Q, Grant SF. The genetics of human obesity. Annals of the New York Academy of Sciences. 2013;1281:178-90.
38. Virtanen M, Kivimaki H, Ervasti J, Oksanen T, Pentti J, Kouvonen A, et al. Fast-food outlets and grocery stores near school and adolescents' eating habits and overweight in Finland. European journal of public health. 2015.
39. Van Hulst A, Roy-Gagnon MH, Gauvin L, Kestens Y, Henderson M, Barnett TA. Identifying risk profiles for childhood obesity using recursive partitioning based on individual, familial, and neighborhood environment factors. The international journal of behavioral nutrition and physical activity. 2015;12(1):175.
40. Fuemmeler BF, Stroo M, Lee CT, Bazemore S, Blocker B, Ostbye T. Racial Differences in Obesity-Related Risk Factors Between 2-Year-Old Children Born of Overweight Mothers. Journal of pediatric psychology. 2015.
41. Bauer KW, Neumark-Sztainer D, Fulkerson JA, Story M. Adolescent girls' weight-related family environments, Minnesota. Preventing chronic disease. 2011;8(3):A68.
42. Bauer KW, Neumark-Sztainer D, Fulkerson JA, Hannan PJ, Story M. Familial correlates of adolescent girls' physical activity, television use, dietary intake, weight, and body composition. The international journal of behavioral nutrition and physical activity. 2011;8:25.
43. Davison KK, Birch LL. Childhood overweight: a contextual model and recommendations for future research. Obesity reviews : an official journal of the International Association for the Study of Obesity. 2001;2(3):159-71.

44. Booth SL, Sallis JF, Ritenbaugh C, Hill JO, Birch LL, Frank LD, et al. Environmental and societal factors affect food choice and physical activity: rationale, influences, and leverage points. Nutrition reviews. 2001;59(3 Pt 2):S21-39; discussion S57-65.
45. El-Sayed Moustafa JS, Froguel P. From obesity genetics to the future of personalized obesity therapy. Nature reviews Endocrinology. 2013;9(7):402-13.
46. Blakemore AI, Froguel P. Investigation of Mendelian forms of obesity holds out the prospect of personalized medicine. Annals of the New York Academy of Sciences. 2010;1214:180-9.
47. O'Rahilly S. Human genetics illuminates the paths to metabolic disease. Nature. 2009;462(7271):307-14.
48. Blakemore AI, Froguel P. Is obesity our genetic legacy? The Journal of clinical endocrinology and metabolism. 2008;93(11 Suppl 1):S51-6.
49. Walley AJ, Asher JE, Froguel P. The genetic contribution to non-syndromic human obesity. Nature reviews Genetics. 2009;10(7):431-42.
50. Saeed S, Butt TA, Anwer M, Arslan M, Froguel P. High prevalence of leptin and melanocortin-4 receptor gene mutations in children with severe obesity from Pakistani consanguineous families. Mol Genet Metab. 2012;106(1):121-6.
51. Dubern B, Clement K. Leptin and leptin receptor-related monogenic obesity. Biochimie. 2012;94(10):2111-5.
52. Ranadive SA, Vaisse C. Lessons from extreme human obesity: monogenic disorders. Endocrinol Metab Clin North Am. 2008;37(3):733-51, x.
53. Santini F, Maffei M, Pelosini C, Salvetti G, Scartabelli G, Pinchera A. Melanocortin-4 receptor mutations in obesity. Adv Clin Chem. 2009;48:95-109.
54. Girardet C, Butler AA. Neural melanocortin receptors in obesity and related metabolic disorders. Biochimica et biophysica acta. 2014;1842(3):482-94.
55. Philippe J, Stijnen P, Meyre D, De Graeve F, Thuillier D, Delplanque J, et al. A nonsense loss-of-function mutation in PCSK1 contributes to dominantly inherited human obesity. International journal of obesity. 2015;39(2):295-302.
56. Traurig M, Mack J, Hanson RL, Ghoussaini M, Meyre D, Knowler WC, et al. Common variation in SIM1 is reproducibly associated with BMI in Pima Indians. Diabetes. 2009;58(7):1682-9.

57. Loos RJ. Recent progress in the genetics of common obesity. British journal of clinical pharmacology. 2009;68(6):811-29.
58. Gerken T, Girard CA, Tung YC, Webby CJ, Saudek V, Hewitson KS, et al. The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase. Science. 2007;318(5855):1469-72.
59. Dina C, Meyre D, Gallina S, Durand E, Korner A, Jacobson P, et al. Variation in FTO contributes to childhood obesity and severe adult obesity. Nature genetics. 2007;39(6):724-6.
60. Melka MG, Gillis J, Bernard M, Abrahamowicz M, Chakravarty MM, Leonard GT, et al. FTO, obesity and the adolescent brain. Human molecular genetics. 2013;22(5):1050-8.
61. Ho AJ, Stein JL, Hua X, Lee S, Hibar DP, Leow AD, et al. A commonly carried allele of the obesity-related FTO gene is associated with reduced brain volume in the healthy elderly. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(18):8404-9.
62. Wen W, Cho YS, Zheng W, Dorajoo R, Kato N, Qi L, et al. Meta-analysis identifies common variants associated with body mass index in east Asians. Nature genetics. 2012;44(3):307-11.
63. Berndt SI, Gustafsson S, Magi R, Ganna A, Wheeler E, Feitosa MF, et al. Genome-wide meta-analysis identifies 11 new loci for anthropometric traits and provides insights into genetic architecture. Nature genetics. 2013;45(5):501-12.
64. Bradfield JP, Taal HR, Timpson NJ, Scherag A, Lecoeur C, Warrington NM, et al. A genome-wide association meta-analysis identifies new childhood obesity loci. Nature genetics. 2012;44(5):526-31.
65. Jiao H, Arner P, Hoffstedt J, Brodin D, Dubern B, Czernichow S, et al. Genome wide association study identifies KCNMA1 contributing to human obesity. BMC Med Genomics. 2011;4:51.
66. Thorleifsson G, Walters GB, Gudbjartsson DF, Steinthorsdottir V, Sulem P, Helgadottir A, et al. Genome-wide association yields new sequence variants at seven loci that associate with measures of obesity. Nature genetics. 2009;41(1):18-24.

67. Okada Y, Kubo M, Ohmiya H, Takahashi A, Kumasaka N, Hosono N, et al. Common variants at CDKAL1 and KLF9 are associated with body mass index in east Asian populations. Nature genetics. 2012;44(3):302-6.
68. Speliotes EK, Willer CJ, Berndt SI, Monda KL, Thorleifsson G, Jackson AU, et al. Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. Nature genetics. 2010;42(11):937-48.
69. Paternoster L, Evans DM, Nohr EA, Holst C, Gaborieau V, Brennan P, et al. Genome-wide population-based association study of extremely overweight young adults--the GOYA study. PloS one. 2011;6(9):e24303.
70. Kim HJ, Yoo YJ, Ju YS, Lee S, Cho SI, Sung J, et al. Combined linkage and association analyses identify a novel locus for obesity near PROX1 in Asians. Obesity. 2013;21(11):2405-12.
71. Lango Allen H, Estrada K, Lettre G, Berndt SI, Weedon MN, Rivadeneira F, et al. Hundreds of variants clustered in genomic loci and biological pathways affect human height. Nature. 2010;467(7317):832-8.
72. Cotsapas C, Speliotes EK, Hatoum IJ, Greenawalt DM, Dobrin R, Lum PY, et al. Common body mass index-associated variants confer risk of extreme obesity. Human molecular genetics. 2009;18(18):3502-7.
73. Meyre D, Delplanque J, Chevre JC, Lecoeur C, Lobbens S, Gallina S, et al. Genome-wide association study for early-onset and morbid adult obesity identifies three new risk loci in European populations. Nature genetics. 2009;41(2):157-9.
74. Dorajoo R, Blakemore AI, Sim X, Ong RT, Ng DP, Seielstad M, et al. Replication of 13 obesity loci among Singaporean Chinese, Malay and Asian-Indian populations. International journal of obesity. 2012;36(1):159-63.
75. Wang K, Li WD, Zhang CK, Wang Z, Glessner JT, Grant SF, et al. A genome-wide association study on obesity and obesity-related traits. PloS one. 2011;6(4):e18939.
76. Kilpelainen TO, Zillikens MC, Stancakova A, Finucane FM, Ried JS, Langenberg C, et al. Genetic variation near IRS1 associates with reduced adiposity and an impaired metabolic profile. Nature genetics. 2011;43(8):753-60.

77. Shuldiner AR, Yang R, Gong DW. Resistin, obesity and insulin resistance--the emerging role of the adipocyte as an endocrine organ. The New England journal of medicine. 2001;345(18):1345-6.
78. Sinha R, Fisch G, Teague B, Tamborlane WV, Banyas B, Allen K, et al. Prevalence of impaired glucose tolerance among children and adolescents with marked obesity. The New England journal of medicine. 2002;346(11):802-10.
79. Gaenzer H. Impaired glucose tolerance in obese children and adolescents. The New England journal of medicine. 2002;347(4):290-2; author reply -2.
80. Wanless IR, Lentz JS. Fatty liver hepatitis (steatohepatitis) and obesity: an autopsy study with analysis of risk factors. Hepatology. 1990;12(5):1106-10.
81. Serafino-Agrusa L, Spatafora M, Scichilone N. Asthma and metabolic syndrome: Current knowledge and future perspectives. World journal of clinical cases. 2015;3(3):285-92.
82. Pakhale S, Baron J, Dent R, Vandemheen K, Aaron SD. Effects of weight loss on airway responsiveness in obese asthmatics: Does weight loss lead to reversibility of asthma? Chest. 2015.
83. Agrawal S, Duseja A, Aggarwal A, Das A, Mehta M, Dhiman RK, et al. Obstructive sleep apnea is an important predictor of hepatic fibrosis in patients with nonalcoholic fatty liver disease in a tertiary care center. Hepatology international. 2015.
84. Hu W, Jin X, Gu J, Zhang P, Yu Q, Yin G, et al. Risk factor panels associated with hypertension in obstructive sleep apnea patients with different body mass indexes. Journal of the American Society of Hypertension : JASH. 2015.
85. Akyuz F, Uyanikoglu A, Ermis F, Arici S, Akyuz U, Baran B, et al. Gastroesophageal reflux in asymptomatic obese subjects: An esophageal impedance-pH study. World journal of gastroenterology : WJG. 2015;21(10):3030-4.
86. Huseini M, Wood GC, Seiler J, Argyropoulos G, Irving BA, Gerhard GS, et al. Gastrointestinal symptoms in morbid obesity. Frontiers in medicine. 2014;1:49.
87. Hershkovich O, Friedlander A, Gordon B, Arzi H, Derazne E, Tzur D, et al. Association between body mass index, body height, and the prevalence of spinal deformities. The spine journal : official journal of the North American Spine Society. 2014;14(8):1581-7.

88. Rosner B, Eliassen AH, Toriola AT, Hankinson SE, Willett WC, Natarajan L, et al. Short-term weight gain and breast cancer risk by hormone receptor classification among pre- and postmenopausal women. Breast cancer research and treatment. 2015.
89. Oberman B, Khaku A, Camacho F, Goldenberg D. Relationship between obesity, diabetes and the risk of thyroid cancer. American journal of otolaryngology. 2015.
90. de Cobelli O, Terracciano D, Tagliabue E, Raimondi S, Galasso G, Cioffi A, et al. Body mass index was associated with upstaging and upgrading in patients with low-risk prostate cancer who met the inclusion criteria for active surveillance. Urologic oncology. 2015.
91. Booth A, Magnuson A, Fouts J, Foster M. Adipose tissue, obesity and adipokines: role in cancer promotion. Hormone molecular biology and clinical investigation. 2015;21(1):57-74.
92. Maciel GA, Moreira RP, Bugano DD, Hayashida SA, Marcondes JA, Gomes LG, et al. Association of glucocorticoid receptor polymorphisms with clinical and metabolic profiles in polycystic ovary syndrome. Clinics. 2014;69(3):179-84.
93. Hammoud AO, Gibson M, Peterson CM, Meikle AW, Carrell DT. Impact of male obesity on infertility: a critical review of the current literature. Fertility and sterility. 2008;90(4):897-904.
94. Bloui CV, N.; Diogo Bary, A.; Hamel, E.; Martel, S. Amadou Diogo Barry Yun Jen Denis Hamel Ernest Lo Sylvie Martel (2015). Les conséquences économiques associées à l'obésité et à l'embonpoint au Québec : les coûts liés à l'hospitalisation et aux consultations médicales. Quebec: Institut National de Santé Publique du Québec; 2015.
95. Hashimoto Y, Hamaguchi M, Fukuda T, Obora A, Kojima T, Fukui M. Weight gain since age of 20 as risk of metabolic syndrome even in non-overweight individuals. Endocrine. 2017.
96. Maffetone PB, Rivera-Dominguez I, Laursen PB. Overfat Adults and Children in Developed Countries: The Public Health Importance of Identifying Excess Body Fat. Front Public Health. 2017;5:190.
97. Yusuf S, Hawken S, Ounpuu S, Dans T, Avezum A, Lanas F, et al. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. Lancet. 2004;364(9438):937-52.

98. Cetin D, Lessig BA, Nasr E. Comprehensive Evaluation for Obesity: Beyond Body Mass Index. J Am Osteopath Assoc. 2016;116(6):376-82.
99. Lee WS. Body fatness charts based on BMI and waist circumference. Obesity. 2016;24(1):245-9.
100. Choi B, Steiss D, Garcia-Rivas J, Kojaku S, Schnall P, Dobson M, et al. Comparison of body mass index with waist circumference and skinfold-based percent body fat in firefighters: adiposity classification and associations with cardiovascular disease risk factors. Int Arch Occup Environ Health. 2016;89(3):435-48.
101. Motamed N, Rabiee B, Hemasi GR, Ajdarkosh H, Khonsari MR, Maadi M, et al. Body Roundness Index and Waist-to-Height Ratio are Strongly Associated With Non-Alcoholic Fatty Liver Disease: A Population-Based Study. Hepatitis monthly. 2016;16(9):e39575.
102. Kalupahana NS, Massiera F, Quignard-Boulange A, Ailhaud G, Voy BH, Wasserman DH, et al. Overproduction of angiotensinogen from adipose tissue induces adipose inflammation, glucose intolerance, and insulin resistance. Obesity. 2012;20(1):48-56.
103. Aronne LJ, Segal KR. Adiposity and fat distribution outcome measures: assessment and clinical implications. Obesity research. 2002;10 Suppl 1:14S-21S.
104. Stears A, O'Rahilly S, Semple RK, Savage DB. Metabolic insights from extreme human insulin resistance phenotypes. Best Pract Res Clin Endocrinol Metab. 2012;26(2):145-57.
105. Bjorntorp P. "Portal" adipose tissue as a generator of risk factors for cardiovascular disease and diabetes. Arteriosclerosis. 1990;10(4):493-6.
106. Item F, Konrad D. Visceral fat and metabolic inflammation: the portal theory revisited. Obesity reviews : an official journal of the International Association for the Study of Obesity. 2012;13 Suppl 2:30-9.
107. Kawai M, de Paula FJ, Rosen CJ. New insights into osteoporosis: the bone-fat connection. J Intern Med. 2012;272(4):317-29.
108. Guebre-Egziabher F, Alix PM, Koppe L, Pelletier CC, Kalbacher E, Fouque D, et al. Ectopic lipid accumulation: A potential cause for metabolic disturbances and a contributor to the alteration of kidney function. Biochimie. 2013;95(11):1971-9.

109. Virtue S, Vidal-Puig A. Adipose tissue expandability, lipotoxicity and the Metabolic Syndrome--an allostatic perspective. Biochimica et biophysica acta. 2010;1801(3):338-49.
110. Garbossa SG, Folli F. Vitamin D, sub-inflammation and insulin resistance. A window on a potential role for the interaction between bone and glucose metabolism. Rev Endocr Metab Disord. 2017;18(2):243-58.
111. Kabir M, Catalano KJ, Ananthnarayan S, Kim SP, Van Citters GW, Dea MK, et al. Molecular evidence supporting the portal theory: a causative link between visceral adiposity and hepatic insulin resistance. American journal of physiology Endocrinology and metabolism. 2005;288(2):E454-61.
112. Garrison RJ, Kannel WB, Stokes J, 3rd, Castelli WP. Incidence and precursors of hypertension in young adults: the Framingham Offspring Study. Prev Med. 1987;16(2):235-51.
113. Stamler R, Stamler J, Riedlinger WF, Algera G, Roberts RH. Weight and blood pressure. Findings in hypertension screening of 1 million Americans. Jama. 1978;240(15):1607-10.
114. Dandona P, Mohanty P, Ghanim H, Aljada A, Browne R, Hamouda W, et al. The suppressive effect of dietary restriction and weight loss in the obese on the generation of reactive oxygen species by leukocytes, lipid peroxidation, and protein carbonylation. The Journal of clinical endocrinology and metabolism. 2001;86(1):355-62.
115. Lin KY, Ito A, Asagami T, Tsao PS, Adimoolam S, Kimoto M, et al. Impaired nitric oxide synthase pathway in diabetes mellitus: role of asymmetric dimethylarginine and dimethylarginine dimethylaminohydrolase. Circulation. 2002;106(8):987-92.
116. Giacchetti G, Faloia E, Sardu C, Camilloni MA, Mariniello B, Gatti C, et al. Gene expression of angiotensinogen in adipose tissue of obese patients. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity. 2000;24 Suppl 2:S142-3.
117. Poirier P, Lemieux I, Mauriege P, Dewailly E, Blanchet C, Bergeron J, et al. Impact of waist circumference on the relationship between blood pressure and insulin: the Quebec Health Survey. Hypertension. 2005;45(3):363-7.

118. Collaboration NCDRF. Worldwide trends in blood pressure from 1975 to 2015: a pooled analysis of 1479 population-based measurement studies with 19.1 million participants. Lancet. 2017;389(10064):37-55.
119. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science. 1993;259(5091):87-91.
120. Dandona P, Aljada A, Ghanim H, Mohanty P, Tripathy C, Hofmeyer D, et al. Increased plasma concentration of macrophage migration inhibitory factor (MIF) and MIF mRNA in mononuclear cells in the obese and the suppressive action of metformin. The Journal of clinical endocrinology and metabolism. 2004;89(10):5043-7.
121. Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. American journal of physiology Endocrinology and metabolism. 2001;280(5):E745-51.
122. Dandona P, Weinstock R, Thusu K, Abdel-Rahman E, Aljada A, Wadden T. Tumor necrosis factor-alpha in sera of obese patients: fall with weight loss. The Journal of clinical endocrinology and metabolism. 1998;83(8):2907-10.
123. Vozarova B, Weyer C, Hanson K, Tataranni PA, Bogardus C, Pratley RE. Circulating interleukin-6 in relation to adiposity, insulin action, and insulin secretion. Obesity research. 2001;9(7):414-7.
124. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. The Journal of clinical investigation. 2003;112(12):1821-30.
125. Morrison MC, Kleemann R. Role of Macrophage Migration Inhibitory Factor in Obesity, Insulin Resistance, Type 2 Diabetes, and Associated Hepatic Co-Morbidities: A Comprehensive Review of Human and Rodent Studies. Front Immunol. 2015;6:308.
126. Verschuren L, Kooistra T, Bernhagen J, Voshol PJ, Ouwens DM, van Erk M, et al. MIF deficiency reduces chronic inflammation in white adipose tissue and impairs the development of insulin resistance, glucose intolerance, and associated atherosclerotic disease. Circulation research. 2009;105(1):99-107.
127. Hirokawa J, Sakaue S, Tagami S, Kawakami Y, Sakai M, Nishi S, et al. Identification of macrophage migration inhibitory factor in adipose tissue and its induction by tumor

necrosis factor-alpha. Biochemical and biophysical research communications. 1997;235(1):94-8.

128. Sakaue S, Nishihira J, Hirokawa J, Yoshimura H, Honda T, Aoki K, et al. Regulation of macrophage migration inhibitory factor (MIF) expression by glucose and insulin in adipocytes in vitro. Mol Med. 1999;5(6):361-71.

129. Zhang N, Zhang N, Song L, Xie H, Zhao C, Li S, et al. Adipokines and free fatty acids regulate insulin sensitivity by increasing microRNA-21 expression in human mature adipocytes. Mol Med Rep. 2017;16(2):2254-8.

130. Fruhbeck G, Catalan V, Rodriguez A, Ramirez B, Becerril S, Salvador J, et al. Involvement of the leptin-adiponectin axis in inflammation and oxidative stress in the metabolic syndrome. Sci Rep. 2017;7(1):6619.

131. Engin A. Adiponectin-Resistance in Obesity. Adv Exp Med Biol. 2017;960:415-41.

132. Anderson EJ, Lustig ME, Boyle KE, Woodlief TL, Kane DA, Lin CT, et al. Mitochondrial H2O2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. The Journal of clinical investigation. 2009;119(3):573-81.

133. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, et al. Increased oxidative stress in obesity and its impact on metabolic syndrome. The Journal of clinical investigation. 2004;114(12):1752-61.

134. Lee H, Lee YJ, Choi H, Ko EH, Kim JW. Reactive oxygen species facilitate adipocyte differentiation by accelerating mitotic clonal expansion. The Journal of biological chemistry. 2009;284(16):10601-9.

135. Higuchi M, Dusting GJ, Peshavariya H, Jiang F, Hsiao ST, Chan EC, et al. Differentiation of human adipose-derived stem cells into fat involves reactive oxygen species and Forkhead box O1 mediated upregulation of antioxidant enzymes. Stem Cells Dev. 2013;22(6):878-88.

136. Horvath TL, Andrews ZB, Diano S. Fuel utilization by hypothalamic neurons: roles for ROS. Trends in endocrinology and metabolism: TEM. 2009;20(2):78-87.

137. Mlinar B, Marc J. New insights into adipose tissue dysfunction in insulin resistance. Clin Chem Lab Med. 2011;49(12):1925-35.

138. Viroonudomphol D, Pongpaew P, Tungtrongchitr R, Phonrat B, Supawan V, Vudhivai N, et al. Erythrocyte antioxidant enzymes and blood pressure in relation to overweight and obese Thai in Bangkok. Southeast Asian J Trop Med Public Health. 2000;31(2):325-34.
139. Nikolaidis MG, Kerksick CM, Lamprecht M, McAnulty SR. Redox biology of exercise. Oxidative medicine and cellular longevity. 2012;2012:407978.
140. D'Archivio M, Annuzzi G, Vari R, Filesi C, Giacco R, Scazzocchio B, et al. Predominant role of obesity/insulin resistance in oxidative stress development. Eur J Clin Invest. 2012;42(1):70-8.
141. Ruperez AI, Olza J, Gil-Campos M, Leis R, Mesa MD, Tojo R, et al. Are catalase -844A/G polymorphism and activity associated with childhood obesity? Antioxid Redox Signal. 2013;19(16):1970-5.
142. Ozgen IT, Tascilar ME, Bilir P, Boyraz M, Guncikan MN, Akay C, et al. Oxidative stress in obese children and its relation with insulin resistance. Journal of pediatric endocrinology & metabolism : JPEM. 2012;25(3-4):261-6.
143. Rodriguez-Iturbe B, Zhan CD, Quiroz Y, Sindhu RK, Vaziri ND. Antioxidant-rich diet relieves hypertension and reduces renal immune infiltration in spontaneously hypertensive rats. Hypertension. 2003;41(2):341-6.
144. Jiang Y, Bao H, Ge Y, Tang W, Cheng D, Luo K, et al. Therapeutic targeting of GSK3beta enhances the Nrf2 antioxidant response and confers hepatic cytoprotection in hepatitis C. Gut. 2015;64(1):168-79.
145. Chiu J, Khan ZA, Farhangkhoee H, Chakrabarti S. Curcumin prevents diabetes-associated abnormalities in the kidneys by inhibiting p300 and nuclear factor-kappaB. Nutrition. 2009;25(9):964-72.
146. Bostan C, Yildiz A, Ozkan AA, Uzunhasan I, Kaya A, Yigit Z. Beneficial effects of rosuvastatin treatment in patients with metabolic syndrome. Angiology. 2015;66(2):122-7.
147. Chrysohoou C, Panagiotakos DB, Pitsavos C, Skoumas I, Papademetriou L, Economou M, et al. The implication of obesity on total antioxidant capacity in apparently healthy men and women: the ATTICA study. Nutrition, metabolism, and cardiovascular diseases : NMCD. 2007;17(8):590-7.

148. Brown LA, Kerr CJ, Whiting P, Finer N, McEneny J, Ashton T. Oxidant stress in healthy normal-weight, overweight, and obese individuals. Obesity. 2009;17(3):460-6.
149. Mueller CF, Laude K, McNally JS, Harrison DG. ATVB in focus: redox mechanisms in blood vessels. Arteriosclerosis, thrombosis, and vascular biology. 2005;25(2):274-8.
150. Youn JY, Siu KL, Lob HE, Itani H, Harrison DG, Cai H. Role of vascular oxidative stress in obesity and metabolic syndrome. Diabetes. 2014;63(7):2344-55.
151. Kang KH, Lemke G, Kim JW. The PI3K-PTEN tug-of-war, oxidative stress and retinal degeneration. Trends Mol Med. 2009;15(5):191-8.
152. Koh KK, Park SM, Quon MJ. Leptin and cardiovascular disease: response to therapeutic interventions. Circulation. 2008;117(25):3238-49.
153. Cai H. NAD(P)H oxidase-dependent self-propagation of hydrogen peroxide and vascular disease. Circulation research. 2005;96(8):818-22.
154. Kuwahara H, Horie T, Ishikawa S, Tsuda C, Kawakami S, Noda Y, et al. Oxidative stress in skeletal muscle causes severe disturbance of exercise activity without muscle atrophy. Free radical biology & medicine. 2010;48(9):1252-62.
155. Seppala-Lindroos A, Vehkavaara S, Hakkinen AM, Goto T, Westerbacka J, Sovijarvi A, et al. Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. The Journal of clinical endocrinology and metabolism. 2002;87(7):3023-8.
156. Lapointe A, Piche ME, Weisnagel SJ, Bergeron J, Lemieux S. Associations between circulating free fatty acids, visceral adipose tissue accumulation, and insulin sensitivity in postmenopausal women. Metabolism: clinical and experimental. 2009;58(2):180-5.
157. Tripathy D, Mohanty P, Dhindsa S, Syed T, Ghanim H, Aljada A, et al. Elevation of free fatty acids induces inflammation and impairs vascular reactivity in healthy subjects. Diabetes. 2003;52(12):2882-7.
158. Mooradian AD, Haas MJ, Wong NC. Transcriptional control of apolipoprotein A-I gene expression in diabetes. Diabetes. 2004;53(3):513-20.
159. Groenendijk M, Cantor RM, Blom NH, Rotter JI, de Bruin TW, Dallinga-Thie GM. Association of plasma lipids and apolipoproteins with the insulin response element in the apoC-III promoter region in familial combined hyperlipidemia. Journal of lipid research. 1999;40(6):1036-44.

160. Kobayashi J, Miyashita K, Nakajima K, Mabuchi H. Hepatic Lipase: a Comprehensive View of its Role on Plasma Lipid and Lipoprotein Metabolism. Journal of atherosclerosis and thrombosis. 2015;22(10):1001-11.
161. Wyszynski DF, Waterworth DM, Barter PJ, Cohen J, Kesaniemi YA, Mahley RW, et al. Relation between atherogenic dyslipidemia and the Adult Treatment Program-III definition of metabolic syndrome (Genetic Epidemiology of Metabolic Syndrome Project). Am J Cardiol. 2005;95(2):194-8.
162. Qu S, Zhang T, Dong HH. Effect of hepatic insulin expression on lipid metabolism in diabetic mice. Journal of diabetes. 2016;8(3):314-23.
163. Hassing HC, Surendran RP, Mooij HL, Stroes ES, Nieuwdorp M, Dallinga-Thie GM. Pathophysiology of hypertriglyceridemia. Biochimica et biophysica acta. 2012;1821(5):826-32.
164. Stan S, Levy E, Delvin EE, Hanley JA, Lamarche B, O'Loughlin J, et al. Distribution of LDL particle size in a population-based sample of children and adolescents and relationship with other cardiovascular risk factors. Clin Chem. 2005;51(7):1192-200.
165. Chan DC, Barrett PH, Watts GF. The metabolic and pharmacologic bases for treating atherogenic dyslipidaemia. Best Pract Res Clin Endocrinol Metab. 2014;28(3):369-85.
166. Jorgensen AB, Frikke-Schmidt R, West AS, Grande P, Nordestgaard BG, Tybjaerg-Hansen A. Genetically elevated non-fasting triglycerides and calculated remnant cholesterol as causal risk factors for myocardial infarction. Eur Heart J. 2013;34(24):1826-33.
167. Johansen CT, Hegele RA. Using Mendelian randomization to determine causative factors in cardiovascular disease. J Intern Med. 2013;273(1):44-7.
168. Zilversmit DB. Atherogenesis: a postprandial phenomenon. Circulation. 1979;60(3):473-85.
169. Chapman MJ, Ginsberg HN, Amarenco P, Andreotti F, Boren J, Catapano AL, et al. Triglyceride-rich lipoproteins and high-density lipoprotein cholesterol in patients at high risk of cardiovascular disease: evidence and guidance for management. Eur Heart J. 2011;32(11):1345-61.

170. Miller M, Stone NJ, Ballantyne C, Bittner V, Criqui MH, Ginsberg HN, et al. Triglycerides and cardiovascular disease: a scientific statement from the American Heart Association. Circulation. 2011;123(20):2292-333.
171. Harmel E, Grenier E, Bendjoudi Ouadda A, El Chebly M, Ziv E, Beaulieu JF, et al. AMPK in the small intestine in normal and pathophysiological conditions. Endocrinology. 2014;155(3):873-88.
172. Levy E, Spahis S, Garofalo C, Marcil V, Montoudis A, Sinnet D, et al. Sar1b transgenic male mice are more susceptible to high-fat diet-induced obesity, insulin insensitivity and intestinal chylomicron overproduction. The Journal of nutritional biochemistry. 2014;25(5):540-8.
173. Levy E, Garofalo C, Thibault L, Dionne S, Daoust L, Lepage G, et al. Intraluminal and intracellular phases of fat absorption are impaired in essential fatty acid deficiency. The American journal of physiology. 1992;262(2 Pt 1):G319-26.
174. Levy E, Zoltowska M. Prolonged post-prandial increment in obese subjects with intra-abdominal visceral fat accumulation. Eur J Clin Invest. 1999;29(4):271-3.
175. Zoltowska M, Ziv E, Delvin E, Sinnett D, Kalman R, Garofalo C, et al. Cellular aspects of intestinal lipoprotein assembly in Psammomys obesus: a model of insulin resistance and type 2 diabetes. Diabetes. 2003;52(10):2539-45.
176. Xu E, Forest MP, Schwab M, Avramoglu RK, St-Amand E, Caron AZ, et al. Hepatocyte-specific Ptpn6 deletion promotes hepatic lipid accretion, but reduces NAFLD in diet-induced obesity: potential role of PPARgamma. Hepatology. 2014;59(5):1803-15.
177. Veilleux A, Mayeur S, Berube JC, Beaulieu JF, Tremblay E, Hould FS, et al. Altered intestinal functions and increased local inflammation in insulin-resistant obese subjects: a gene-expression profile analysis. BMC gastroenterology. 2015;15:119.
178. Marcil V, Seidman E, Sinnett D, Sanchez R, Spahis S, Sane A, et al. Tissue distribution and regulation of the small Sar1b GTPase in mice. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology. 2014;33(6):1815-26.
179. Sane A, Seidman E, Spahis S, Lamantia V, Garofalo C, Montoudis A, et al. New Insights In Intestinal Sar1B GTPase Regulation and Role in Cholesterol Homeostasis. J Cell Biochem. 2015;116(10):2270-82.

180. Kalderon B, Adler JH, Levy E, Gutman A. Lipogenesis in the sand rat (Psammomys obesus). The American journal of physiology. 1983;244(5):E480-6.
181. Ben Djoudi Ouadda A, Levy E, Ziv E, Lalonde G, Sane AT, Delvin E, et al. Increased hepatic lipogenesis in insulin resistance and Type 2 diabetes is associated with AMPK signalling pathway up-regulation in Psammomys obesus. Biosci Rep. 2009;29(5):283-92.
182. Sikaris KA. The clinical biochemistry of obesity. Clin Biochem Rev. 2004;25(3):165-81.
183. Bays HE. "Sick fat," metabolic disease, and atherosclerosis. Am J Med. 2009;122(1 Suppl):S26-37.
184. Inoguchi T, Li P, Umeda F, Yu HY, Kakimoto M, Imamura M, et al. High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C--dependent activation of NAD(P)H oxidase in cultured vascular cells. Diabetes. 2000;49(11):1939-45.
185. Spahis S, Borys JM, Levy E. Metabolic Syndrome as a Multifaceted Risk Factor for Oxidative Stress. Antioxid Redox Signal. 2017;26(9):445-61.
186. Bloch-Damti A, Bashan N. Proposed mechanisms for the induction of insulin resistance by oxidative stress. Antioxid Redox Signal. 2005;7(11-12):1553-67.
187. Tan M, Li S, Swaroop M, Guan K, Oberley LW, Sun Y. Transcriptional activation of the human glutathione peroxidase promoter by p53. The Journal of biological chemistry. 1999;274(17):12061-6.
188. Sablina AA, Budanov AV, Ilyinskaya GV, Agapova LS, Kravchenko JE, Chumakov PM. The antioxidant function of the p53 tumor suppressor. Nature medicine. 2005;11(12):1306-13.
189. Liu B, Chen Y, St Clair DK. ROS and p53: a versatile partnership. Free radical biology & medicine. 2008;44(8):1529-35.
190. Kruiswijk F, Labuschagne CF, Vousden KH. p53 in survival, death and metabolic health: a lifeguard with a licence to kill. Nature reviews Molecular cell biology. 2015;16(7):393-405.

191. Nair J, Kakkar VV, Shanker J. Comparative analysis of inflammatory gene expression levels in metabolic syndrome & coronary artery disease. The Indian journal of medical research. 2017;145(6):777-85.
192. Mozos I, Malainer C, Horbanczuk J, Gug C, Stoian D, Luca CT, et al. Inflammatory Markers for Arterial Stiffness in Cardiovascular Diseases. Front Immunol. 2017;8:1058.
193. Gustafson B, Hammarstedt A, Andersson CX, Smith U. Inflamed adipose tissue: a culprit underlying the metabolic syndrome and atherosclerosis. Arteriosclerosis, thrombosis, and vascular biology. 2007;27(11):2276-83.
194. Hsueh WA, Quinones MJ. Role of endothelial dysfunction in insulin resistance. Am J Cardiol. 2003;92(4A):10J-7J.
195. Phillips CM, Perry IJ. Does inflammation determine metabolic health status in obese and nonobese adults? The Journal of clinical endocrinology and metabolism. 2013;98(10):E1610-9.
196. Danesh J, Wheeler JG, Hirschfield GM, Eda S, Eiriksdottir G, Rumley A, et al. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. The New England journal of medicine. 2004;350(14):1387-97.
197. Ridker PM, Rifai N, Rose L, Buring JE, Cook NR. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. The New England journal of medicine. 2002;347(20):1557-65.
198. Rizzo M, Rizvi AA, Rini GB, Berneis K. The therapeutic modulation of atherogenic dyslipidemia and inflammatory markers in the metabolic syndrome: what is the clinical relevance? Acta Diabetol. 2009;46(1):1-11.
199. O'Rourke RW, White AE, Metcalf MD, Olivas AS, Mitra P, Larison WG, et al. Hypoxia-induced inflammatory cytokine secretion in human adipose tissue stromovascular cells. Diabetologia. 2011;54(6):1480-90.
200. Chawla A, Nguyen KD, Goh YP. Macrophage-mediated inflammation in metabolic disease. Nat Rev Immunol. 2011;11(11):738-49.
201. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. The Journal of clinical investigation. 2003;112(12):1796-808.

202. Skurk T, Alberti-Huber C, Herder C, Hauner H. Relationship between adipocyte size and adipokine expression and secretion. The Journal of clinical endocrinology and metabolism. 2007;92(3):1023-33.
203. Esser N, L'Homme L, De Roover A, Kohnen L, Scheen AJ, Moutschen M, et al. Obesity phenotype is related to NLRP3 inflammasome activity and immunological profile of visceral adipose tissue. Diabetologia. 2013;56(11):2487-97.
204. Al Rifai M, Silverman MG, Nasir K, Budoff MJ, Blankstein R, Szklo M, et al. The association of nonalcoholic fatty liver disease, obesity, and metabolic syndrome, with systemic inflammation and subclinical atherosclerosis: the Multi-Ethnic Study of Atherosclerosis (MESA). Atherosclerosis. 2015;239(2):629-33.
205. Huang W, Metlakunta A, Dedousis N, Zhang P, Sipula I, Dube JJ, et al. Depletion of liver Kupffer cells prevents the development of diet-induced hepatic steatosis and insulin resistance. Diabetes. 2010;59(2):347-57.
206. Stienstra R, van Diepen JA, Tack CJ, Zaki MH, van de Veerdonk FL, Perera D, et al. Inflammasome is a central player in the induction of obesity and insulin resistance. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(37):15324-9.
207. Vandanmagsar B, Youm YH, Ravussin A, Galgani JE, Stadler K, Mynatt RL, et al. The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. Nature medicine. 2011;17(2):179-88.
208. Strowig T, Henao-Mejia J, Elinav E, Flavell R. Inflammasomes in health and disease. Nature. 2012;481(7381):278-86.
209. Lezutekong JN, Nikhanj A, Oudit GY. Imbalance of gut microbiome and intestinal epithelial barrier dysfunction in cardiovascular disease. Clinical science. 2018;132(8):901-4.
210. Crosnier C, Stamataki D, Lewis J. Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. Nature reviews Genetics. 2006;7(5):349-59.
211. Veldhoen M, Brucklacher-Waldert V. Dietary influences on intestinal immunity. Nat Rev Immunol. 2012;12(10):696-708.

212. Qiu Y, Yu M, Yang Y, Sheng H, Wang W, Sun L, et al. Disturbance of intraepithelial lymphocytes in a murine model of acute intestinal ischemia/reperfusion. J Mol Histol. 2014;45(2):217-27.
213. Muniz LR, Knosp C, Yeretssian G. Intestinal antimicrobial peptides during homeostasis, infection, and disease. Front Immunol. 2012;3:310.
214. Parker A, Maclaren OJ, Fletcher AG, Muraro D, Kreuzaler PA, Byrne HM, et al. Cell proliferation within small intestinal crypts is the principal driving force for cell migration on villi. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2017;31(2):636-49.
215. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature. 2007;449(7165):1003-7.
216. Kaemmerer E, Kuhn P, Schneider U, Clahsen T, Jeon MK, Klaus C, et al. Beta-7 integrin controls enterocyte migration in the small intestine. World journal of gastroenterology : WJG. 2015;21(6):1759-64.
217. Rankin CR, Hilgarth RS, Leoni G, Kwon M, Den Beste KA, Parkos CA, et al. Annexin A2 regulates beta1 integrin internalization and intestinal epithelial cell migration. The Journal of biological chemistry. 2013;288(21):15229-39.
218. Wang Y, Wang GZ, Rabinovitch PS, Tabas I. Macrophage mitochondrial oxidative stress promotes atherosclerosis and nuclear factor-kappaB-mediated inflammation in macrophages. Circulation research. 2014;114(3):421-33.
219. Durer U, Hartig R, Bang S, Thim L, Hoffmann W. TFF3 and EGF induce different migration patterns of intestinal epithelial cells in vitro and trigger increased internalization of E-cadherin. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology. 2007;20(5):329-46.
220. Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, et al. IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. American journal of physiology Gastrointestinal and liver physiology. 2006;290(4):G827-38.

221. Smith JM, Johanesen PA, Wendt MK, Binion DG, Dwinell MB. CXCL12 activation of CXCR4 regulates mucosal host defense through stimulation of epithelial cell migration and promotion of intestinal barrier integrity. American journal of physiology Gastrointestinal and liver physiology. 2005;288(2):G316-26.
222. Begnaud S, Chen T, Delacour D, Mege RM, Ladoux B. Mechanics of epithelial tissues during gap closure. Current opinion in cell biology. 2016;42:52-62.
223. Schmitz H, Barmeyer C, Fromm M, Runkel N, Foss HD, Bentzel CJ, et al. Altered tight junction structure contributes to the impaired epithelial barrier function in ulcerative colitis. Gastroenterology. 1999;116(2):301-9.
224. Farquhar MG, Palade GE. Junctional complexes in various epithelia. J Cell Biol. 1963;17:375-412.
225. Zihni C, Mills C, Matter K, Balda MS. Tight junctions: from simple barriers to multifunctional molecular gates. Nature reviews Molecular cell biology. 2016;17(9):564-80.
226. Mineta K, Yamamoto Y, Yamazaki Y, Tanaka H, Tada Y, Saito K, et al. Predicted expansion of the claudin multigene family. FEBS letters. 2011;585(4):606-12.
227. Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. Nature reviews Molecular cell biology. 2001;2(4):285-93.
228. Goodrich KM, Fundaro G, Griffin LE, Grant A, Hulver MW, Ponder MA, et al. Chronic administration of dietary grape seed extract increases colonic expression of gut tight junction protein occludin and reduces fecal calprotectin: a secondary analysis of healthy Wistar Furth rats. Nutrition research. 2012;32(10):787-94.
229. de Cossio LF, Fourrier C, Sauvant J, Everard A, Capuron L, Cani PD, et al. Impact of prebiotics on metabolic and behavioral alterations in a mouse model of metabolic syndrome. Brain Behav Immun. 2017;64:33-49.
230. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes. 2008;57(6):1470-81.
231. Frisard MI, McMillan RP, Marchand J, Wahlberg KA, Wu Y, Voelker KA, et al. Toll-like receptor 4 modulates skeletal muscle substrate metabolism. American journal of physiology Endocrinology and metabolism. 2010;298(5):E988-98.

232. de Kort S, Keszthelyi D, Masclee AA. Leaky gut and diabetes mellitus: what is the link? Obesity reviews : an official journal of the International Association for the Study of Obesity. 2011;12(6):449-58.
233. Cani PD. Interactions between gut microbes and host cells control gut barrier and metabolism. Int J Obes Suppl. 2016;6(Suppl 1):S28-S31.
234. Cani PD. Gut cell metabolism shapes the microbiome. Science. 2017;357(6351):548-9.
235. Cani PD. Gut microbiota - at the intersection of everything? Nature reviews Gastroenterology & hepatology. 2017;14(6):321-2.
236. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes. 2007;56(7):1761-72.
237. Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, et al. Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia. 2007;50(11):2374-83.
238. Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, et al. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut. 2009;58(8):1091-103.
239. Larsen N, Vogensen FK, van den Berg FW, Nielsen DS, Andreasen AS, Pedersen BK, et al. Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. PloS one. 2010;5(2):e9085.
240. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(31):11070-5.
241. Menard S, Cerf-Bensussan N, Heyman M. Multiple facets of intestinal permeability and epithelial handling of dietary antigens. Mucosal Immunol. 2010;3(3):247-59.
242. Wells JM, Brummer RJ, Derrien M, MacDonald TT, Troost F, Cani PD, et al. Homeostasis of the gut barrier and potential biomarkers. American journal of physiology Gastrointestinal and liver physiology. 2017;312(3):G171-G93.
243. de Vos WM, de Vos EA. Role of the intestinal microbiome in health and disease: from correlation to causation. Nutrition reviews. 2012;70 Suppl 1:S45-56.

244. Savage DC. Microbial ecology of the gastrointestinal tract. Annu Rev Microbiol. 1977;31:107-33.
245. Holdeman LV, Good IJ, Moore WE. Human fecal flora: variation in bacterial composition within individuals and a possible effect of emotional stress. Applied and environmental microbiology. 1976;31(3):359-75.
246. Nielsen HB, Almeida M, Juncker AS, Rasmussen S, Li J, Sunagawa S, et al. Identification and assembly of genomes and genetic elements in complex metagenomic samples without using reference genomes. Nat Biotechnol. 2014;32(8):822-8.
247. Roggenbuck M, Baerholm Schnell I, Blom N, Baelum J, Bertelsen MF, Sicheritz-Ponten T, et al. The microbiome of New World vultures. Nat Commun. 2014;5:5498.
248. Davis SC, Yadav JS, Barrow SD, Robertson BK. Gut microbiome diversity influenced more by the Westernized dietary regime than the body mass index as assessed using effect size statistic. Microbiologyopen. 2017;6(4).
249. Beaumont M, Portune KJ, Steuer N, Lan A, Cerrudo V, Audebert M, et al. Quantity and source of dietary protein influence metabolite production by gut microbiota and rectal mucosa gene expression: a randomized, parallel, double-blind trial in overweight humans. The American journal of clinical nutrition. 2017;106(4):1005-19.
250. van de Wouw M, Schellekens H, Dinan TG, Cryan JF. Microbiota-Gut-Brain Axis: Modulator of Host Metabolism and Appetite. The Journal of nutrition. 2017;147(5):727-45.
251. Vaughn AC, Cooper EM, DiLorenzo PM, O'Loughlin LJ, Konkel ME, Peters JH, et al. Energy-dense diet triggers changes in gut microbiota, reorganization of gutbrain vagal communication and increases body fat accumulation. Acta Neurobiol Exp (Wars). 2017;77(1):18-30.
252. Fischer MM, Kessler AM, Kieffer DA, Knotts TA, Kim K, Wei A, et al. Effects of obesity, energy restriction and neutering on the faecal microbiota of cats. The British journal of nutrition. 2017;118(7):513-24.
253. Welly RJ, Liu TW, Zidon TM, Rowles JL, 3rd, Park YM, Smith TN, et al. Comparison of Diet versus Exercise on Metabolic Function and Gut Microbiota in Obese Rats. Med Sci Sports Exerc. 2016;48(9):1688-98.

254. Chassaing B, Vijay-Kumar M, Gewirtz AT. How diet can impact gut microbiota to promote or endanger health. Curr Opin Gastroenterol. 2017;33(6):417-21.
255. Gonzalez-Correa CA, Mulett-Vasquez E, Miranda DA, Gonzalez-Correa CH, Gomez-Buitrago PA. The colon revisited or the key to wellness, health and disease. Med Hypotheses. 2017;108:133-43.
256. Marungruang N, Tovar J, Bjorck I, Hallenius FF. Improvement in cardiometabolic risk markers following a multifunctional diet is associated with gut microbial taxa in healthy overweight and obese subjects. Eur J Nutr. 2017.
257. Dore J, Blottiere H. The influence of diet on the gut microbiota and its consequences for health. Curr Opin Biotechnol. 2015;32:195-9.
258. Li D, Wang P, Wang P, Hu X, Chen F. The gut microbiota: A treasure for human health. Biotechnol Adv. 2016;34(7):1210-24.
259. Tolhurst G, Heffron H, Lam YS, Parker HE, Habib AM, Diakogiannaki E, et al. Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2. Diabetes. 2012;61(2):364-71.
260. Kobayashi M, Mikami D, Kimura H, Kamiyama K, Morikawa Y, Yokoi S, et al. Short-chain fatty acids, GPR41 and GPR43 ligands, inhibit TNF-alpha-induced MCP-1 expression by modulating p38 and JNK signaling pathways in human renal cortical epithelial cells. Biochemical and biophysical research communications. 2017;486(2):499-505.
261. Matheus VA, Monteiro L, Oliveira RB, Maschio DA, Collares-Buzato CB. Butyrate reduces high-fat diet-induced metabolic alterations, hepatic steatosis and pancreatic beta cell and intestinal barrier dysfunctions in prediabetic mice. Exp Biol Med (Maywood). 2017:1535370217708188.
262. Everard A, Lazarevic V, Derrien M, Girard M, Muccioli GG, Neyrinck AM, et al. Responses of gut microbiota and glucose and lipid metabolism to prebiotics in genetic obese and diet-induced leptin-resistant mice. Diabetes. 2011;60(11):2775-86.
263. Hur KY, Lee MS. Gut Microbiota and Metabolic Disorders. Diabetes Metab J. 2015;39(3):198-203.

264. Stappenbeck TS, Hooper LV, Gordon JI. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. Proceedings of the National Academy of Sciences of the United States of America. 2002;99(24):15451-5.
265. Compare D, Coccoli P, Rocco A, Nardone OM, De Maria S, Carteni M, et al. Gut--liver axis: the impact of gut microbiota on non alcoholic fatty liver disease. Nutrition, metabolism, and cardiovascular diseases : NMCD. 2012;22(6):471-6.
266. Li DY, Yang M, Edwards S, Ye SQ. Nonalcoholic fatty liver disease: for better or worse, blame the gut microbiota? JPEN Journal of parenteral and enteral nutrition. 2013;37(6):787-93.
267. Miele L, Marrone G, Lauritano C, Cefalo C, Gasbarrini A, Day C, et al. Gut-liver axis and microbiota in NAFLD: insight pathophysiology for novel therapeutic target. Current pharmaceutical design. 2013;19(29):5314-24.
268. Vajro P, Paolella G, Fasano A. Microbiota and gut-liver axis: their influences on obesity and obesity-related liver disease. Journal of pediatric gastroenterology and nutrition. 2013;56(5):461-8.
269. Thomson AW, Knolle PA. Antigen-presenting cell function in the tolerogenic liver environment. Nat Rev Immunol. 2010;10(11):753-66.
270. Son G, Kremer M, Hines IN. Contribution of gut bacteria to liver pathobiology. Gastroenterology research and practice. 2010;2010.
271. Robinson CJ, Bohannan BJ, Young VB. From structure to function: the ecology of host-associated microbial communities. Microbiol Mol Biol Rev. 2010;74(3):453-76.
272. Crispe IN. The liver as a lymphoid organ. Annu Rev Immunol. 2009;27:147-63.
273. Henao-Mejia J, Elinav E, Thaiss CA, Licona-Limon P, Flavell RA. Role of the intestinal microbiome in liver disease. J Autoimmun. 2013;46:66-73.
274. Calne RY, Sells RA, Pena JR, Davis DR, Millard PR, Herbertson BM, et al. Induction of immunological tolerance by porcine liver allografts. Nature. 1969;223(5205):472-6.
275. Hartmann P, Haimerl M, Mazagova M, Brenner DA, Schnabl B. Toll-like receptor 2-mediated intestinal injury and enteric tumor necrosis factor receptor I contribute to liver fibrosis in mice. Gastroenterology. 2012;143(5):1330-40 e1.

276. Seki E, De Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, et al. TLR4 enhances TGF-beta signaling and hepatic fibrosis. Nature medicine. 2007;13(11):1324-32.
277. Abu-Shanab A, Quigley EM. The role of the gut microbiota in nonalcoholic fatty liver disease. Nature reviews Gastroenterology & hepatology. 2010;7(12):691-701.
278. Machado MV, Cortez-Pinto H. Gut microbiota and nonalcoholic fatty liver disease. Annals of hepatology. 2012;11(4):440-9.
279. Liberal R, Grant CR, Longhi MS, Mieli-Vergani G, Vergani D. Diagnostic criteria of autoimmune hepatitis. Autoimmun Rev. 2014;13(4-5):435-40.
280. Fujii H, Kawada N. Inflammation and fibrogenesis in steatohepatitis. J Gastroenterol. 2012;47(3):215-25.
281. Forbes SJ, Parola M. Liver fibrogenic cells. Best practice & research Clinical gastroenterology. 2011;25(2):207-17.
282. Alexopoulou A, Agiasotelli D, Vasilieva LE, Dourakis SP. Bacterial translocation markers in liver cirrhosis. Ann Gastroenterol. 2017;30(5):486-97.
283. Sanduzzi Zamparelli M, Rocco A, Compare D, Nardone G. The gut microbiota: A new potential driving force in liver cirrhosis and hepatocellular carcinoma. United European Gastroenterol J. 2017;5(7):944-53.
284. Slocum MM, Sittig KM, Specian RD, Deitch EA. Absence of intestinal bile promotes bacterial translocation. Am Surg. 1992;58(5):305-10.
285. Lorenzo-Zuniga V, Bartoli R, Planas R, Hofmann AF, Vinado B, Hagey LR, et al. Oral bile acids reduce bacterial overgrowth, bacterial translocation, and endotoxemia in cirrhotic rats. Hepatology. 2003;37(3):551-7.
286. Chu CJ, Lee FY, Wang SS, Lu RH, Tsai YT, Lin HC, et al. Hyperdynamic circulation of cirrhotic rats with ascites: role of endotoxin, tumour necrosis factor-alpha and nitric oxide. Clinical science. 1997;93(3):219-25.
287. Rahman K, Desai C, Iyer SS, Thorn NE, Kumar P, Liu Y, et al. Loss of Junctional Adhesion Molecule A Promotes Severe Steatohepatitis in Mice on a Diet High in Saturated Fat, Fructose, and Cholesterol. Gastroenterology. 2016;151(4):733-46 e12.
288. Wiest R, Albillos A, Trauner M, Bajaj JS, Jalan R. Targeting the gut-liver axis in liver disease. Journal of hepatology. 2017;67(5):1084-103.

289. Chiang JYL. Bile acid metabolism and signaling in liver disease and therapy. Liver Res. 2017;1(1):3-9.
290. Gonzalez-Regueiro JA, Moreno-Castaneda L, Uribe M, Chavez-Tapia NC. The Role of Bile Acids in Glucose Metabolism and Their Relation with Diabetes. Annals of hepatology. 2017;16(0):16-21.
291. Singh R, Bullard J, Kalra M, Assefa S, Kaul AK, Vonfeldt K, et al. Status of bacterial colonization, Toll-like receptor expression and nuclear factor-kappa B activation in normal and diseased human livers. Clin Immunol. 2011;138(1):41-9.
292. Bajaj JS, Heuman DM, Hylemon PB, Sanyal AJ, White MB, Monteith P, et al. Altered profile of human gut microbiome is associated with cirrhosis and its complications. Journal of hepatology. 2014;60(5):940-7.
293. Qin N, Yang F, Li A, Prifti E, Chen Y, Shao L, et al. Alterations of the human gut microbiome in liver cirrhosis. Nature. 2014;513(7516):59-64.
294. Gkolfakis P, Dimitriadis G, Triantafyllou K. Gut microbiota and non-alcoholic fatty liver disease. Hepatobiliary Pancreat Dis Int. 2015;14(6):572-81.
295. Hara E. Relationship between Obesity, Gut Microbiome and Hepatocellular Carcinoma Development. Digestive diseases. 2015;33(3):346-50.
296. Ma HD, Wang YH, Chang C, Gershwin ME, Lian ZX. The intestinal microbiota and microenvironment in liver. Autoimmun Rev. 2015;14(3):183-91.
297. Bril F, Sninsky JJ, Baca AM, Robert Superko H, Portillo Sanchez P, Biernacki D, et al. Hepatic Steatosis and Insulin Resistance, but not Steatohepatitis, Promote Atherogenic Dyslipidemia in NAFLD. The Journal of clinical endocrinology and metabolism. 2015:jc20153111.
298. Del Ben M, Baratta F, Polimeni L, Angelico F. Non-alcoholic fatty liver disease and cardiovascular disease: epidemiological, clinical and pathophysiological evidences. Internal and emergency medicine. 2012;7 Suppl 3:S291-6.
299. Carotti S, Vespasiani-Gentilucci U, Perrone G, Picardi A, Morini S. Portal inflammation during NAFLD is frequent and associated with the early phases of putative hepatic progenitor cell activation. J Clin Pathol. 2015;68(11):883-90.
300. Bellentani S, Scaglioni F, Marino M, Bedogni G. Epidemiology of non-alcoholic fatty liver disease. Digestive diseases. 2010;28(1):155-61.

301. Browning JD, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, et al. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. Hepatology. 2004;40(6):1387-95.
302. Dowman JK, Armstrong MJ, Tomlinson JW, Newsome PN. Current therapeutic strategies in non-alcoholic fatty liver disease. Diabetes, obesity & metabolism. 2011;13(8):692-702.
303. Dowman JK, Tomlinson JW, Newsome PN. Pathogenesis of non-alcoholic fatty liver disease. QJM : monthly journal of the Association of Physicians. 2010;103(2):71-83.
304. Zhang T, Zhang C, Zhang Y, Tang F, Li H, Zhang Q, et al. Metabolic syndrome and its components as predictors of nonalcoholic fatty liver disease in a northern urban Han Chinese population: A prospective cohort study. Atherosclerosis. 2015;240(1):144-8.
305. Leite NC, Salles GF, Araujo AL, Villela-Nogueira CA, Cardoso CR. Prevalence and associated factors of non-alcoholic fatty liver disease in patients with type-2 diabetes mellitus. Liver international : official journal of the International Association for the Study of the Liver. 2009;29(1):113-9.
306. Serviddio G, Bellanti F, Vendemiale G. Free radical biology for medicine: learning from nonalcoholic fatty liver disease. Free radical biology & medicine. 2013;65:952-68.
307. Schwimmer JB, Deutsch R, Kahen T, Lavine JE, Stanley C, Behling C. Prevalence of fatty liver in children and adolescents. Pediatrics. 2006;118(4):1388-93.
308. Angulo P. Nonalcoholic fatty liver disease. The New England journal of medicine. 2002;346(16):1221-31.
309. Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, et al. The diagnosis and management of non-alcoholic fatty liver disease: practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. Hepatology. 2012;55(6):2005-23.
310. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global Epidemiology of Non-Alcoholic Fatty Liver Disease-Meta-Analytic Assessment of Prevalence, Incidence and Outcomes. Hepatology. 2015.
311. Patton HM, Yates K, Unalp-Arida A, Behling CA, Huang TT, Rosenthal P, et al. Association between metabolic syndrome and liver histology among children with

nonalcoholic Fatty liver disease. The American journal of gastroenterology. 2010;105(9):2093-102.

312. Patton HM, Sirlin C, Behling C, Middleton M, Schwimmer JB, Lavine JE. Pediatric nonalcoholic fatty liver disease: a critical appraisal of current data and implications for future research. Journal of pediatric gastroenterology and nutrition. 2006;43(4):413-27.

313. Feldstein AE, Charatcharoenwitthaya P, Treeprasertsuk S, Benson JT, Enders FB, Angulo P. The natural history of non-alcoholic fatty liver disease in children: a follow-up study for up to 20 years. Gut. 2009;58(11):1538-44.

314. McCullough AJ. Epidemiology of the metabolic syndrome in the USA. J Dig Dis. 2011;12(5):333-40.

315. Anty RC, C. M.; Gual, P.; TRAN, A. Non-alcoholic fatty liver disease: natural history, mechanisms and diagnostic guidelines Hépato-Gastro & Oncologie Digestive 2017;24(7):702-18.

316. Allen AM, Terry TM, Larson JJ, Coward A, Somers VK, Kamath PS. Nonalcoholic Fatty Liver Disease Incidence and Impact on Metabolic Burden and Death: a 20 Year-Community Study. Hepatology. 2017.

317. Gupte P, Amarapurkar D, Agal S, Baijal R, Kulshrestha P, Pramanik S, et al. Non-alcoholic steatohepatitis in type 2 diabetes mellitus. Journal of gastroenterology and hepatology. 2004;19(8):854-8.

318. Jou J, Choi SS, Diehl AM. Mechanisms of disease progression in nonalcoholic fatty liver disease. Seminars in liver disease. 2008;28(4):370-9.

319. Altinbas A, Sowa JP, Hasenberg T, Canbay A. The diagnosis and treatment of non-alcoholic fatty liver disease. Minerva Gastroenterol Dietol. 2015;61(3):159-69.

320. Buzzetti E, Pinzani M, Tsochatzis EA. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). Metabolism: clinical and experimental. 2016;65(8):1038-48.

321. Jornayvaz FR, Samuel VT, Shulman GI. The role of muscle insulin resistance in the pathogenesis of atherogenic dyslipidemia and nonalcoholic fatty liver disease associated with the metabolic syndrome. Annual review of nutrition. 2010;30:273-90.

322. Smith BW, Adams LA. Nonalcoholic fatty liver disease and diabetes mellitus: pathogenesis and treatment. Nature reviews Endocrinology. 2011;7(8):456-65.

323. Tariq Z, Green CJ, Hodson L. Are oxidative stress mechanisms the common denominator in the progression from hepatic steatosis towards non-alcoholic steatohepatitis (NASH)? Liver international : official journal of the International Association for the Study of the Liver. 2014;34(7):e180-90.
324. Vajro P, Lenta S, Socha P, Dhawan A, McKiernan P, Baumann U, et al. Diagnosis of nonalcoholic fatty liver disease in children and adolescents: position paper of the ESPGHAN Hepatology Committee. Journal of pediatric gastroenterology and nutrition. 2012;54(5):700-13.
325. Rodriguez G, Gallego S, Breidenassel C, Moreno LA, Gottrand F. Is liver transaminases assessment an appropriate tool for the screening of non-alcoholic fatty liver disease in at risk obese children and adolescents? Nutricion hospitalaria. 2010;25(5):712-7.
326. Nobili V, Reale A, Alisi A, Morino G, Trenta I, Pisani M, et al. Elevated serum ALT in children presenting to the emergency unit: Relationship with NAFLD. Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver. 2009;41(10):749-52.
327. Saadeh S, Younossi ZM, Remer EM, Gramlich T, Ong JP, Hurley M, et al. The utility of radiological imaging in nonalcoholic fatty liver disease. Gastroenterology. 2002;123(3):745-50.
328. Weiss R, Bremer AA, Lustig RH. What is metabolic syndrome, and why are children getting it? Annals of the New York Academy of Sciences. 2013;1281:123-40.
329. Pacifico L, Nobili V, Anania C, Verdecchia P, Chiesa C. Pediatric nonalcoholic fatty liver disease, metabolic syndrome and cardiovascular risk. World journal of gastroenterology : WJG. 2011;17(26):3082-91.
330. Giorgio V, Prono F, Graziano F, Nobili V. Pediatric non alcoholic fatty liver disease: old and new concepts on development, progression, metabolic insight and potential treatment targets. BMC pediatrics. 2013;13:40.
331. Manco M, Marcellini M, Devito R, Comparcola D, Sartorelli MR, Nobili V. Metabolic syndrome and liver histology in paediatric non-alcoholic steatohepatitis. International journal of obesity. 2008;32(2):381-7.

332. Lee JJ, Lambert JE, Hovhannisyan Y, Ramos-Roman MA, Trombold JR, Wagner DA, et al. Palmitoleic acid is elevated in fatty liver disease and reflects hepatic lipogenesis. The American journal of clinical nutrition. 2015;101(1):34-43.

333. Marchesini G, Brizi M, Bianchi G, Tomassetti S, Bugianesi E, Lenzi M, et al. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. Diabetes. 2001;50(8):1844-50.

334. Sanyal AJ, Campbell-Sargent C, Mirshahi F, Rizzo WB, Contos MJ, Sterling RK, et al. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. Gastroenterology. 2001;120(5):1183-92.

335. Asrih M, Jornayvaz FR. Metabolic syndrome and nonalcoholic fatty liver disease: Is insulin resistance the link? Molecular and cellular endocrinology. 2015.

336. James OF, Day CP. Non-alcoholic steatohepatitis (NASH): a disease of emerging identity and importance. Journal of hepatology. 1998;29(3):495-501.

337. Pagano G, Pacini G, Musso G, Gambino R, Mecca F, Depetris N, et al. Nonalcoholic steatohepatitis, insulin resistance, and metabolic syndrome: further evidence for an etiologic association. Hepatology. 2002;35(2):367-72.

338. Cruz ML, Bergman RN, Goran MI. Unique effect of visceral fat on insulin sensitivity in obese Hispanic children with a family history of type 2 diabetes. Diabetes care. 2002;25(9):1631-6.

339. Zheng J, Woo SL, Hu X, Botchlett R, Chen L, Huo Y, et al. Metformin and metabolic diseases: a focus on hepatic aspects. Frontiers of medicine. 2015.

340. Wree A, Schlattjan M, Bechmann LP, Claudel T, Sowa JP, Stojakovic T, et al. Adipocyte cell size, free fatty acids and apolipoproteins are associated with non-alcoholic liver injury progression in severely obese patients. Metabolism: clinical and experimental. 2014;63(12):1542-52.

341. Koeck ES, Iordanskaia T, Sevilla S, Ferrante SC, Hubal MJ, Freishtat RJ, et al. Adipocyte exosomes induce transforming growth factor beta pathway dysregulation in hepatocytes: a novel paradigm for obesity-related liver disease. The Journal of surgical research. 2014;192(2):268-75.

342. Armstrong MJ, Hazlehurst JM, Hull D, Guo K, Borrows S, Yu J, et al. Abdominal subcutaneous adipose tissue insulin resistance and lipolysis in patients with non-alcoholic steatohepatitis. Diabetes, obesity & metabolism. 2014;16(7):651-60.
343. Park SH, Kim BI, Yun JW, Kim JW, Park DI, Cho YK, et al. Insulin resistance and C-reactive protein as independent risk factors for non-alcoholic fatty liver disease in non-obese Asian men. Journal of gastroenterology and hepatology. 2004;19(6):694-8.
344. Hong F, Radaeva S, Pan HN, Tian Z, Veech R, Gao B. Interleukin 6 alleviates hepatic steatosis and ischemia/reperfusion injury in mice with fatty liver disease. Hepatology. 2004;40(4):933-41.
345. Pessayre D, Berson A, Fromenty B, Mansouri A. Mitochondria in steatohepatitis. Seminars in liver disease. 2001;21(1):57-69.
346. Crespo J, Cayon A, Fernandez-Gil P, Hernandez-Guerra M, Mayorga M, Dominguez-Diez A, et al. Gene expression of tumor necrosis factor alpha and TNF-receptors, p55 and p75, in nonalcoholic steatohepatitis patients. Hepatology. 2001;34(6):1158-63.
347. Sumida Y, Niki E, Naito Y, Yoshikawa T. Involvement of free radicals and oxidative stress in NAFLD/NASH. Free radical research. 2013;47(11):869-80.
348. Podrini C, Borghesan M, Greco A, Pazienza V, Mazzoccoli G, Vinciguerra M. Redox homeostasis and epigenetics in non-alcoholic fatty liver disease (NAFLD). Current pharmaceutical design. 2013;19(15):2737-46.
349. Rolo AP, Teodoro JS, Palmeira CM. Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. Free radical biology & medicine. 2012;52(1):59-69.
350. Mehta K, Van Thiel DH, Shah N, Mobarhan S. Nonalcoholic fatty liver disease: pathogenesis and the role of antioxidants. Nutrition reviews. 2002;60(9):289-93.
351. Nobili V, Manco M, Ciampalini P, Diciommo V, Devito R, Piemonte F, et al. Leptin, free leptin index, insulin resistance and liver fibrosis in children with non-alcoholic fatty liver disease. European journal of endocrinology / European Federation of Endocrine Societies. 2006;155(5):735-43.
352. Silveira LS, Monteiro PA, Antunes Bde M, Seraphim PM, Fernandes RA, Christofaro DG, et al. Intra-abdominal fat is related to metabolic syndrome and non-alcoholic fat liver disease in obese youth. BMC pediatrics. 2013;13:115.

353. Pessayre D. Role of mitochondria in non-alcoholic fatty liver disease. Journal of gastroenterology and hepatology. 2007;22 Suppl 1:S20-7.
354. Novo E, Busletta C, Bonzo LV, Povero D, Paternostro C, Mareschi K, et al. Intracellular reactive oxygen species are required for directional migration of resident and bone marrow-derived hepatic pro-fibrogenic cells. Journal of hepatology. 2011;54(5):964-74.
355. Singal AK, Jampana SC, Weinman SA. Antioxidants as therapeutic agents for liver disease. Liver international : official journal of the International Association for the Study of the Liver. 2011;31(10):1432-48.
356. Caldwell SH, Swerdlow RH, Khan EM, Iezzoni JC, Hespenheide EE, Parks JK, et al. Mitochondrial abnormalities in non-alcoholic steatohepatitis. Journal of hepatology. 1999;31(3):430-4.
357. Le TH, Caldwell SH, Redick JA, Sheppard BL, Davis CA, Arseneau KO, et al. The zonal distribution of megamitochondria with crystalline inclusions in nonalcoholic steatohepatitis. Hepatology. 2004;39(5):1423-9.
358. Berson A, De Beco V, Letteron P, Robin MA, Moreau C, El Kahwaji J, et al. Steatohepatitis-inducing drugs cause mitochondrial dysfunction and lipid peroxidation in rat hepatocytes. Gastroenterology. 1998;114(4):764-74.
359. Cortez-Pinto H, Chatham J, Chacko VP, Arnold C, Rashid A, Diehl AM. Alterations in liver ATP homeostasis in human nonalcoholic steatohepatitis: a pilot study. Jama. 1999;282(17):1659-64.
360. Hardwick RN, Fisher CD, Canet MJ, Lake AD, Cherrington NJ. Diversity in antioxidant response enzymes in progressive stages of human nonalcoholic fatty liver disease. Drug metabolism and disposition: the biological fate of chemicals. 2010;38(12):2293-301.
361. Perez-Carreras M, Del Hoyo P, Martin MA, Rubio JC, Martin A, Castellano G, et al. Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. Hepatology. 2003;38(4):999-1007.
362. Kushnareva Y, Murphy AN, Andreyev A. Complex I-mediated reactive oxygen species generation: modulation by cytochrome c and NAD(P)+ oxidation-reduction state. The Biochemical journal. 2002;368(Pt 2):545-53.

363. Aharoni-Simon M, Hann-Obercyger M, Pen S, Madar Z, Tirosh O. Fatty liver is associated with impaired activity of PPARgamma-coactivator 1alpha (PGC1alpha) and mitochondrial biogenesis in mice. Lab Invest. 2011;91(7):1018-28.

364. Wang S, Kamat A, Pergola P, Swamy A, Tio F, Cusi K. Metabolic factors in the development of hepatic steatosis and altered mitochondrial gene expression in vivo. Metabolism: clinical and experimental. 2011;60(8):1090-9.

365. Nadal-Casellas A, Amengual-Cladera E, Proenza AM, Llado I, Gianotti M. Long-term high-fat-diet feeding impairs mitochondrial biogenesis in liver of male and female rats. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology. 2010;26(3):291-302.

366. St-Pierre J, Drori S, Uldry M, Silvaggi JM, Rhee J, Jager S, et al. Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. Cell. 2006;127(2):397-408.

367. Alisi A, Manco M, Vania A, Nobili V. Pediatric nonalcoholic fatty liver disease in 2009. The Journal of pediatrics. 2009;155(4):469-74.

368. Alisi A, Cianfarani S, Manco M, Agostoni C, Nobili V. Non-alcoholic fatty liver disease and metabolic syndrome in adolescents: pathogenetic role of genetic background and intrauterine environment. Ann Med. 2012;44(1):29-40.

369. Dongiovanni P, Anstee QM, Valenti L. Genetic predisposition in NAFLD and NASH: impact on severity of liver disease and response to treatment. Current pharmaceutical design. 2013;19(29):5219-38.

370. Romeo S, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nature genetics. 2008;40(12):1461-5.

371. Lin YC, Chang PF, Chang MH, Ni YH. Genetic variants in GCKR and PNPLA3 confer susceptibility to nonalcoholic fatty liver disease in obese individuals. The American journal of clinical nutrition. 2014;99(4):869-74.

372. Santoro N, Savoye M, Kim G, Marotto K, Shaw MM, Pierpont B, et al. Hepatic fat accumulation is modulated by the interaction between the rs738409 variant in the PNPLA3 gene and the dietary omega6/omega3 PUFA intake. PloS one. 2012;7(5):e37827.

373. Day CP. Genetic and environmental susceptibility to non-alcoholic fatty liver disease. Digestive diseases. 2010;28(1):255-60.
374. Miele L, Beale G, Patman G, Nobili V, Leathart J, Grieco A, et al. The Kruppel-like factor 6 genotype is associated with fibrosis in nonalcoholic fatty liver disease. Gastroenterology. 2008;135(1):282-91 e1.
375. Yoneda M, Hotta K, Nozaki Y, Endo H, Uchiyama T, Mawatari H, et al. Association between angiotensin II type 1 receptor polymorphisms and the occurrence of nonalcoholic fatty liver disease. Liver international : official journal of the International Association for the Study of the Liver. 2009;29(7):1078-85.
376. Zain SM, Mohamed Z, Mahadeva S, Rampal S, Basu RC, Cheah PL, et al. Susceptibility and gene interaction study of the angiotensin II type 1 receptor (AGTR1) gene polymorphisms with non-alcoholic fatty liver disease in a multi-ethnic population. PloS one. 2013;8(3):e58538.
377. Sookoian S, Castano G, Gianotti TF, Gemma C, Pirola CJ. Polymorphisms of MRP2 (ABCC2) are associated with susceptibility to nonalcoholic fatty liver disease. The Journal of nutritional biochemistry. 2009;20(10):765-70.
378. Oliveira CP, Stefano JT, Cavaleiro AM, Zanella Fortes MA, Vieira SM, Rodrigues Lima VM, et al. Association of polymorphisms of glutamate-cystein ligase and microsomal triglyceride transfer protein genes in non-alcoholic fatty liver disease. Journal of gastroenterology and hepatology. 2010;25(2):357-61.
379. George DK, Goldwurm S, MacDonald GA, Cowley LL, Walker NI, Ward PJ, et al. Increased hepatic iron concentration in nonalcoholic steatohepatitis is associated with increased fibrosis. Gastroenterology. 1998;114(2):311-8.
380. Nelson JE, Bhattacharya R, Lindor KD, Chalasani N, Raaka S, Heathcote EJ, et al. HFE C282Y mutations are associated with advanced hepatic fibrosis in Caucasians with nonalcoholic steatohepatitis. Hepatology. 2007;46(3):723-9.
381. Valenti L, Fracanzani AL, Bugianesi E, Dongiovanni P, Galmozzi E, Vanni E, et al. HFE genotype, parenchymal iron accumulation, and liver fibrosis in patients with nonalcoholic fatty liver disease. Gastroenterology. 2010;138(3):905-12.
382. Al-Serri A, Anstee QM, Valenti L, Nobili V, Leathart JB, Dongiovanni P, et al. The SOD2 C47T polymorphism influences NAFLD fibrosis severity: evidence from case-

control and intra-familial allele association studies. Journal of hepatology. 2012;56(2):448-54.

383. Tokushige K, Takakura M, Tsuchiya-Matsushita N, Taniai M, Hashimoto E, Shiratori K. Influence of TNF gene polymorphisms in Japanese patients with NASH and simple steatosis. Journal of hepatology. 2007;46(6):1104-10.

384. Valenti L, Fracanzani AL, Dongiovanni P, Santorelli G, Branchi A, Taioli E, et al. Tumor necrosis factor alpha promoter polymorphisms and insulin resistance in nonalcoholic fatty liver disease. Gastroenterology. 2002;122(2):274-80.

385. Carulli L, Canedi I, Rondinella S, Lombardini S, Ganazzi D, Fargion S, et al. Genetic polymorphisms in non-alcoholic fatty liver disease: interleukin-6-174G/C polymorphism is associated with non-alcoholic steatohepatitis. Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver. 2009;41(11):823-8.

386. Gupta AC, Misra R, Sakhuja P, Singh Y, Basir SF, Sarin SK. Association of adiponectin gene functional polymorphisms (-11377C/G and +45T/G) with nonalcoholic fatty liver disease. Gene. 2012;496(1):63-7.

387. Aller R, De Luis DA, Izaola O, Gonzalez Sagrado M, Conde R, Pacheco D, et al. Lys656Asn polymorphism of leptin receptor, leptin levels and insulin resistance in patients with non alcoholic fatty liver disease. European review for medical and pharmacological sciences. 2012;16(3):335-41.

388. Zain SM, Mohamed Z, Mahadeva S, Cheah PL, Rampal S, Chin KF, et al. Impact of leptin receptor gene variants on risk of non-alcoholic fatty liver disease and its interaction with adiponutrin gene. Journal of gastroenterology and hepatology. 2013;28(5):873-9.

389. Baldini M, Lohman IC, Halonen M, Erickson RP, Holt PG, Martinez FD. A Polymorphism* in the 5' flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum immunoglobulin E. Am J Respir Cell Mol Biol. 1999;20(5):976-83.

390. Mohammad Alizadeh AH, Ranjbar M, Hajilooi M, Fallahian F. Association of promoter polymorphism of the CD14 C (-159) T endotoxin receptor gene with chronic hepatitis B. World journal of gastroenterology : WJG. 2006;12(35):5717-20.

391. Bernard S, Touzet S, Personne I, Lapras V, Bondon PJ, Berthezene F, et al. Association between microsomal triglyceride transfer protein gene polymorphism and the biological features of liver steatosis in patients with type II diabetes. Diabetologia. 2000;43(8):995-9.

392. Lonardo A, Lombardini S, Scaglioni F, Carulli L, Ricchi M, Ganazzi D, et al. Hepatic steatosis and insulin resistance: does etiology make a difference? Journal of hepatology. 2006;44(1):190-6.

393. Zhou J, Zhai Y, Mu Y, Gong H, Uppal H, Toma D, et al. A novel pregnane X receptor-mediated and sterol regulatory element-binding protein-independent lipogenic pathway. The Journal of biological chemistry. 2006;281(21):15013-20.

394. Sookoian S, Castano GO, Burgueno AL, Gianotti TF, Rosselli MS, Pirola CJ. The nuclear receptor PXR gene variants are associated with liver injury in nonalcoholic fatty liver disease. Pharmacogenet Genomics. 2010;20(1):1-8.

395. Song J, da Costa KA, Fischer LM, Kohlmeier M, Kwock L, Wang S, et al. Polymorphism of the PEMT gene and susceptibility to nonalcoholic fatty liver disease (NAFLD). FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2005;19(10):1266-71.

396. Dong H, Wang J, Li C, Hirose A, Nozaki Y, Takahashi M, et al. The phosphatidylethanolamine N-methyltransferase gene V175M single nucleotide polymorphism confers the susceptibility to NASH in Japanese population. Journal of hepatology. 2007;46(5):915-20.

397. Valenti L, Al-Serri A, Daly AK, Galmozzi E, Rametta R, Dongiovanni P, et al. Homozygosity for the patatin-like phospholipase-3/adiponutrin I148M polymorphism influences liver fibrosis in patients with nonalcoholic fatty liver disease. Hepatology. 2010;51(4):1209-17.

398. Dongiovanni P, Rametta R, Fracanzani AL, Benedan L, Borroni V, Maggioni P, et al. Lack of association between peroxisome proliferator-activated receptors alpha and gamma2 polymorphisms and progressive liver damage in patients with non-alcoholic fatty liver disease: a case control study. BMC gastroenterology. 2010;10:102.

399. Doege H, Grimm D, Falcon A, Tsang B, Storm TA, Xu H, et al. Silencing of hepatic fatty acid transporter protein 5 in vivo reverses diet-induced non-alcoholic fatty liver

disease and improves hyperglycemia. The Journal of biological chemistry. 2008;283(32):22186-92.

400. Sentinelli F, Romeo S, Maglio C, Incani M, Burza MA, Scano F, et al. Lack of effect of apolipoprotein C3 polymorphisms on indices of liver steatosis, lipid profile and insulin resistance in obese Southern Europeans. Lipids in health and disease. 2011;10:93.

401. Kozlitina J, Boerwinkle E, Cohen JC, Hobbs HH. Dissociation between APOC3 variants, hepatic triglyceride content and insulin resistance. Hepatology. 2011;53(2):467-74.

402. Petersen KF, Dufour S, Hariri A, Nelson-Williams C, Foo JN, Zhang XM, et al. Apolipoprotein C3 gene variants in nonalcoholic fatty liver disease. The New England journal of medicine. 2010;362(12):1082-9.

403. Sazci A, Akpinar G, Aygun C, Ergul E, Senturk O, Hulagu S. Association of apolipoprotein E polymorphisms in patients with non-alcoholic steatohepatitis. Digestive diseases and sciences. 2008;53(12):3218-24.

404. Demirag MD, Onen HI, Karaoguz MY, Dogan I, Karakan T, Ekmekci A, et al. Apolipoprotein E gene polymorphism in nonalcoholic fatty liver disease. Digestive diseases and sciences. 2007;52(12):3399-403.

405. Utermann G, Hees M, Steinmetz A. Polymorphism of apolipoprotein E and occurrence of dysbetalipoproteinaemia in man. Nature. 1977;269(5629):604-7.

406. Wang Y, Tong J, Chang B, Wang BF, Zhang D, Wang BY. Relationship of SREBP-2 rs2228314 G>C polymorphism with nonalcoholic fatty liver disease in a Han Chinese population. Genet Test Mol Biomarkers. 2014;18(9):653-7.

407. Musso G, Bo S, Cassader M, De Michieli F, Gambino R. Impact of sterol regulatory element-binding factor-1c polymorphism on incidence of nonalcoholic fatty liver disease and on the severity of liver disease and of glucose and lipid dysmetabolism. The American journal of clinical nutrition. 2013;98(4):895-906.

408. Wang X, Liu Z, Peng Z, Liu W. The TM6SF2 rs58542926 T allele is significantly associated with non-alcoholic fatty liver disease in Chinese. Journal of hepatology. 2015;62(6):1438-9.

409. McGettrick AJ, Feener EP, Kahn CR. Human insulin receptor substrate-1 (IRS-1) polymorphism G972R causes IRS-1 to associate with the insulin receptor and inhibit

receptor autophosphorylation. The Journal of biological chemistry. 2005;280(8):6441-6.

410. Grarup N, Urhammer SA, Ek J, Albrechtsen A, Glumer C, Borch-Johnsen K, et al. Studies of the relationship between the ENPP1 K121Q polymorphism and type 2 diabetes, insulin resistance and obesity in 7,333 Danish white subjects. Diabetologia. 2006;49(9):2097-104.

411. Palmer ND, Musani SK, Yerges-Armstrong LM, Feitosa MF, Bielak LF, Hernaez R, et al. Characterization of European ancestry nonalcoholic fatty liver disease-associated variants in individuals of African and Hispanic descent. Hepatology. 2013;58(3):966-75.

412. Speliotes EK, Yerges-Armstrong LM, Wu J, Hernaez R, Kim LJ, Palmer CD, et al. Genome-wide association analysis identifies variants associated with nonalcoholic fatty liver disease that have distinct effects on metabolic traits. PLoS Genet. 2011;7(3):e1001324.

413. Reue K. The lipin family: mutations and metabolism. Current opinion in lipidology. 2009;20(3):165-70.

414. Reue K, Zhang P. The lipin protein family: dual roles in lipid biosynthesis and gene expression. FEBS letters. 2008;582(1):90-6.

415. Tonjes A, Scholz M, Loeffler M, Stumvoll M. Association of Pro12Ala polymorphism in peroxisome proliferator-activated receptor gamma with Pre-diabetic phenotypes: meta-analysis of 57 studies on nondiabetic individuals. Diabetes care. 2006;29(11):2489-97.

416. Vazquez-Chantada M, Gonzalez-Lahera A, Martinez-Arranz I, Garcia-Monzon C, Regueiro MM, Garcia-Rodriguez JL, et al. Solute carrier family 2 member 1 is involved in the development of nonalcoholic fatty liver disease. Hepatology. 2013;57(2):505-14.

417. Musso G, Gambino R, Pacini G, Pagano G, Durazzo M, Cassader M. Transcription factor 7-like 2 polymorphism modulates glucose and lipid homeostasis, adipokine profile, and hepatocyte apoptosis in NASH. Hepatology. 2009;49(2):426-35.

418. Postic C, Girard J. The role of the lipogenic pathway in the development of hepatic steatosis. Diabetes & metabolism. 2008;34(6 Pt 2):643-8.

419. Lopaschuk GD. Fatty Acid Oxidation and Its Relation with Insulin Resistance and Associated Disorders. Annals of nutrition & metabolism. 2016;68 Suppl 3:15-20.
420. Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. The Journal of clinical investigation. 2005;115(5):1343-51.
421. Enoch HG, Catala A, Strittmatter P. Mechanism of rat liver microsomal stearyl-CoA desaturase. Studies of the substrate specificity, enzyme-substrate interactions, and the function of lipid. The Journal of biological chemistry. 1976;251(16):5095-103.
422. Moon YA, Shah NA, Mohapatra S, Warrington JA, Horton JD. Identification of a mammalian long chain fatty acyl elongase regulated by sterol regulatory element-binding proteins. The Journal of biological chemistry. 2001;276(48):45358-66.
423. Kawano Y, Cohen DE. Mechanisms of hepatic triglyceride accumulation in non-alcoholic fatty liver disease. J Gastroenterol. 2013;48(4):434-41.
424. Ido-Kitamura Y, Sasaki T, Kobayashi M, Kim HJ, Lee YS, Kikuchi O, et al. Hepatic FoxO1 integrates glucose utilization and lipid synthesis through regulation of Chrebp O-glycosylation. PloS one. 2012;7(10):e47231.
425. Deng X, Zhang W, I OS, Williams JB, Dong Q, Park EA, et al. FoxO1 inhibits sterol regulatory element-binding protein-1c (SREBP-1c) gene expression via transcription factors Sp1 and SREBP-1c. The Journal of biological chemistry. 2012;287(24):20132-43.
426. Menke A, Casagrande S, Geiss L, Cowie CC. Prevalence of and Trends in Diabetes Among Adults in the United States, 1988-2012. Jama. 2015;314(10):1021-9.
427. Collaborators GBDO, Afshin A, Forouzanfar MH, Reitsma MB, Sur P, Estep K, et al. Health Effects of Overweight and Obesity in 195 Countries over 25 Years. The New England journal of medicine. 2017;377(1):13-27.
428. Williams CD, Stengel J, Asike MI, Torres DM, Shaw J, Contreras M, et al. Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. Gastroenterology. 2011;140(1):124-31.

429. Stoeckman AK, Ma L, Towle HC. Mlx is the functional heteromeric partner of the carbohydrate response element-binding protein in glucose regulation of lipogenic enzyme genes. The Journal of biological chemistry. 2004;279(15):15662-9.
430. Kawaguchi T, Osatomi K, Yamashita H, Kabashima T, Uyeda K. Mechanism for fatty acid "sparing" effect on glucose-induced transcription: regulation of carbohydrate-responsive element-binding protein by AMP-activated protein kinase. The Journal of biological chemistry. 2002;277(6):3829-35.
431. Foretz M, Guichard C, Ferre P, Foufelle F. Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(22):12737-42.
432. Horton JD, Bashmakov Y, Shimomura I, Shimano H. Regulation of sterol regulatory element binding proteins in livers of fasted and refed mice. Proceedings of the National Academy of Sciences of the United States of America. 1998;95(11):5987-92.
433. Geidl-Flueck B, Gerber PA. Insights into the Hexose Liver Metabolism-Glucose versus Fructose. Nutrients. 2017;9(9).
434. Massillon D, Barzilai N, Hawkins M, Prus-Wertheimer D, Rossetti L. Induction of hepatic glucose-6-phosphatase gene expression by lipid infusion. Diabetes. 1997;46(1):153-7.
435. Im SS, Kim MY, Kwon SK, Kim TH, Bae JS, Kim H, et al. Peroxisome proliferator-activated receptor {alpha} is responsible for the up-regulation of hepatic glucose-6-phosphatase gene expression in fasting and db/db Mice. The Journal of biological chemistry. 2011;286(2):1157-64.
436. Patsouris D, Reddy JK, Muller M, Kersten S. Peroxisome proliferator-activated receptor alpha mediates the effects of high-fat diet on hepatic gene expression. Endocrinology. 2006;147(3):1508-16.
437. Kim S, Sohn I, Ahn JI, Lee KH, Lee YS, Lee YS. Hepatic gene expression profiles in a long-term high-fat diet-induced obesity mouse model. Gene. 2004;340(1):99-109.
438. Shoji I, Deng L, Hotta H. Molecular mechanism of hepatitis C virus-induced glucose metabolic disorders. Front Microbiol. 2011;2:278.

439. Eaton S, Bartlett K, Pourfarzam M. Mammalian mitochondrial beta-oxidation. The Biochemical journal. 1996;320 (Pt 2):345-57.
440. Tolwani RJ, Hamm DA, Tian L, Sharer JD, Vockley J, Rinaldo P, et al. Medium-chain acyl-CoA dehydrogenase deficiency in gene-targeted mice. PLoS Genet. 2005;1(2):e23.
441. Houtkooper RH, Pirinen E, Auwerx J. Sirtuins as regulators of metabolism and healthspan. Nature reviews Molecular cell biology. 2012;13(4):225-38.
442. Foster DW. Malonyl-CoA: the regulator of fatty acid synthesis and oxidation. The Journal of clinical investigation. 2012;122(6):1958-9.
443. Koo SH. Nonalcoholic fatty liver disease: molecular mechanisms for the hepatic steatosis. Clin Mol Hepatol. 2013;19(3):210-5.
444. Wei Y, Rector RS, Thyfault JP, Ibdah JA. Nonalcoholic fatty liver disease and mitochondrial dysfunction. World journal of gastroenterology : WJG. 2008;14(2):193-9.
445. Hsu MH, Savas U, Griffin KJ, Johnson EF. Identification of peroxisome proliferator-responsive human genes by elevated expression of the peroxisome proliferator-activated receptor alpha in HepG2 cells. The Journal of biological chemistry. 2001;276(30):27950-8.
446. Stienstra R, Duval C, Muller M, Kersten S. PPARs, Obesity, and Inflammation. PPAR Res. 2007;2007:95974.
447. Reddy JK. Nonalcoholic steatosis and steatohepatitis. III. Peroxisomal beta-oxidation, PPAR alpha, and steatohepatitis. American journal of physiology Gastrointestinal and liver physiology. 2001;281(6):G1333-9.
448. Latorre J, Moreno-Navarrete JM, Mercader JM, Sabater M, Rovira O, Girones J, et al. Decreased lipid metabolism but increased FA biosynthesis are coupled with changes in liver microRNAs in obese subjects with NAFLD. International journal of obesity. 2017;41(4):620-30.
449. Tyagi S, Gupta P, Saini AS, Kaushal C, Sharma S. The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases. J Adv Pharm Technol Res. 2011;2(4):236-40.

450. Aithal GP, Thomas JA, Kaye PV, Lawson A, Ryder SD, Spendlove I, et al. Randomized, placebo-controlled trial of pioglitazone in nondiabetic subjects with nonalcoholic steatohepatitis. Gastroenterology. 2008;135(4):1176-84.

451. Belfort R, Harrison SA, Brown K, Darland C, Finch J, Hardies J, et al. A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis. The New England journal of medicine. 2006;355(22):2297-307.

452. Promrat K, Lutchman G, Uwaifo GI, Freedman RJ, Soza A, Heller T, et al. A pilot study of pioglitazone treatment for nonalcoholic steatohepatitis. Hepatology. 2004;39(1):188-96.

453. Sanyal AJ, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, et al. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. The New England journal of medicine. 2010;362(18):1675-85.

454. Lewis JH, Mortensen ME, Zweig S, Fusco MJ, Medoff JR, Belder R, et al. Efficacy and safety of high-dose pravastatin in hypercholesterolemic patients with well-compensated chronic liver disease: Results of a prospective, randomized, double-blind, placebo-controlled, multicenter trial. Hepatology. 2007;46(5):1453-63.

455. Neuschwander-Tetri BA, Loomba R, Sanyal AJ, Lavine JE, Van Natta ML, Abdelmalek MF, et al. Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. Lancet. 2015;385(9972):956-65.

456. Ratziu V, Harrison SA, Francque S, Bedossa P, Lehert P, Serfaty L, et al. Elafibranor, an Agonist of the Peroxisome Proliferator-Activated Receptor-alpha and -delta, Induces Resolution of Nonalcoholic Steatohepatitis Without Fibrosis Worsening. Gastroenterology. 2016;150(5):1147-59 e5.

457. Armstrong MJ, Gaunt P, Aithal GP, Barton D, Hull D, Parker R, et al. Liraglutide safety and efficacy in patients with non-alcoholic steatohepatitis (LEAN): a multicentre, double-blind, randomised, placebo-controlled phase 2 study. Lancet. 2016;387(10019):679-90.

458. Lavine JE. Vitamin E treatment of nonalcoholic steatohepatitis in children: a pilot study. The Journal of pediatrics. 2000;136(6):734-8.

459. Lavine JE, Schwimmer JB, Van Natta ML, Molleston JP, Murray KF, Rosenthal P, et al. Effect of vitamin E or metformin for treatment of nonalcoholic fatty liver disease in children and adolescents: the TONIC randomized controlled trial. Jama. 2011;305(16):1659-68.

460. Schwimmer JB, Middleton MS, Deutsch R, Lavine JE. A phase 2 clinical trial of metformin as a treatment for non-diabetic paediatric non-alcoholic steatohepatitis. Alimentary pharmacology & therapeutics. 2005;21(7):871-9.

461. Nobili V, Alisi A, Della Corte C, Rise P, Galli C, Agostoni C, et al. Docosahexaenoic acid for the treatment of fatty liver: randomised controlled trial in children. Nutrition, metabolism, and cardiovascular diseases : NMCD. 2013;23(11):1066-70.

462. Nobili V, Bedogni G, Alisi A, Pietrobattista A, Rise P, Galli C, et al. Docosahexaenoic acid supplementation decreases liver fat content in children with non-alcoholic fatty liver disease: double-blind randomised controlled clinical trial. Archives of disease in childhood. 2011;96(4):350-3.

463. Nobili V, Carpino G, Alisi A, De Vito R, Franchitto A, Alpini G, et al. Role of docosahexaenoic acid treatment in improving liver histology in pediatric nonalcoholic fatty liver disease. PloS one. 2014;9(2):e88005.

464. Nobili V, Carter-Kent C, Feldstein AE. The role of lifestyle changes in the management of chronic liver disease. BMC medicine. 2011;9:70.

465. Nobili V, Giorgio V, Liccardo D, Bedogni G, Morino G, Alisi A, et al. Vitamin D levels and liver histological alterations in children with nonalcoholic fatty liver disease. European journal of endocrinology / European Federation of Endocrine Societies. 2014;170(4):547-53.

466. Nobili V, Manco M, Devito R, Ciampalini P, Piemonte F, Marcellini M. Effect of vitamin E on aminotransferase levels and insulin resistance in children with non-alcoholic fatty liver disease. Alimentary pharmacology & therapeutics. 2006;24(11-12):1553-61.

467. Spahis S, Alvarez F, Dubois J, Ahmed N, Peretti N, Levy E. Plasma fatty acid composition in French-Canadian children with non-alcoholic fatty liver disease: Effect of n-3 PUFA supplementation. Prostaglandins, leukotrienes, and essential fatty acids. 2015;99:25-34.

468. Wensheng L, Baker SS, Baker RD, Zhu L. Antioxidant Mechanisms in Nonalcoholic Fatty Liver Disease. Current drug targets. 2015.
469. Hashemian Z, Hashemi A, Fateminasab M. The Benefits of vitamin E on liver function and the hemopoietic System in thalassemia Patients. Iranian journal of pediatric hematology and oncology. 2012;2(4):153-8.
470. Chung MY, Yeung SF, Park HJ, Volek JS, Bruno RS. Dietary alpha- and gamma-tocopherol supplementation attenuates lipopolysaccharide-induced oxidative stress and inflammatory-related responses in an obese mouse model of nonalcoholic steatohepatitis. The Journal of nutritional biochemistry. 2010;21(12):1200-6.
471. Hoofnagle JH, Van Natta ML, Kleiner DE, Clark JM, Kowdley KV, Loomba R, et al. Vitamin E and changes in serum alanine aminotransferase levels in patients with non-alcoholic steatohepatitis. Alimentary pharmacology & therapeutics. 2013;38(2):134-43.
472. Hasegawa T, Yoneda M, Nakamura K, Makino I, Terano A. Plasma transforming growth factor-beta1 level and efficacy of alpha-tocopherol in patients with non-alcoholic steatohepatitis: a pilot study. Alimentary pharmacology & therapeutics. 2001;15(10):1667-72.
473. Corey KE, Vuppalanchi R, Vos M, Kohli R, Molleston JP, Wilson L, et al. Improvement in liver histology is associated with reduction in dyslipidemia in children with nonalcoholic fatty liver disease. Journal of pediatric gastroenterology and nutrition. 2015;60(3):360-7.
474. Harrison SA, Torgerson S, Hayashi P, Ward J, Schenker S. Vitamin E and vitamin C treatment improves fibrosis in patients with nonalcoholic steatohepatitis. The American journal of gastroenterology. 2003;98(11):2485-90.
475. Niki E. Role of vitamin E as a lipid-soluble peroxyl radical scavenger: in vitro and in vivo evidence. Free radical biology & medicine. 2014;66:3-12.
476. Miller ER, 3rd, Pastor-Barriuso R, Dalal D, Riemersma RA, Appel LJ, Guallar E. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. Annals of internal medicine. 2005;142(1):37-46.
477. DeLuca HF. Overview of general physiologic features and functions of vitamin D. The American journal of clinical nutrition. 2004;80(6 Suppl):1689S-96S.

478. Moore DD, Kato S, Xie W, Mangelsdorf DJ, Schmidt DR, Xiao R, et al. International Union of Pharmacology. LXII. The NR1H and NR1I receptors: constitutive androstane receptor, pregnene X receptor, farnesoid X receptor alpha, farnesoid X receptor beta, liver X receptor alpha, liver X receptor beta, and vitamin D receptor. Pharmacological reviews. 2006;58(4):742-59.

479. Roth CL, Elfers CT, Figlewicz DP, Melhorn SJ, Morton GJ, Hoofnagle A, et al. Vitamin D deficiency in obese rats exacerbates nonalcoholic fatty liver disease and increases hepatic resistin and Toll-like receptor activation. Hepatology. 2012;55(4):1103-11.

480. Abramovitch S, Dahan-Bachar L, Sharvit E, Weisman Y, Ben Tov A, Brazowski E, et al. Vitamin D inhibits proliferation and profibrotic marker expression in hepatic stellate cells and decreases thioacetamide-induced liver fibrosis in rats. Gut. 2011;60(12):1728-37.

481. Nakano T, Cheng YF, Lai CY, Hsu LW, Chang YC, Deng JY, et al. Impact of artificial sunlight therapy on the progress of non-alcoholic fatty liver disease in rats. Journal of hepatology. 2011;55(2):415-25.

482. Bugianesi E, Moscatiello S, Ciaravella MF, Marchesini G. Insulin resistance in nonalcoholic fatty liver disease. Current pharmaceutical design. 2010;16(17):1941-51.

483. Barchetta I, Carotti S, Labbadia G, Gentilucci UV, Muda AO, Angelico F, et al. Liver vitamin D receptor, CYP2R1, and CYP27A1 expression: relationship with liver histology and vitamin D3 levels in patients with nonalcoholic steatohepatitis or hepatitis C virus. Hepatology. 2012;56(6):2180-7.

484. Manco M, Ciampalini P, Nobili V. Low levels of 25-hydroxyvitamin D(3) in children with biopsy-proven nonalcoholic fatty liver disease. Hepatology. 2010;51(6):2229; author reply 30.

485. Sharifi N, Amani R, Hajiani E, Cheraghian B. Does vitamin D improve liver enzymes, oxidative stress, and inflammatory biomarkers in adults with non-alcoholic fatty liver disease? A randomized clinical trial. Endocrine. 2014;47(1):70-80.

486. Grattagliano I, Palmieri VO, Portincasa P, Moschetta A, Palasciano G. Oxidative stress-induced risk factors associated with the metabolic syndrome: a unifying hypothesis. The Journal of nutritional biochemistry. 2008;19(8):491-504.

487. Bentinger M, Brismar K, Dallner G. The antioxidant role of coenzyme Q. Mitochondrion. 2007;7 Suppl:S41-50.
488. Petrosillo G, Portincasa P, Grattagliano I, Casanova G, Matera M, Ruggiero FM, et al. Mitochondrial dysfunction in rat with nonalcoholic fatty liver Involvement of complex I, reactive oxygen species and cardiolipin. Biochimica et biophysica acta. 2007;1767(10):1260-7.
489. Yesilova Z, Yaman H, Oktenli C, Ozcan A, Uygun A, Cakir E, et al. Systemic markers of lipid peroxidation and antioxidants in patients with nonalcoholic Fatty liver disease. The American journal of gastroenterology. 2005;100(4):850-5.
490. Farhangi MA, Alipour B, Jafarvand E, Khoshbaten M. Oral coenzyme Q10 supplementation in patients with nonalcoholic fatty liver disease: effects on serum vaspin, chemerin, pentraxin 3, insulin resistance and oxidative stress. Arch Med Res. 2014;45(7):589-95.
491. Van De Wier B, Koek GH, Bast A, Haenen GR. The Potential of Flavonoids in the Treatment of Non-alcoholic Fatty Liver Disease. Critical reviews in food science and nutrition. 2015:0.
492. Bast A, Haenen GR. Ten misconceptions about antioxidants. Trends in pharmacological sciences. 2013;34(8):430-6.
493. Liu Y, Wang D, Zhang D, Lv Y, Wei Y, Wu W, et al. Inhibitory effect of blueberry polyphenolic compounds on oleic acid-induced hepatic steatosis in vitro. Journal of agricultural and food chemistry. 2011;59(22):12254-63.
494. Wu KC, Cui JY, Klaassen CD. Beneficial role of Nrf2 in regulating NADPH generation and consumption. Toxicol Sci. 2011;123(2):590-600.
495. Liu Y, Zhang L, Song H, Ji G. Update on berberine in nonalcoholic Fatty liver disease. Evidence-based complementary and alternative medicine : eCAM. 2013;2013:308134.
496. Ge Y, Zhang Y, Li R, Chen W, Li Y, Chen G. Berberine regulated Gck, G6pc, Pck1 and Srebp-1c expression and activated AMP-activated protein kinase in primary rat hepatocytes. International journal of biological sciences. 2011;7(5):673-84.
497. Leng SH, Lu FE, Xu LJ. Therapeutic effects of berbe{Leng, 2004 #286}rine in impaired glucose tolerance rats and its influence on insulin secretion. Acta pharmacologica Sinica. 2004;25(4):496-502.

498. Lou T, Zhang Z, Xi Z, Liu K, Li L, Liu B, et al. Berberine inhibits inflammatory response and ameliorates insulin resistance in hepatocytes. Inflammation. 2011;34(6):659-67.
499. Kong WJ, Zhang H, Song DQ, Xue R, Zhao W, Wei J, et al. Berberine reduces insulin resistance through protein kinase C-dependent up-regulation of insulin receptor expression. Metabolism: clinical and experimental. 2009;58(1):109-19.
500. Zhou JY, Zhou SW, Zhang KB, Tang JL, Guang LX, Ying Y, et al. Chronic effects of berberine on blood, liver glucolipid metabolism and liver PPARs expression in diabetic hyperlipidemic rats. Biological & pharmaceutical bulletin. 2008;31(6):1169-76.
501. Shen N, Huan Y, Shen ZF. Berberine inhibits mouse insulin gene promoter through activation of AMP activated protein kinase and may exert beneficial effect on pancreatic beta-cell. European journal of pharmacology. 2012;694(1-3):120-6.
502. Li Y, Xu S, Mihaylova MM, Zheng B, Hou X, Jiang B, et al. AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. Cell metabolism. 2011;13(4):376-88.
503. Li H, Dong B, Park SW, Lee HS, Chen W, Liu J. Hepatocyte nuclear factor 1alpha plays a critical role in PCSK9 gene transcription and regulation by the natural hypocholesterolemic compound berberine. The Journal of biological chemistry. 2009;284(42):28885-95.
504. Goldstein JL, DeBose-Boyd RA, Brown MS. Protein sensors for membrane sterols. Cell. 2006;124(1):35-46.
505. Yang QH, Hu SP, Zhang YP, Xie WN, Li N, Ji GY, et al. Effect of berberine on expressions of uncoupling protein-2 mRNA and protein in hepatic tissue of non-alcoholic fatty liver disease in rats. Chinese journal of integrative medicine. 2011;17(3):205-11.
506. Lao-ong T, Chatuphonprasert W, Nemoto N, Jarukamjorn K. Alteration of hepatic glutathione peroxidase and superoxide dismutase expression in streptozotocin-induced diabetic mice by berberine. Pharmaceutical biology. 2012;50(8):1007-12.
507. Zhang X, Zhao Y, Zhang M, Pang X, Xu J, Kang C, et al. Structural changes of gut microbiota during berberine-mediated prevention of obesity and insulin resistance in high-fat diet-fed rats. PloS one. 2012;7(8):e42529.

508. Chang X, Yan H, Fei J, Jiang M, Zhu H, Lu D, et al. Berberine reduces methylation of the MTTP promoter and alleviates fatty liver induced by a high-fat diet in rats. Journal of lipid research. 2010;51(9):2504-15.

509. Teodoro JS, Duarte FV, Gomes AP, Varela AT, Peixoto FM, Rolo AP, et al. Berberine reverts hepatic mitochondrial dysfunction in high-fat fed rats: a possible role for SirT3 activation. Mitochondrion. 2013;13(6):637-46.

510. Kim WS, Lee YS, Cha SH, Jeong HW, Choe SS, Lee MR, et al. Berberine improves lipid dysregulation in obesity by controlling central and peripheral AMPK activity. American journal of physiology Endocrinology and metabolism. 2009;296(4):E812-9.

511. Brusq JM, Ancellin N, Grondin P, Guillard R, Martin S, Saintillan Y, et al. Inhibition of lipid synthesis through activation of AMP kinase: an additional mechanism for the hypolipidemic effects of berberine. Journal of lipid research. 2006;47(6):1281-8.

512. Zhang BJ, Xu D, Guo Y, Ping J, Chen LB, Wang H. Protection by and anti-oxidant mechanism of berberine against rat liver fibrosis induced by multiple hepatotoxic factors. Clinical and experimental pharmacology & physiology. 2008;35(3):303-9.

513. Marazzi G, Cacciotti L, Pelliccia F, Iaia L, Volterrani M, Caminiti G, et al. Long-term effects of nutraceuticals (berberine, red yeast rice, policosanol) in elderly hypercholesterolemic patients. Advances in therapy. 2011;28(12):1105-13.

514. Di Pierro F, Villanova N, Agostini F, Marzocchi R, Soverini V, Marchesini G. Pilot study on the additive effects of berberine and oral type 2 diabetes agents for patients with suboptimal glycemic control. Diabetes, metabolic syndrome and obesity : targets and therapy. 2012;5:213-7.

515. Liu YT, Hao HP, Xie HG, Lai L, Wang Q, Liu CX, et al. Extensive intestinal first-pass elimination and predominant hepatic distribution of berberine explain its low plasma levels in rats. Drug metabolism and disposition: the biological fate of chemicals. 2010;38(10):1779-84.

516. Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, et al. Resveratrol improves health and survival of mice on a high-calorie diet. Nature. 2006;444(7117):337-42.

517. Baur JA, Sinclair DA. Therapeutic potential of resveratrol: the in vivo evidence. Nat Rev Drug Discov. 2006;5(6):493-506.

518. Ahn J, Cho I, Kim S, Kwon D, Ha T. Dietary resveratrol alters lipid metabolism-related gene expression of mice on an atherogenic diet. Journal of hepatology. 2008;49(6):1019-28.

519. Franco JG, Lisboa PC, Lima NS, Amaral TA, Peixoto-Silva N, Resende AC, et al. Resveratrol attenuates oxidative stress and prevents steatosis and hypertension in obese rats programmed by early weaning. The Journal of nutritional biochemistry. 2013;24(6):960-6.

520. Cho SJ, Jung UJ, Choi MS. Differential effects of low-dose resveratrol on adiposity and hepatic steatosis in diet-induced obese mice. The British journal of nutrition. 2012;108(12):2166-75.

521. Bujanda L, Hijona E, Larzabal M, Beraza M, Aldazabal P, Garcia-Urkia N, et al. Resveratrol inhibits nonalcoholic fatty liver disease in rats. BMC gastroenterology. 2008;8:40.

522. Shang J, Chen LL, Xiao FX, Sun H, Ding HC, Xiao H. Resveratrol improves non-alcoholic fatty liver disease by activating AMP-activated protein kinase. Acta pharmacologica Sinica. 2008;29(6):698-706.

523. Do GM, Jung UJ, Park HJ, Kwon EY, Jeon SM, McGregor RA, et al. Resveratrol ameliorates diabetes-related metabolic changes via activation of AMP-activated protein kinase and its downstream targets in db/db mice. Molecular nutrition & food research. 2012;56(8):1282-91.

524. Zhou M, Wang S, Zhao A, Wang K, Fan Z, Yang H, et al. Transcriptomic and metabonomic profiling reveal synergistic effects of quercetin and resveratrol supplementation in high fat diet fed mice. Journal of proteome research. 2012;11(10):4961-71.

525. Cho IJ, Ahn JY, Kim S, Choi MS, Ha TY. Resveratrol attenuates the expression of HMG-CoA reductase mRNA in hamsters. Biochemical and biophysical research communications. 2008;367(1):190-4.

526. Gomez-Zorita S, Fernandez-Quintela A, Macarulla MT, Aguirre L, Hijona E, Bujanda L, et al. Resveratrol attenuates steatosis in obese Zucker rats by decreasing fatty acid availability and reducing oxidative stress. The British journal of nutrition. 2012;107(2):202-10.

527. Xin P, Han H, Gao D, Cui W, Yang X, Ying C, et al. Alleviative effects of resveratrol on nonalcoholic fatty liver disease are associated with up regulation of hepatic low density lipoprotein receptor and scavenger receptor class B type I gene expressions in rats. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2013;52:12-8.
528. Faghihzadeh F, Adibi P, Rafiei R, Hekmatdoost A. Resveratrol supplementation improves inflammatory biomarkers in patients with nonalcoholic fatty liver disease. Nutrition research. 2014;34(10):837-43.
529. Timmers S, Konings E, Bilet L, Houtkooper RH, van de Weijer T, Goossens GH, et al. Calorie restriction-like effects of 30 days of resveratrol supplementation on energy metabolism and metabolic profile in obese humans. Cell metabolism. 2011;14(5):612-22.
530. Abenavoli L, Milic N, Capasso F. Anti-oxidant therapy in non-alcoholic fatty liver disease: the role of silymarin. Endocrine. 2012;42(3):754-5.
531. El-Kamary SS, Shardell MD, Abdel-Hamid M, Ismail S, El-Ateek M, Metwally M, et al. A randomized controlled trial to assess the safety and efficacy of silymarin on symptoms, signs and biomarkers of acute hepatitis. Phytomedicine : international journal of phytotherapy and phytopharmacology. 2009;16(5):391-400.
532. Serviddio G, Bellanti F, Giudetti AM, Gnoni GV, Petrella A, Tamborra R, et al. A silybin-phospholipid complex prevents mitochondrial dysfunction in a rodent model of nonalcoholic steatohepatitis. The Journal of pharmacology and experimental therapeutics. 2010;332(3):922-32.
533. Haddad Y, Vallerand D, Brault A, Haddad PS. Antioxidant and hepatoprotective effects of silibinin in a rat model of nonalcoholic steatohepatitis. Evidence-based complementary and alternative medicine : eCAM. 2011;2011:nep164.
534. Kim M, Yang SG, Kim JM, Lee JW, Kim YS, Lee JI. Silymarin suppresses hepatic stellate cell activation in a dietary rat model of non-alcoholic steatohepatitis: analysis of isolated hepatic stellate cells. International journal of molecular medicine. 2012;30(3):473-9.

535. Qin R, Zhang J, Li C, Zhang X, Xiong A, Huang F, et al. Protective effects of gypenosides against fatty liver disease induced by high fat and cholesterol diet and alcohol in rats. Archives of pharmacal research. 2012;35(7):1241-50.

536. Grattagliano I, Diogo CV, Mastrodonato M, de Bari O, Persichella M, Wang DQ, et al. A silybin-phospholipids complex counteracts rat fatty liver degeneration and mitochondrial oxidative changes. World journal of gastroenterology : WJG. 2013;19(20):3007-17.

537. Daubioul CA, Horsmans Y, Lambert P, Danse E, Delzenne NM. Effects of oligofructose on glucose and lipid metabolism in patients with nonalcoholic steatohepatitis: results of a pilot study. European journal of clinical nutrition. 2005;59(5):723-6.

538. Aller R, De Luis DA, Izaola O, Conde R, Gonzalez Sagrado M, Primo D, et al. Effect of a probiotic on liver aminotransferases in nonalcoholic fatty liver disease patients: a double blind randomized clinical trial. European review for medical and pharmacological sciences. 2011;15(9):1090-5.

539. Parnell JA, Raman M, Rioux KP, Reimer RA. The potential role of prebiotic fibre for treatment and management of non-alcoholic fatty liver disease and associated obesity and insulin resistance. Liver international : official journal of the International Association for the Study of the Liver. 2012;32(5):701-11.

540. Fan JG, Xu ZJ, Wang GL. Effect of lactulose on establishment of a rat non-alcoholic steatohepatitis model. World journal of gastroenterology : WJG. 2005;11(32):5053-6.

541. Salminen S, Salminen E. Lactulose, lactic acid bacteria, intestinal microecology and mucosal protection. Scandinavian journal of gastroenterology Supplement. 1997;222:45-8.

542. Wong VW, Won GL, Chim AM, Chu WC, Yeung DK, Li KC, et al. Treatment of nonalcoholic steatohepatitis with probiotics. A proof-of-concept study. Annals of hepatology. 2013;12(2):256-62.

543. Eslamparast T, Poustchi H, Zamani F, Sharafkhah M, Malekzadeh R, Hekmatdoost A. Synbiotic supplementation in nonalcoholic fatty liver disease: a randomized, double-blind, placebo-controlled pilot study. The American journal of clinical nutrition. 2014;99(3):535-42.

544. Malaguarnera M, Vacante M, Antic T, Giordano M, Chisari G, Acquaviva R, et al. Bifidobacterium longum with fructo-oligosaccharides in patients with non alcoholic steatohepatitis. Digestive diseases and sciences. 2012;57(2):545-53.

545. Frasinariu OE, Ceccarelli S, Alisi A, Moraru E, Nobili V. Gut-liver axis and fibrosis in nonalcoholic fatty liver disease: an input for novel therapies. Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver. 2013;45(7):543-51.

546. Mouzaki M, Comelli EM, Arendt BM, Bonengel J, Fung SK, Fischer SE, et al. Intestinal microbiota in patients with nonalcoholic fatty liver disease. Hepatology. 2013;58(1):120-7.

547. Kelishadi R, Farajian S, Mirlohi M. Probiotics as a novel treatment for non-alcoholic Fatty liver disease; a systematic review on the current evidences. Hepatitis monthly. 2013;13(4):e7233.

548. Henao-Mejia J, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, et al. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature. 2012;482(7384):179-85.

549. Zhu L, Baker SS, Gill C, Liu W, Alkhouri R, Baker RD, et al. Characterization of gut microbiomes in nonalcoholic steatohepatitis (NASH) patients: a connection between endogenous alcohol and NASH. Hepatology. 2013;57(2):601-9.

550. Esposito E, Iacono A, Bianco G, Autore G, Cuzzocrea S, Vajro P, et al. Probiotics reduce the inflammatory response induced by a high-fat diet in the liver of young rats. The Journal of nutrition. 2009;139(5):905-11.

551. Li Z, Yang S, Lin H, Huang J, Watkins PA, Moser AB, et al. Probiotics and antibodies to TNF inhibit inflammatory activity and improve nonalcoholic fatty liver disease. Hepatology. 2003;37(2):343-50.

552. Xu RY, Wan YP, Fang QY, Lu W, Cai W. Supplementation with probiotics modifies gut flora and attenuates liver fat accumulation in rat nonalcoholic fatty liver disease model. Journal of clinical biochemistry and nutrition. 2012;50(1):72-7.

553. Alisi A, Bedogni G, Baviera G, Giorgio V, Porro E, Paris C, et al. Randomised clinical trial: The beneficial effects of VSL#3 in obese children with non-alcoholic steatohepatitis. Alimentary pharmacology & therapeutics. 2014;39(11):1276-85.

554. Loguercio C, Federico A, Tuccillo C, Terracciano F, D'Auria MV, De Simone C, et al. Beneficial effects of a probiotic VSL#3 on parameters of liver dysfunction in chronic liver diseases. Journal of clinical gastroenterology. 2005;39(6):540-3.
555. Miele L, Valenza V, La Torre G, Montalto M, Cammarota G, Ricci R, et al. Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. Hepatology. 2009;49(6):1877-87.
556. Hennig B, Petriello MC, Gamble MV, Surh YJ, Kresty LA, Frank N, et al. The role of nutrition in influencing mechanisms involved in environmentally mediated diseases. Rev Environ Health. 2018;33(1):87-97.
557. Parodi PW. Distribution of isomeric octadecenoic fatty acids in milk fat. Journal of dairy science. 1976;59(11):1870-3.
558. Mozaffarian D, Abdollahi M, Campos H, Houshiarrad A, Willett WC. Consumption of trans fats and estimated effects on coronary heart disease in Iran. European journal of clinical nutrition. 2007;61(8):1004-10.
559. Colandre ME, Diez RS, Bernal CA. Metabolic effects of trans fatty acids on an experimental dietary model. The British journal of nutrition. 2003;89(5):631-9.
560. Dhaka V, Gulia N, Ahlawat KS, Khatkar BS. Trans fats-sources, health risks and alternative approach - A review. Journal of food science and technology. 2011;48(5):534-41.
561. Dorfman SE, Laurent D, Gounarides JS, Li X, Mullarkey TL, Rocheford EC, et al. Metabolic implications of dietary trans-fatty acids. Obesity. 2009;17(6):1200-7.
562. Arcand J, Scourboutakos MJ, Au JT, L'Abbe MR. trans Fatty acids in the Canadian food supply: an updated analysis. The American journal of clinical nutrition. 2014;100(4):1116-23.
563. Bahrami G, Ghanbarian E, Masoumi M, Rahimi Z, Rezwan Madani F. Comparison of fatty acid profiles of aorta and internal mammary arteries in patients with coronary artery disease. Clin Chim Acta. 2006;370(1-2):143-6.
564. Williams CM. Beneficial nutritional properties of olive oil: implications for postprandial lipoproteins and factor VII. Nutrition, metabolism, and cardiovascular diseases : NMCD. 2001;11(4 Suppl):51-6.

565. Shin S, Ajuwon KM. Effects of Diets Differing in Composition of 18-C Fatty Acids on Adipose Tissue Thermogenic Gene Expression in Mice Fed High-Fat Diets. Nutrients. 2018;10(2).

566. Pischon T, Hankinson SE, Hotamisligil GS, Rifai N, Willett WC, Rimm EB. Habitual dietary intake of n-3 and n-6 fatty acids in relation to inflammatory markers among US men and women. Circulation. 2003;108(2):155-60.

567. Harbige LS. Fatty acids, the immune response, and autoimmunity: a question of n-6 essentiality and the balance between n-6 and n-3. Lipids. 2003;38(4):323-41.

568. Zarate R, El Jaber-Vazdekis N, Tejera N, Perez JA, Rodriguez C. Significance of long chain polyunsaturated fatty acids in human health. Clin Transl Med. 2017;6(1):25.

569. Thomas MH, Pelleieux S, Vitale N, Olivier JL. Dietary arachidonic acid as a risk factor for age-associated neurodegenerative diseases: Potential mechanisms. Biochimie. 2016;130:168-77.

570. Fattahi MJ, Mirshafiey A. Prostaglandins and rheumatoid arthritis. Arthritis. 2012;2012:239310.

571. Korotkova M, Lundberg IE. The skeletal muscle arachidonic acid cascade in health and inflammatory disease. Nat Rev Rheumatol. 2014;10(5):295-303.

572. Laster MJ, Gong D, Kerschmann RL, Eger EI, 2nd, Martin JL. Acetaminophen predisposes to renal and hepatic injury from compound A in the fasting rat. Anesth Analg. 1997;84(1):169-72.

573. Sorokin AV, Domenichiello A, Dey AK, Goyal A, Rose SM, Playford MP, et al. Bioactive Lipid Mediators Profiles In Human Psoriasis Skin And Blood. J Invest Dermatol. 2018.

574. Hu XF, Laird BD, Chan HM. Mercury diminishes the cardiovascular protective effect of omega-3 polyunsaturated fatty acids in the modern diet of Inuit in Canada. Environ Res. 2017;152:470-7.

575. Wang Y, Crawford MA, Chen J, Li J, Ghebremeskel K, Campbell TC, et al. Fish consumption, blood docosahexaenoic acid and chronic diseases in Chinese rural populations. Comp Biochem Physiol A Mol Integr Physiol. 2003;136(1):127-40.

576. Gucalp A, Zhou XK, Cook ED, Garber JE, Crew KD, Nangia JR, et al. A Randomized Multicenter Phase II Study of Docosahexaenoic Acid in Patients with a History of Breast Cancer, Premalignant Lesions or Benign Breast Disease. Cancer Prev Res (Phila). 2018.
577. Belanger SA, Vanasse M, Spahis S, Sylvestre MP, Lippe S, L'Heureux F, et al. Omega-3 fatty acid treatment of children with attention-deficit hyperactivity disorder: A randomized, double-blind, placebo-controlled study. Paediatr Child Health. 2009;14(2):89-98.
578. Spahis S, Vanasse M, Belanger SA, Ghadirian P, Grenier E, Levy E. Lipid profile, fatty acid composition and pro- and anti-oxidant status in pediatric patients with attention-deficit/hyperactivity disorder. Prostaglandins, leukotrienes, and essential fatty acids. 2008;79(1-2):47-53.
579. Rezende FA, Lapalme E, Qian CX, Smith LE, SanGiovanni JP, Sapieha P. Omega-3 supplementation combined with anti-vascular endothelial growth factor lowers vitreal levels of vascular endothelial growth factor in wet age-related macular degeneration. Am J Ophthalmol. 2014;158(5):1071-78.
580. Sanchez-Borrego R, von Schacky C, Osorio MJA, Llaneza P, Pinto X, Losa F, et al. Recommendations of the Spanish Menopause Society on the consumption of omega-3 polyunsaturated fatty acids by postmenopausal women. Maturitas. 2017;103:71-7.
581. Zehr KR, Walker MK. Omega-3 polyunsaturated fatty acids improve endothelial function in humans at risk for atherosclerosis: A review. Prostaglandins Other Lipid Mediat. 2018;134:131-40.
582. Micha R, Penalvo JL, Cudhea F, Imamura F, Rehm CD, Mozaffarian D. Association Between Dietary Factors and Mortality From Heart Disease, Stroke, and Type 2 Diabetes in the United States. Jama. 2017;317(9):912-24.
583. Siscovick DS, Barringer TA, Fretts AM, Wu JH, Lichtenstein AH, Costello RB, et al. Omega-3 Polyunsaturated Fatty Acid (Fish Oil) Supplementation and the Prevention of Clinical Cardiovascular Disease: A Science Advisory From the American Heart Association. Circulation. 2017;135(15):e867-e84.
584. Spahis S, Delvin E, Borys JM, Levy E. Oxidative Stress as a Critical Factor in Nonalcoholic Fatty Liver Disease Pathogenesis. Antioxid Redox Signal. 2017;26(10):519-41.

585. Bouzianas DG, Bouziana SD, Hatzitolios AI. Potential treatment of human nonalcoholic fatty liver disease with long-chain omega-3 polyunsaturated fatty acids. Nutrition reviews. 2013;71(11):753-71.
586. Deckelbaum RJ, Worgall TS, Seo T. n-3 fatty acids and gene expression. The American journal of clinical nutrition. 2006;83(6 Suppl):1520S-5S.
587. Bang HO, Dyerberg J, Sinclair HM. The composition of the Eskimo food in north western Greenland. The American journal of clinical nutrition. 1980;33(12):2657-61.
588. Okuda N, Ueshima H, Okayama A, Saitoh S, Nakagawa H, Rodriguez BL, et al. Relation of long chain n-3 polyunsaturated fatty acid intake to serum high density lipoprotein cholesterol among Japanese men in Japan and Japanese-American men in Hawaii: the INTERLIPID study. Atherosclerosis. 2005;178(2):371-9.
589. Yoshino T, Kakunaka N, Liang Y, Ito Y, Maeda Y, Nomaguchi T, et al. Production of omega3 fatty acids in marine cyanobacterium Synechococcus sp. strain NKBG 15041c via genetic engineering. Appl Microbiol Biotechnol. 2017;101(18):6899-905.
590. Peltomaa E, Johnson MD, Taipale SJ. Marine Cryptophytes Are Great Sources of EPA and DHA. Mar Drugs. 2017;16(1).
591. Shahidi F, Ambigaipalan P. Omega-3 Polyunsaturated Fatty Acids and Their Health Benefits. Annu Rev Food Sci Technol. 2018.
592. Griffin BA. How relevant is the ratio of dietary n-6 to n-3 polyunsaturated fatty acids to cardiovascular disease risk? Evidence from the OPTILIP study. Current opinion in lipidology. 2008;19(1):57-62.
593. Tani S, Takahashi A, Nagao K, Hirayama A. Association of Fish Consumption-Derived Ratio of Serum n-3 to n-6 Polyunsaturated Fatty Acids and Cardiovascular Risk With the Prevalence of Coronary Artery Disease. Int Heart J. 2015;56(3):260-8.
594. Harris WS. The omega-3 index: from biomarker to risk marker to risk factor. Current atherosclerosis reports. 2009;11(6):411-7.
595. Harris WS, Von Schacky C. The Omega-3 Index: a new risk factor for death from coronary heart disease? Prev Med. 2004;39(1):212-20.
596. Dyerberg J, Bang HO, Stoffersen E, Moncada S, Vane JR. Eicosapentaenoic acid and prevention of thrombosis and atherosclerosis? Lancet. 1978;2(8081):117-9.
597. Mozaffarian D. JELIS, fish oil, and cardiac events. Lancet. 2007;369(9567):1062-3.

598. Iso H, Kobayashi M, Ishihara J, Sasaki S, Okada K, Kita Y, et al. Intake of fish and n3 fatty acids and risk of coronary heart disease among Japanese: the Japan Public Health Center-Based (JPHC) Study Cohort I. Circulation. 2006;113(2):195-202.
599. Nakamura Y, Ueshima H, Okuda N, Higashiyama A, Kita Y, Kadowaki T, et al. Relation of dietary and other lifestyle traits to difference in serum adiponectin concentration of Japanese in Japan and Hawaii: the INTERLIPID Study. The American journal of clinical nutrition. 2008;88(2):424-30.
600. Hibbeln JR, Nieminen LR, Blasbalg TL, Riggs JA, Lands WE. Healthy intakes of n-3 and n-6 fatty acids: estimations considering worldwide diversity. The American journal of clinical nutrition. 2006;83(6 Suppl):1483S-93S.
601. Yagi S, Fukuda D, Aihara KI, Akaike M, Shimabukuro M, Sata M. n-3 Polyunsaturated Fatty Acids: Promising Nutrients for Preventing Cardiovascular Disease. Journal of atherosclerosis and thrombosis. 2017;24(10):999-1010.
602. Adkins Y, Kelley DS. Mechanisms underlying the cardioprotective effects of omega-3 polyunsaturated fatty acids. The Journal of nutritional biochemistry. 2010;21(9):781-92.
603. Calder PC. Omega-3 polyunsaturated fatty acids and inflammatory processes: nutrition or pharmacology? British journal of clinical pharmacology. 2013;75(3):645-62.
604. McLennan P, Howe P, Abeywardena M, Muggli R, Raederstorff D, Mano M, et al. The cardiovascular protective role of docosahexaenoic acid. European journal of pharmacology. 1996;300(1-2):83-9.
605. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. Lancet. 1999;354(9177):447-55.
606. Casula M, Soranna D, Catapano AL, Corrao G. Long-term effect of high dose omega-3 fatty acid supplementation for secondary prevention of cardiovascular outcomes: A meta-analysis of randomized, placebo controlled trials [corrected]. Atherosclerosis Supplements. 2013;14(2):243-51.
607. Musa-Veloso K, Binns MA, Kocenas A, Chung C, Rice H, Oppedal-Olsen H, et al. Impact of low v. moderate intakes of long-chain n-3 fatty acids on risk of coronary heart disease. The British journal of nutrition. 2011;106(8):1129-41.

608. Mozaffarian D, Wu JH. Omega-3 fatty acids and cardiovascular disease: effects on risk factors, molecular pathways, and clinical events. J Am Coll Cardiol. 2011;58(20):2047-67.

609. Zhao YT, Chen Q, Sun YX, Li XB, Zhang P, Xu Y, et al. Prevention of sudden cardiac death with omega-3 fatty acids in patients with coronary heart disease: a meta-analysis of randomized controlled trials. Ann Med. 2009;41(4):301-10.

610. Mozaffarian D, Bryson CL, Lemaitre RN, Burke GL, Siscovick DS. Fish intake and risk of incident heart failure. J Am Coll Cardiol. 2005;45(12):2015-21.

611. Hooper L, Griffiths E, Abrahams B, Alexander W, Atkins S, Atkinson G, et al. Dietetic guidelines: diet in secondary prevention of cardiovascular disease (first update, June 2003). J Hum Nutr Diet. 2004;17(4):337-49.

612. Bucher HC, Hengstler P, Schindler C, Meier G. N-3 polyunsaturated fatty acids in coronary heart disease: a meta-analysis of randomized controlled trials. Am J Med. 2002;112(4):298-304.

613. Liu N, Zhang W, Ma W, Shang W, Zheng J, Sun L. Risk factors for hypoxemia following surgical repair of acute type A aortic dissection. Interact Cardiovasc Thorac Surg. 2017;24(2):251-6.

614. Hooper L, Bartlett C, Davey SG, Ebrahim S. Advice to reduce dietary salt for prevention of cardiovascular disease. The Cochrane database of systematic reviews. 2004(1):CD003656.

615. Hooper L, Thompson RL, Harrison RA, Summerbell CD, Ness AR, Moore HJ, et al. Risks and benefits of omega 3 fats for mortality, cardiovascular disease, and cancer: systematic review. BMJ. 2006;332(7544):752-60.

616. Shearer GC, Savinova OV, Harris WS. Fish oil -- how does it reduce plasma triglycerides? Biochimica et biophysica acta. 2012;1821(5):843-51.

617. Schirmer SH, Werner CM, Binder SB, Faas ME, Custodis F, Bohm M, et al. Effects of omega-3 fatty acids on postprandial triglycerides and monocyte activation. Atherosclerosis. 2012;225(1):166-72.

618. Harris WS, Connor WE, Alam N, Illingworth DR. Reduction of postprandial triglyceridemia in humans by dietary n-3 fatty acids. Journal of lipid research. 1988;29(11):1451-60.

619. Miyoshi T, Noda Y, Ohno Y, Sugiyama H, Oe H, Nakamura K, et al. Omega-3 fatty acids improve postprandial lipemia and associated endothelial dysfunction in healthy individuals - a randomized cross-over trial. Biomed Pharmacother. 2014;68(8):1071-7.
620. Ooi EM, Lichtenstein AH, Millar JS, Diffenderfer MR, Lamon-Fava S, Rasmussen H, et al. Effects of Therapeutic Lifestyle Change diets high and low in dietary fish-derived FAs on lipoprotein metabolism in middle-aged and elderly subjects. Journal of lipid research. 2012;53(9):1958-67.
621. Tholstrup T, Hellgren LI, Petersen M, Basu S, Straarup EM, Schnohr P, et al. A solid dietary fat containing fish oil redistributes lipoprotein subclasses without increasing oxidative stress in men. The Journal of nutrition. 2004;134(5):1051-7.
622. Schenk S, Saberi M, Olefsky JM. Insulin sensitivity: modulation by nutrients and inflammation. The Journal of clinical investigation. 2008;118(9):2992-3002.
623. Ruiz-Nunez B, Dijck-Brouwer DA, Muskiet FA. The relation of saturated fatty acids with low-grade inflammation and cardiovascular disease. The Journal of nutritional biochemistry. 2016;36:1-20.
624. Karaderi T, Drong AW, Lindgren CM. Insights into the Genetic Susceptibility to Type 2 Diabetes from Genome-Wide Association Studies of Obesity-Related Traits. Current diabetes reports. 2015;15(10):83.
625. Di Meo S, Iossa S, Venditti P. Skeletal muscle insulin resistance: role of mitochondria and other ROS sources. The Journal of endocrinology. 2017;233(1):R15-R42.
626. Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, et al. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell. 2010;142(5):687-98.
627. Bhaswant M, Poudyal H, Brown L. Mechanisms of enhanced insulin secretion and sensitivity with n-3 unsaturated fatty acids. The Journal of nutritional biochemistry. 2015;26(6):571-84.
628. Martins AR, Crisma AR, Masi LN, Amaral CL, Marzuca-Nassr GN, Bomfim LHM, et al. Attenuation of obesity and insulin resistance by fish oil supplementation is associated with improved skeletal muscle mitochondrial function in mice fed a high-fat diet. The Journal of nutritional biochemistry. 2017;55:76-88.

629. Pahlavani M, Ramalho T, Koboziev I, LeMieux MJ, Jayarathne S, Ramalingam L, et al. Adipose tissue inflammation in insulin resistance: review of mechanisms mediating anti-inflammatory effects of omega-3 polyunsaturated fatty acids. J Investig Med. 2017;65(7):1021-7.
630. Abbott KA, Burrows TL, Thota RN, Acharya S, Garg ML. Do omega-3 PUFAs affect insulin resistance in a sex-specific manner? A systematic review and meta-analysis of randomized controlled trials. The American journal of clinical nutrition. 2016;104(5):1470-84.
631. Figueras M, Olivan M, Busquets S, Lopez-Soriano FJ, Argiles JM. Effects of eicosapentaenoic acid (EPA) treatment on insulin sensitivity in an animal model of diabetes: improvement of the inflammatory status. Obesity. 2011;19(2):362-9.
632. Lamping KG, Nuno DW, Coppey LJ, Holmes AJ, Hu S, Oltman CL, et al. Modification of high saturated fat diet with n-3 polyunsaturated fat improves glucose intolerance and vascular dysfunction. Diabetes, obesity & metabolism. 2013;15(2):144-52.
633. Anna Z, Angela S, Barbara B, Jana R, Tamara B, Csilla V, et al. Heart-protective effect of n-3 PUFA demonstrated in a rat model of diabetic cardiomyopathy. Molecular and cellular biochemistry. 2014;389(1-2):219-27.
634. Coelho OGL, da Silva BP, Rocha D, Lopes LL, Alfenas RCG. Polyunsaturated fatty acids and type 2 diabetes: Impact on the glycemic control mechanism. Critical reviews in food science and nutrition. 2017;57(17):3614-9.
635. Vilar L, Oliveira CP, Faintuch J, Mello ES, Nogueira MA, Santos TE, et al. High-fat diet: a trigger of non-alcoholic steatohepatitis? Preliminary findings in obese subjects. Nutrition. 2008;24(11-12):1097-102.
636. de Wit NJ, Afman LA, Mensink M, Muller M. Phenotyping the effect of diet on non-alcoholic fatty liver disease. Journal of hepatology. 2012;57(6):1370-3.
637. Masterton GS, Plevris JN, Hayes PC. Review article: omega-3 fatty acids - a promising novel therapy for non-alcoholic fatty liver disease. Alimentary pharmacology & therapeutics. 2010;31(7):679-92.
638. Di Minno MN, Tremoli E, Tufano A, Russolillo A, Lupoli R, Di Minno G. Exploring newer cardioprotective strategies: omega-3 fatty acids in perspective. Thrombosis and haemostasis. 2010;104(4):664-80.

639. Gerber PA, Gouni-Berthold I, Berneis K. Omega-3 fatty acids: role in metabolism and cardiovascular disease. Current pharmaceutical design. 2013;19(17):3074-93.
640. Borengasser SJ, Rector RS, Uptergrove GM, Morris EM, Perfield JW, 2nd, Booth FW, et al. Exercise and Omega-3 Polyunsaturated Fatty Acid Supplementation for the Treatment of Hepatic Steatosis in Hyperphagic OLETF Rats. Journal of nutrition and metabolism. 2012;2012:268680.
641. Assy N. Nutritional recommendations for patients with non-alcoholic fatty liver diseases. World journal of gastroenterology : WJG. 2011;17(29):3375-6.
642. Takeuchi Y, Yahagi N, Izumida Y, Nishi M, Kubota M, Teraoka Y, et al. Polyunsaturated fatty acids selectively suppress sterol regulatory element-binding protein-1 through proteolytic processing and autoloop regulatory circuit. The Journal of biological chemistry. 2010;285(15):11681-91.
643. Mehta SR. Advances in the treatment of nonalcoholic fatty liver disease. Therapeutic advances in endocrinology and metabolism. 2010;1(3):101-15.
644. Neschen S, Morino K, Dong J, Wang-Fischer Y, Cline GW, Romanelli AJ, et al. n-3 Fatty acids preserve insulin sensitivity in vivo in a peroxisome proliferator-activated receptor-alpha-dependent manner. Diabetes. 2007;56(4):1034-41.
645. Sato A, Kawano H, Notsu T, Ohta M, Nakakuki M, Mizuguchi K, et al. Antiobesity effect of eicosapentaenoic acid in high-fat/high-sucrose diet-induced obesity: importance of hepatic lipogenesis. Diabetes. 2010;59(10):2495-504.
646. Zhao Y, Joshi-Barve S, Barve S, Chen LH. Eicosapentaenoic acid prevents LPS-induced TNF-alpha expression by preventing NF-kappaB activation. Journal of the American College of Nutrition. 2004;23(1):71-8.
647. Molendi-Coste O, Legry V, Leclercq IA. Why and How Meet n-3 PUFA Dietary Recommendations? Gastroenterology research and practice. 2011;2011:364040.
648. Gonzalez-Periz A, Horrillo R, Ferre N, Gronert K, Dong B, Moran-Salvador E, et al. Obesity-induced insulin resistance and hepatic steatosis are alleviated by omega-3 fatty acids: a role for resolvins and protectins. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2009;23(6):1946-57.

649. Dentin R, Benhamed F, Hainault I, Fauveau V, Foufelle F, Dyck JR, et al. Liver-specific inhibition of ChREBP improves hepatic steatosis and insulin resistance in ob/ob mice. Diabetes. 2006;55(8):2159-70.

650. Claria J, Gonzalez-Periz A, Lopez-Vicario C, Rius B, Titos E. New insights into the role of macrophages in adipose tissue inflammation and Fatty liver disease: modulation by endogenous omega-3 Fatty Acid-derived lipid mediators. Front Immunol. 2011;2:49.

651. Georgiadi A, Kersten S. Mechanisms of gene regulation by fatty acids. Adv Nutr. 2012;3(2):127-34.

652. Pettinelli P, Del Pozo T, Araya J, Rodrigo R, Araya AV, Smok G, et al. Enhancement in liver SREBP-1c/PPAR-alpha ratio and steatosis in obese patients: correlations with insulin resistance and n-3 long-chain polyunsaturated fatty acid depletion. Biochimica et biophysica acta. 2009;1792(11):1080-6.

653. Parker HM, Johnson NA, Burdon CA, Cohn JS, O'Connor HT, George J. Omega-3 supplementation and non-alcoholic fatty liver disease: a systematic review and meta-analysis. Journal of hepatology. 2012;56(4):944-51.

654. Yu L, Yuan M, Wang L. The effect of omega-3 unsaturated fatty acids on non-alcoholic fatty liver disease: A systematic review and meta-analysis of RCTs. Pak J Med Sci. 2017;33(4):1022-8.

655. Pacifico L, Bonci E, Di Martino M, Versacci P, Andreoli G, Silvestri LM, et al. A double-blind, placebo-controlled randomized trial to evaluate the efficacy of docosahexaenoic acid supplementation on hepatic fat and associated cardiovascular risk factors in overweight children with nonalcoholic fatty liver disease. Nutrition, metabolism, and cardiovascular diseases : NMCD. 2015;25(8):734-41.

656. Tanaka N, Sano K, Horiuchi A, Tanaka E, Kiyosawa K, Aoyama T. Highly purified eicosapentaenoic acid treatment improves nonalcoholic steatohepatitis. Journal of clinical gastroenterology. 2008;42(4):413-8.

657. Al-Gayyar MM, Shams ME, Barakat EA. Fish oil improves lipid metabolism and ameliorates inflammation in patients with metabolic syndrome: impact of nonalcoholic fatty liver disease. Pharmaceutical biology. 2012;50(3):297-303.

658. Di Minno MN, Russolillo A, Lupoli R, Ambrosino P, Di Minno A, Tarantino G. Omega-3 fatty acids for the treatment of non-alcoholic fatty liver disease. World journal of gastroenterology : WJG. 2012;18(41):5839-47.
659. Wachira JK, Larson MK, Harris WS. n-3 Fatty acids affect haemostasis but do not increase the risk of bleeding: clinical observations and mechanistic insights. The British journal of nutrition. 2014;111(9):1652-62.
660. Shapiro H, Tehilla M, Attal-Singer J, Bruck R, Luzzatti R, Singer P. The therapeutic potential of long-chain omega-3 fatty acids in nonalcoholic fatty liver disease. Clinical nutrition. 2011;30(1):6-19.
661. Li Y, Chen D. The optimal dose of omega-3 supplementation for non-alcoholic fatty liver disease. Journal of hepatology. 2012;57(2):468-9; author reply 9-70.
662. Pedron T, Nigro G, Sansonetti PJ. From homeostasis to pathology: decrypting microbe-host symbiotic signals in the intestinal crypt. Philos Trans R Soc Lond B Biol Sci. 2016;371(1707).
663. Avetisyan M, Schill EM, Heuckeroth RO. Building a second brain in the bowel. The Journal of clinical investigation. 2015;125(3):899-907.
664. Stan S, Lambert M, Delvin E, Paradis G, O'Loughlin J, Hanley JA, et al. Intestinal fatty acid binding protein and microsomal triglyceride transfer protein polymorphisms in French-Canadian youth. Journal of lipid research. 2005;46(2):320-7.
665. Levy E, Stan S, Delvin E, Menard D, Shoulders C, Garofalo C, et al. Localization of microsomal triglyceride transfer protein in the Golgi: possible role in the assembly of chylomicrons. The Journal of biological chemistry. 2002;277(19):16470-7.
666. Levy E, Stan S, Garofalo C, Delvin EE, Seidman EG, Menard D. Immunolocalization, ontogeny, and regulation of microsomal triglyceride transfer protein in human fetal intestine. American journal of physiology Gastrointestinal and liver physiology. 2001;280(4):G563-71.
667. Levy E. Insights from human congenital disorders of intestinal lipid metabolism. Journal of lipid research. 2015;56(5):945-62.
668. Levy E, Roy CC, Thibault L, Bonin A, Brochu P, Seidman EG. Variable expression of familial heterozygous hypobetalipoproteinemia: transient malabsorption during infancy. Journal of lipid research. 1994;35(12):2170-7.

669. Levy E, Seidman EG. Genetic aspects of disorders in beta-lipoproteins. Journal of pediatric gastroenterology and nutrition. 1992;14(4):472-3.
670. Levy E, Marcel YL, Milne RW, Grey VL, Roy CC. Absence of intestinal synthesis of apolipoprotein B-48 in two cases of abetalipoproteinemia. Gastroenterology. 1987;93(5):1119-26.
671. Charcosset M, Sassolas A, Peretti N, Roy CC, Deslandres C, Sinnett D, et al. Anderson or chylomicron retention disease: molecular impact of five mutations in the SAR1B gene on the structure and the functionality of Sar1b protein. Mol Genet Metab. 2008;93(1):74-84.
672. Levy E, Marcel Y, Deckelbaum RJ, Milne R, Lepage G, Seidman E, et al. Intestinal apoB synthesis, lipids, and lipoproteins in chylomicron retention disease. Journal of lipid research. 1987;28(11):1263-74.
673. Peretti N, Roy CC, Sassolas A, Deslandres C, Drouin E, Rasquin A, et al. Chylomicron retention disease: a long term study of two cohorts. Mol Genet Metab. 2009;97(2):136-42.
674. Peretti N, Sassolas A, Roy CC, Deslandres C, Charcosset M, Castagnetti J, et al. Guidelines for the diagnosis and management of chylomicron retention disease based on a review of the literature and the experience of two centers. Orphanet journal of rare diseases. 2010;5:24.
675. Roy CC, Levy E, Green PH, Sniderman A, Letarte J, Buts JP, et al. Malabsorption, hypocholesterolemia, and fat-filled enterocytes with increased intestinal apoprotein B. Chylomicron retention disease. Gastroenterology. 1987;92(2):390-9.
676. Sane AT, Seidman E, Peretti N, Kleme ML, Delvin E, Deslandres C, et al. Understanding Chylomicron Retention Disease Through Sar1b Gtpase Gene Disruption: Insight From Cell Culture. Arteriosclerosis, thrombosis, and vascular biology. 2017;37(12):2243-51.
677. Setayeshgar S, Ekwaru JP, Maximova K, Majumdar SR, Storey KE, McGavock J, et al. Dietary intake and prospective changes in cardiometabolic risk factors in children and youth. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme. 2017;42(1):39-45.

678. Taskinen MR, Soderlund S, Bogl LH, Hakkarainen A, Matikainen N, Pietilainen KH, et al. Adverse effects of fructose on cardiometabolic risk factors and hepatic lipid metabolism in subjects with abdominal obesity. J Intern Med. 2017;282(2):187-201.
679. Kant AK, Graubard BI. A prospective study of frequency of eating restaurant prepared meals and subsequent 9-year risk of all-cause and cardiometabolic mortality in US adults. PloS one. 2018;13(1):e0191584.
680. Boden G, Chen X, Ruiz J, White JV, Rossetti L. Mechanisms of fatty acid-induced inhibition of glucose uptake. The Journal of clinical investigation. 1994;93(6):2438-46.
681. Morales PE, Bucarey JL, Espinosa A. Muscle Lipid Metabolism: Role of Lipid Droplets and Perilipins. J Diabetes Res. 2017;2017:1789395.
682. Faasse S, Braun H, Vos M. The role of NAFLD in cardiometabolic disease: an update. F1000Res. 2018;7:170.
683. Lu FB, Hu ED, Xu LM, Chen L, Wu JL, Li H, et al. The relationship between obesity and the severity of non-alcoholic fatty liver disease: systematic review and meta-analysis. Expert Rev Gastroenterol Hepatol. 2018:1-12.
684. Karaman H, Karaman A, Donmez-Altuntas H, Bitgen N, Hamurcu Z, Oguz A, et al. Investigation of genome instability in patients with non-alcoholic steatohepatitis. World journal of gastroenterology : WJG. 2013;19(32):5295-301.
685. Del Chierico F, Gnani D, Vernocchi P, Petrucca A, Alisi A, Dallapiccola B, et al. Meta-omic platforms to assist in the understanding of NAFLD gut microbiota alterations: tools and applications. Int J Mol Sci. 2014;15(1):684-711.
686. Doycheva I, Watt KD, Alkhouri N. Nonalcoholic fatty liver disease in adolescents and young adults: The next frontier in the epidemic. Hepatology. 2017;65(6):2100-9.
687. Hamabe A, Uto H, Imamura Y, Kusano K, Mawatari S, Kumagai K, et al. Impact of cigarette smoking on onset of nonalcoholic fatty liver disease over a 10-year period. J Gastroenterol. 2011;46(6):769-78.
688. Kelishadi R, Poursafa P. Obesity and air pollution: global risk factors for pediatric non-alcoholic fatty liver disease. Hepatitis monthly. 2011;11(10):794-802.
689. Parekh S, Anania FA. Abnormal lipid and glucose metabolism in obesity: implications for nonalcoholic fatty liver disease. Gastroenterology. 2007;132(6):2191-207.

690. Ferguson AE, Xanthakos SA, Siegel RM. Challenges in Screening for Pediatric Nonalcoholic Fatty Liver Disease. Clin Pediatr (Phila). 2018;57(5):558-62.
691. Alkhouri N, Feldstein AE. Treating nonalcoholic steatohepatitis in children: Not a cinch task. Hepatology. 2017;65(4):1407-9.
692. Kotronen A, Seppanen-Laakso T, Westerbacka J, Kiviluoto T, Arola J, Ruskeepaa AL, et al. Hepatic stearoyl-CoA desaturase (SCD)-1 activity and diacylglycerol but not ceramide concentrations are increased in the nonalcoholic human fatty liver. Diabetes. 2009;58(1):203-8.
693. Narce M, Bellenger J, Rialland M, Bellenger S. Recent advances on stearoyl-Coa desaturase regulation in fatty liver diseases. Curr Drug Metab. 2012;13(10):1454-63.
694. Wree A, Broderick L, Canbay A, Hoffman HM, Feldstein AE. From NAFLD to NASH to cirrhosis-new insights into disease mechanisms. Nature reviews Gastroenterology & hepatology. 2013;10(11):627-36.
695. Ameer F, Scandiuzzi L, Hasnain S, Kalbacher H, Zaidi N. De novo lipogenesis in health and disease. Metabolism: clinical and experimental. 2014;63(7):895-902.
696. Eaton S. Control of mitochondrial beta-oxidation flux. Progress in lipid research. 2002;41(3):197-239.
697. Nakamura MT, Yudell BE, Loor JJ. Regulation of energy metabolism by long-chain fatty acids. Progress in lipid research. 2014;53:124-44.
698. Souza-Mello V. Peroxisome proliferator-activated receptors as targets to treat non-alcoholic fatty liver disease. World journal of hepatology. 2015;7(8):1012-9.
699. Veiga FMS, Graus-Nunes F, Rachid TL, Barreto AB, Mandarim-de-Lacerda CA, Souza-Mello V. Anti-obesogenic effects of WY14643 (PPAR-alpha agonist): Hepatic mitochondrial enhancement and suppressed lipogenic pathway in diet-induced obese mice. Biochimie. 2017;140:106-16.
700. Lamichane S, Dahal Lamichane B, Kwon SM. Pivotal Roles of Peroxisome Proliferator-Activated Receptors (PPARs) and Their Signal Cascade for Cellular and Whole-Body Energy Homeostasis. Int J Mol Sci. 2018;19(4).
701. Louet JF, Chatelain F, Decaux JF, Park EA, Kohl C, Pineau T, et al. Long-chain fatty acids regulate liver carnitine palmitoyltransferase I gene (L-CPT I) expression through

a peroxisome-proliferator-activated receptor alpha (PPARalpha)-independent pathway. The Biochemical journal. 2001;354(Pt 1):189-97.

702. Yao D, Luo J, He Q, Shi H, Li J, Wang H, et al. SCD1 Alters Long-Chain Fatty Acid (LCFA) Composition and Its Expression Is Directly Regulated by SREBP-1 and PPARgamma 1 in Dairy Goat Mammary Cells. J Cell Physiol. 2017;232(3):635-49.

703. Hamel FG, Bennett RG, Upward JL, Duckworth WC. Insulin inhibits peroxisomal fatty acid oxidation in isolated rat hepatocytes. Endocrinology. 2001;142(6):2702-6.

704. Naimi AI, Paquet C, Gauvin L, Daniel M. Associations between area-level unemployment, body mass index, and risk factors for cardiovascular disease in an urban area. Int J Environ Res Public Health. 2009;6(12):3082-96.

705. Le Ven F, Bibeau K, De Larochelliere E, Tizon-Marcos H, Deneault-Bissonnette S, Pibarot P, et al. Cardiac morphology and function reference values derived from a large subset of healthy young Caucasian adults by magnetic resonance imaging. Eur Heart J Cardiovasc Imaging. 2016;17(9):981-90.

706. Canada S. Body mass index, self-reported, youth (12 to 17 years old), overweight or obese. Table 105-0501 and Catalogue no. 82-221-X. Canada: CANSIM; 2016.

707. Denzer C, Thiere D, Muche R, Koenig W, Mayer H, Kratzer W, et al. Gender-specific prevalences of fatty liver in obese children and adolescents: roles of body fat distribution, sex steroids, and insulin resistance. The Journal of clinical endocrinology and metabolism. 2009;94(10):3872-81.

708. Fraser A, Longnecker MP, Lawlor DA. Prevalence of elevated alanine aminotransferase among US adolescents and associated factors: NHANES 1999-2004. Gastroenterology. 2007;133(6):1814-20.

709. Wiegand S, Keller KM, Robl M, L'Allemand D, Reinehr T, Widhalm K, et al. Obese boys at increased risk for nonalcoholic liver disease: evaluation of 16,390 overweight or obese children and adolescents. International journal of obesity. 2010;34(10):1468-74.

710. Paradies G, Paradies V, Ruggiero FM, Petrosillo G. Oxidative stress, cardiolipin and mitochondrial dysfunction in nonalcoholic fatty liver disease. World journal of gastroenterology : WJG. 2014;20(39):14205-18.

711. Kohli R, Pan X, Malladi P, Wainwright MS, Whitington PF. Mitochondrial reactive oxygen species signal hepatocyte steatosis by regulating the phosphatidylinositol 3-

kinase cell survival pathway. The Journal of biological chemistry. 2007;282(29):21327-36.

712. Serviddio G, Bellanti F, Vendemiale G, Altomare E. Mitochondrial dysfunction in nonalcoholic steatohepatitis. Expert Rev Gastroenterol Hepatol. 2011;5(2):233-44.

713. Tessari P, Coracina A, Cosma A, Tiengo A. Hepatic lipid metabolism and non-alcoholic fatty liver disease. Nutrition, metabolism, and cardiovascular diseases : NMCD. 2009;19(4):291-302.

714. Musso G, Gambino R, De Michieli F, Durazzo M, Pagano G, Cassader M. Adiponectin gene polymorphisms modulate acute adiponectin response to dietary fat: Possible pathogenetic role in NASH. Hepatology. 2008;47(4):1167-77.

715. Targher G. Non-alcoholic fatty liver disease, the metabolic syndrome and the risk of cardiovascular disease: the plot thickens. Diabetic medicine : a journal of the British Diabetic Association. 2007;24(1):1-6.

716. Klebanoff SJ. Myeloperoxidase: friend and foe. J Leukoc Biol. 2005;77(5):598-625.

717. Santos CX, Tanaka LY, Wosniak J, Laurindo FR. Mechanisms and implications of reactive oxygen species generation during the unfolded protein response: roles of endoplasmic reticulum oxidoreductases, mitochondrial electron transport, and NADPH oxidase. Antioxid Redox Signal. 2009;11(10):2409-27.

718. Levy E, Spahis S, Bigras JL, Delvin E, Borys JM. The Epigenetic Machinery in Vascular Dysfunction and Hypertension. Curr Hypertens Rep. 2017;19(6):52.

719. Derdak Z, Villegas KA, Harb R, Wu AM, Sousa A, Wands JR. Inhibition of p53 attenuates steatosis and liver injury in a mouse model of non-alcoholic fatty liver disease. Journal of hepatology. 2013;58(4):785-91.

720. Weber M, Baker MB, Moore JP, Searles CD. MiR-21 is induced in endothelial cells by shear stress and modulates apoptosis and eNOS activity. Biochemical and biophysical research communications. 2010;393(4):643-8.

721. Wu W, Xiao H, Laguna-Fernandez A, Villarreal G, Jr., Wang KC, Geary GG, et al. Flow-Dependent Regulation of Kruppel-Like Factor 2 Is Mediated by MicroRNA-92a. Circulation. 2011;124(5):633-41.

722. Lin YC, Chang PF, Hu FC, Chang MH, Ni YH. Variants in the UGT1A1 gene and the risk of pediatric nonalcoholic fatty liver disease. Pediatrics. 2009;124(6):e1221-7.