

Université de Montréal

Identifying PGC-1 α -dependent hepatokines in a non-alcoholic
fatty liver disease murine model.

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Résumé

La stéatose hépatique non alcoolique (SHNA) est maintenant une des principales causes de cancer du foie. Cependant, les mécanismes physiopathologiques contribuant à son développement ou à la progression de la maladie sont peu connus. Il a été démontré que le niveau d'expression du coactivateur transcriptionnel PGC-1 α est inversement proportionnel avec la sévérité de la stéatose hépatique le stress oxydatif et la résistance à l'insuline dans les foies de souris. Chez l'humain, on observe aussi une diminution de PGC-1 α dans les foies de patients atteints de SHNA. De plus, il a été démontré que les souris avec une réduction de 50% des niveaux hépatique de PGC-1 α mène à une sensibilité à l'insuline et à une tolérance au glucose altérée dans les tissus périphériques. Ces découvertes suggèrent qu'en plus d'être associés au développement de la SHNA, les niveaux hépatiques de PGC-1 α altèrent l'expression de facteurs sécrétoires du foie afin d'influencer la régulation métabolique de tout le corps. **Nous proposons qu'une réduction de l'expression de PGC-1 α dans le foie influence les protéines sécrétées par le foie en situation de stress métabolique, révélant l'importance de PGC-1 α dans la réponse adaptative du foie.** L'analyse du sécrétome hépatique effectuée par spectrométrie de masse sur le milieu conditionné d'hépatocytes primaires a identifié SERPINA3N, une protéine sécrétée, dont les niveaux corrèlent avec les niveaux hépatiques de PGC-1 α et sont influencés par la diète obésogène. Dans ce projet, les niveaux sanguins de cette protéine ont été quantifiés par western blot chez des souris mâles et femelles, sauvages ou hétérozygotes pour PGC-1 α dans le foie et nourris avec une diète control ou riche en gras et en fructose. Nos résultats démontrent que les niveaux circulatoires de SERPINA3N augmentent avec la diète et corrèlent avec les niveaux hépatiques de PGC-1 α de manière dépendante à la diète et le sexe. De plus, les niveaux sanguins de SERPINA3N diminuent avec la progression de la maladie. L'expression hépatique de SERPINA3N est grandement influencée par les niveaux de PGC-1 α , mais indépendamment du facteur transcriptionnel NF- κ B. Nous avons montré que les glucocorticoïdes augmentent les niveaux protéiques et circulatoires de SERPINA3N dans les hépatocytes primaires. De plus, cette augmentation par les glucocorticoïdes est influencée par les niveaux de PGC-1 α . Ces résultats révèlent une nouvelle interaction entre PGC-1 α et le

récepteur des glucocorticoïdes sur l'expression hépatique et la sécrétion de SERPINA3N. Pour conclure, l'identification de protéines circulatoires régulées par PGC-1 α nous aidera à mieux comprendre comment la perte d'expression de PGC-1 α dans le foie affecte le métabolisme de tout le corps dans le contexte de la SHNA.

Mots-clés : SHNA, PGC-1 α , sécrétome hépatique, protéines circulatoires, SERPINA3N, sexe, progression, glucocorticoïdes, diète.

Abstract

Non-alcoholic fatty liver disease (NAFLD) is becoming a serious public health problem and is now one of the leading causes of liver cancer. Although NAFLD is known to be associated with obesity, insulin resistance, metabolic syndrome and type II diabetes, the mechanisms contributing to its development are not fully understood. It is shown that hepatic PGC-1 α levels correlate negatively with NAFLD development, oxidative liver damage and hepatic insulin resistance in murine models. In humans, decrease PGC-1 α expression in NAFLD and NASH patients. Moreover, liver-specific PGC-1 α reduction in mice also disrupts glucose tolerance and insulin sensitivity in muscle and adipose tissue, likely due to altered secretion of hepatic hormones. These findings suggest that in addition to contributing to NAFLD development, the hepatic disruption of PGC-1 α alters the liver secretome, thereby influencing the whole-body energy metabolism. **We hypothesize that decreased expression of PGC-1 α in the liver alters the expression of hepatokines under metabolic challenges, revealing a potential novel role for PGC-1 α in the adaptive response of the liver.** The hepatocyte-specific secretome was analyzed by mass spectrometry (iTRAQ) in conditioned media from primary hepatocytes. We identified SERPINA3N, a secreted protein whose secreted levels correlated with hepatic PGC-1 α levels in a diet-dependent manner. This hepatokine was measured in serum from male, female, wildtype and liver-specific PGC-1 α heterozygote mice fed chow or high-fat, high-fructose diet using western blot. SERPINA3N circulating levels increased with the western diet and correlated with hepatic PGC-1 α levels in a diet and sex-dependent manner. Its serum levels decreased with the progression of the disease. The hepatic SERPINA3N expression was greatly influenced by PGC-1 α levels independently of NF- κ B transcription factor. We showed that glucocorticoids increased SERPINA3N protein and secreted levels in primary hepatocytes. This increase was influenced by PGC-1 α levels, revealing a novel interaction of PGC-1 α and the glucocorticoid receptor on SERPINA3N expression and secretion. In conclusion, this project reveals a novel impact of hepatic PGC-1 α levels on the liver secretome during NAFLD development. This work will provide insights on the role of hepatic PGC-1 α levels on the regulation of hepatokines and how it influences the whole-body energy homeostasis in a context of NAFLD.

Keywords: NAFLD, PGC-1 α , liver secretome, hepatokines, glucocorticoids, circulating, sex, diet, progression.

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List of abbreviations

AAV: adeno-associated virus
ACTA2: actin-alpha 2
ALT: alanine aminotransferase
APOA4: Apolipoprotein A4
APOB: Apolipoprotein B
APOC2: Apolipoprotein C2
APOC3: Apolipoprotein C3
AST: aspartate aminotransferase
B220: B cell isoform of 220kDa of CD45R protein.
BIP: binding immunoglobulin protein
BMI: body mass index
CCL2: chemokine (C-C motif) ligand 2
CCL3: chemokine (C-C motif) ligand 3
CD4: cluster of differentiation 4
CD45R: cluster of differentiation 45 receptor
CD68: cluster of differentiation 68
CHOP: C/EBP homologous protein
COL1A1: collagen type 1 alpha 1
CT: computerized tomography
ECM: extracellular matrix
ECT: electron transport chain
ER: endoplasmic reticulum
ERR α : estrogen-related receptor alpha
FAO: fatty acid β -oxidation
FDA: federal drug administration
FFA: free fatty acids
FGF-21: fibroblast growth factor 21
FLI: fatty liver index

FOXO1: Forkhead box protein O1
G-6-Pase: Glucose-6-phosphatase
GCKR: glucokinase regulatory protein
GR: glucocorticoids receptor
H&E: hematoxylin and eosin
HFHF: high-fat, high-fructose
HNF4 α : hepatocyte nuclear factor 4 alpha
IL-1 β : interleukin-1 beta
IL-6: interleukin-6
ITRAQ: isobaric tags for relative and absolute quantification
LHet: liver-specific heterozygote of PGC-1 α
LKO: liver-specific knock-out of PGC-1 α
LPS: lipopolysaccharides
MRI: magnetic resonance imaging
mTOR: mammalian target of rapamycin
MTTP: Microsomal triglyceride transfer protein
NAFL: non-alcoholic fatty liver
NAFLD: non-alcoholic fatty liver disease
NASH: non-alcoholic steatohepatitis
NF- κ B: nuclear factor-kappa B
NR1D1: nuclear receptor subfamily 1 group D member 1
NRF-1: nuclear respiratory factor 1
NRF-2: nuclear respiratory factor 2
NUR77: nuclear receptor 77, nerve growth factor IB
PEPCK: Phosphoenolpyruvate carboxykinase
PGC-1 α : peroxisome proliferator-activated receptor gamma coactivator-1 alpha protein
PGC-1 β : peroxisome proliferator-activated receptor gamma coactivator-1 beta
PNPLA3: patatin-like phospholipase domain-containing 3
PPAR α : peroxisome proliferator-activated receptor alpha

PPAR γ : peroxisome proliferator-activated receptor gamma

PRC: PGC-related coactivator

PRX5: peroxiredoxin 5

ROS: reactive oxygen species

SHNA: stéatose hépatique non alcoolique

SNP: single nucleotide polymorphism

SOD2: superoxide dismutase 2

STAT3: signal transducer and activator of transcription 3

TFAM: transcription factor A, mitochondrial

TFB: transcription factor B

TGF β 1: transforming growth factor beta 1

TLR4: toll like receptor 4

TM6SF2: transmembrane 6 superfamily 2

TMT: tandem mass tag

TNF-A: tumor necrosis factor alpha

TR: thyroid hormone receptor

UCP1: uncoupling protein 1

VLDL: very low-density lipoprotein

WAT: white adipose tissue

WT: wildtype

XBP1 SP: X-box binding protein 1spliced

XBP1: X-box binding protein 1

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Introduction

The liver

Structural organization of the liver

The liver is the largest visceral organ and gland of the human body capable of multiple functions in metabolism, detoxification and regulation of the body's nutrients (1). The liver is supplied in blood from two different sources. The hepatic artery brings oxygenated blood from peripheral tissues, while the hepatic portal vein brings deoxygenated blood from the gastrointestinal tract (2). The blood then passes through sinusoids to reach the hepatocytes to regulate nutrient levels, detoxify the body from chemicals and metabolize drugs (3). The multiple functions of the liver are important in maintaining the body homeostasis and supporting peripheral tissues. Regulation by the liver consists in balancing nutrients levels in circulation in the blood by metabolizing, storing, and producing metabolites such as glucose (4). These multiple functions are possible due to the complex structure and organization of the liver. The main tissue of the liver, the parenchyma, is composed of multiple cell types; hepatocytes, cholangiocytes, sinusoidal endothelial cells, macrophages, lymphocyte, dendritic cells and stellate cells.

Metabolic functions of the liver

The liver is involved in metabolism and regulation of the levels of sugars, amino acids and fats in the bloodstream. We can group the metabolic functions of the liver under three categories: carbohydrate, protein, and lipid metabolism. Carbohydrate metabolism consists of storing excess blood glucose into glycogen in the fed state; and producing new glucose from amino acids (gluconeogenesis) or breaking down the glycogen into glucose (glycogenolysis) to be released in the circulation under the fasting state (5). Protein metabolism consists of catabolizing proteins into amino acids by a process called proteolysis in the small intestines and then transporting these amino acids to the liver to be either transformed into glucose or ketones or degraded into nitrogen substrates. The amino acid glutamine is transported from peripheral tissues to the liver, to be degraded into glutamate and ammonia. Since high levels of ammonia is toxic for the organism, the cells need to eliminate this nitrogen substrate. Hepatocytes are able to transform ammonia into urea via the urea cycle (6). The resulting urea

is then released in the blood and eliminated from the organism in urine (5). The last main function of the liver is lipid metabolism, which consists of lipolysis and lipogenesis. Lipolysis is the breaking of the dietary triglycerides into fatty acids. Fatty acids can then be oxidized, yielding acetyl-CoA, a substrate for cellular energy production better known as the Krebs's cycle. Lipogenesis is the production of triglycerides, cholesterol and lipoproteins from fatty acid substrates, in context of excess glucose and glycogen storage. These are produced using acetyl-CoA derived from pyruvate, a product of glycolysis during carbohydrate metabolism (7). The liver is crucial in modulating and adapting to changing nutrient substrates such as lipids, sugars and amino acids in response to the physiological state of the organism (fasting, starvation, ketogenic diets, nutrient excess) (7).

Endocrine function of the liver

Considered an endocrine gland, the liver contributes to the synthesis and secretion of multiple circulating proteins and metabolites, essential for peripheral tissue function, organ crosstalk and whole-body energy homeostasis. The liver is well known to secrete bile, which is stored in the gallbladder and contains bile acids important for the digestion of fats in the small intestine (4). Through the hepatic vein, the liver releases glucose, cholesterol, urea and plasma proteins such as albumin and clotting factors into the bloodstream (7, 8). Many hepatokines and hormones secreted by the liver contribute to organ cross-talk and peripheral tissues growth and development (7, 9). This highlights the importance of the liver on the whole-body metabolism through these secreted factors.

The liver is a complex organ of the human body with multiple functions crucial to maintain homeostasis. The liver is in constant adaptation to our dietary habits, revealing a strong association between liver health and malnutrition. When the liver is no longer able to adapt to the high energy demands and metabolic challenges, there is disruption of hepatic metabolic functions. This hepatic disruption leads to development of metabolic disorders such as non-alcoholic fatty liver disease (10). Non-alcoholic fatty liver disease (NAFLD) is closely associated

to obesity, type 2 diabetes (T2D) and insulin resistance (11). It is the hepatic manifestation of obesity and the metabolic syndrome (12).

Non-alcoholic fatty liver disease

Characteristics of NAFLD

Non-alcoholic fatty liver disease (NAFLD) has become a serious public health problem and is now considered as the major cause of liver disease (13). NAFLD is a spectrum of liver diseases starting with non-alcoholic fatty liver (NAFL), also known as simple steatosis. This stage is characterized by triglyceride accumulation in more than 5% of the hepatocytes. Scientists estimate that around 30% of fatty liver cases will develop into non-alcoholic steatohepatitis (NASH), a NAFLD stage characterized by steatosis with evidence of inflammation and various levels of fibrosis. Different levels of fibrosis are observed during the progression of NAFLD, and can eventually progress to cirrhosis, the formation of scarring tissue (14). Hepatocellular carcinoma (HCC) can then emerge from the cirrhotic livers. However, more studies show evidence of NASH-related HCC development in absence of cirrhosis (15, 16). In addition, NASH is now known as one of the main causes for liver transplantation and HCC (Figure 1) (17). The distinctive pathophysiology of the different stages, the patient variability and the environmental factors contributing to NAFLD makes this disease complex and difficult to study. NAFLD is closely associated with obesity, insulin resistance, metabolic syndrome and type 2 diabetes, yet the mechanisms contributing to its development are still not fully understood.

Prevalence

The prevalence of NAFLD is parallel to the global epidemic of obesity, type 2 diabetes and the metabolic syndrome, affecting 25% of the global population (17). In Canada, it is estimated that 50% of Canadians are overweighted and approximately 25% of Canadians are obese (18, 19). Moreover, scientists estimate that 75% of obese subjects will develop a fatty liver. Canadian children are also at risk, estimating that 10% may develop NAFLD in correlation with increasing rates of obesity at reported at young age (20). The increased prevalence of NAFLD is higher in type 2 diabetics and as mentioned obesity. However, multiple studies report lean subjects with

NAFLD, suggesting that besides environmental factors such as diet and lifestyle, genetic predisposition also contributes to the development of liver disease (21).

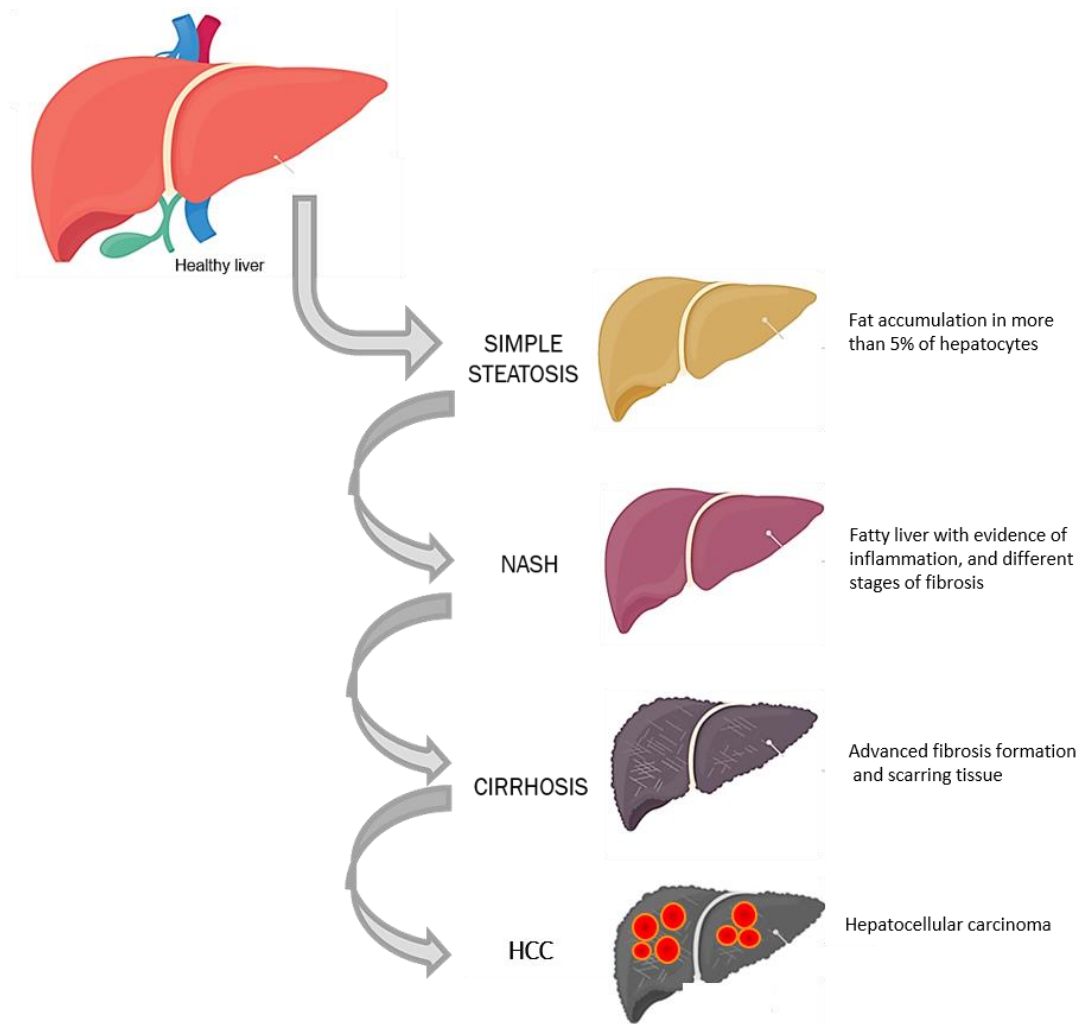


Figure 1.- The spectrum of liver pathologies in non-alcoholic fatty liver disease.

The first stage of disease is simple steatosis, affecting approximately 25% of the worldwide population. 30% of patients with simple steatosis progress to NASH, characterized by inflammation and fibrosis formation in addition to fat accumulation. 20% of NASH patients develop cirrhosis. Finally, many patients with fatty livers will develop hepatocellular carcinoma (1-2%). (Adapted from : Drew, 2017, *Nature*) (14)

Diagnosis methods

One of the main difficulties in treating and preventing the development of non-alcoholic fatty liver disease is the lack of early diagnosis methods. The struggle to detect the early stages of NAFLD is due to the absence of non-invasive and accurate methods. In addition, it is even more difficult to detect this disease since early stages are asymptomatic. Even though NAFLD research has incredibly advanced over the last decades, the gold standard method remains liver biopsy, an invasive and risky methods (22). Since NAFLD patients do not present symptoms, the diagnosis is often accidental after blood tests, medical exams or during surgery when the doctor notices the liver is fatty. Non-alcoholic fatty liver disease is then diagnosed by exclusion when there are no evident causes of alcohol drinking, hepatitis infection or steatogenic medication (23).

Measurements of liver enzymes in the blood, such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are used as indicators of liver damage; however, these are more indicative of the late and irreversible stages of liver disease. Measurements of circulating liver enzymes can be combined with imaging technologies such as ultrasound, MRI, CT scan and FibroScan. The FibroScan uses transient elastography, which is an ultrasound to measure liver stiffness as a surrogate marker of fibrotic and cirrhotic livers (24). The fatty liver index (FLI) is a recent method combining both imaging technologies and liver histology, which consists of measuring BMI, waist circumference, circulating liver enzymes and hepatic triglyceride content (17). However, not all obese individuals develop fatty liver disease and this tool is not designed to differentiate the early stages of NAFLD. As listed above, a multitude of diagnosis methods are available, but the sensitivity and specificity of these techniques are limited. The imaging technologies are non-invasive tools but remain a high cost method and are not applicable for large-scale NAFLD screening. Therefore, there is an urgent need to find non-invasive methods to potentially screen the whole population and diagnose the early stages of NAFLD to prevent the progression of the disease by drug-free treatments such as exercise and diets (24).

Treatments

As of today, there are no approved drugs by the Food and Drug Administration (FDA) to treat NAFLD and NASH (25). Current treatments focus on comorbidities associated with NAFLD, such as type 2 diabetes, insulin resistance, metabolic syndrome and obesity with the hope of slowing down progression. Finding treatments to improve health and reduce mortality of patients is key to prevent NAFLD progression (26). Current drugs in development to treat specifically NAFLD are target hallmarks of the disease, such as hepatic inflammation, oxidative damage, insulin resistance and dyslipidemia (25). However, administration of drugs to patients depend on comorbidities and the severity of the disease. Some stages of NAFLD are reversible, such as simple steatosis, early stages of NASH and some levels of fibrosis (27). However, whether advanced stages such as cirrhosis is reversible is still a debate, since no efficient treatment has proven to reverse completely and restore the liver health (28, 29). Current first-line treatments for NAFLD include:

Weight loss

NAFLD is strongly associated with obesity, therefore it is important to submit obese patients to strict, healthy diets and increased physical activity. It was reported that weight loss can decrease cardiovascular and diabetes risk and that more than 10% weight loss can almost resolve NAFLD and improve fibrosis. In morbidly obese patients, the best option appears to be bariatric surgery (30).

Insulin sensitising drugs

Metformin administration to animal models is found to decrease hepatic steatosis and improve inflammation (26). Metformin improves the insulin sensitivity and restores ALT and AST levels in NAFLD patients, but the impact at the histological level of NAFLD and NASH livers are not convincing (31). More human trials on the efficacy of Metformin to treat NAFLD are needed, since the current observations result from small cohorts (26, 32).

Antioxidants

Oxidative stress and lipid peroxidation are important hallmarks of NAFLD and NASH (25). Pilot studies have showed the efficacy of vitamin E and C in reducing hepatic inflammation, significantly improving the fibrosis score and restored the ALT and AST blood levels in children with NASH (26, 33, 34).

Lipid controlling drugs

Statins are widely-used drugs that reduce hepatic production of cholesterol and low-density lipoproteins, preventing cardiovascular disease and heart attacks (35). In NAFLD, statins are administered to patients with hyperlipidemia and to reduce the hepatic fat content (25). However, statins can have important secondary effects, such as myopathy and increased risk for type 2 diabetes (35).

Pathogenesis

The biological mechanisms contributing to the development and progression of non-alcoholic fatty liver disease are still unclear. NAFLD is a complex and multifactorial disease due to the spectrum of liver pathologies. The different NAFLD stages are well characterized, but the mechanisms contributing to these manifestations throughout the disease progression are still theories. Indeed, a “two-hit hypothesis” was proposed over two decades ago, explaining the pathogenesis of NAFLD (36). The first hit is fat accumulation in hepatocytes, with evidence of systemic and hepatic insulin resistance (37). The insulin resistance results from unhealthy diets, sedentary lifestyle combined with environmental and genetic factors. This insulin resistance and hepatic lipid accumulation leads to alterations of metabolic functions in the liver, adipose tissue and muscle (38). This is concomitant with a defect in the hepatic lipid clearance, such as efflux of cholesterol-related lipids or inefficiency in fatty acid β -oxidation (39). The second hit can come from multiple mechanisms, all potentially contributing to the progression from NAFLD to non-alcoholic steatohepatitis (NASH). Liver metabolism that is disrupted by excessive lipid content and insulin resistance becomes vulnerable to other insults. These second insults consist of oxidative stress, increased inflammatory signaling, fibrosis development and/or

genetic susceptibility. Disruption of these biological pathways greatly impacts liver metabolism and homeostasis, worsening of the disease (38). Here follows, the main biological pathways involved in the pathogenesis of NAFLD.

Lipid metabolism

As mentioned, sedentary lifestyle and unhealthy diets such as high sugars and high fat diets can induce insulin resistance. Insulin normally inhibits adipose tissue lipolysis and promotes fatty acid storage into triglycerides. The insulin resistance observed in NAFLD induces free fatty acid (FFA) release into the blood from the adipose tissue lipolysis. This increase FFA in the blood is taken by the liver, where the hepatic insulin resistance induces hepatic *de novo* lipogenesis and hypertriglyceridemia and disruption of the triglycerides secretion through cholesterol-related lipoproteins (40).

Mitochondrial dysfunction

In the early phases of hepatic fat accumulation, mitochondrial activity is increased as an adaptive response to the fatty acid influx. This compensation results in enhanced transformation of free fatty acids into triglycerides stocks to prevent lipotoxicity (41). However, long-term and excessive fat influx can overload the mitochondria and leads to disruption of fatty acid β -oxidation (FAO) and the electron transport chain (ETC). (42). Multiple studies show evidence of mitochondrial dysfunction and decreased expression of fatty acid β -oxidation and electron chain transport pathways in diabetic and NAFLD patients (42-44).

Oxidative stress

Long-term excessive lipid accumulation leads to alterations in mitochondrial activity and structure. Imbalances in the mitochondrial respiratory chain activity can cause accumulation of reactive oxygen species (ROS) (45). This ROS accumulation induces oxidative stress, leading to lipid peroxidation and mitochondrial DNA damage. In some cases, genetic factors contribute to decrease expression of ROS detoxification enzymes, leading to more oxidative damage. This

hepatic impairment known as a hallmark of NASH, is now considered as the central process in the development and progression of NAFLD (41).

Inflammation

Bacterial lipopolysaccharides can enter the circulation due to a leaky gut (related to diet). These reach the liver and bind to the toll-like receptor 4 (TLR4) at the surface of hepatocytes and can also activate Kupffer cells, the hepatic immune cells, causing secretion of pro-inflammatory cytokines (38). In addition to these extrahepatic sources of inflammation, production of toxic or inflammatory metabolites can occur due to inappropriate hepatic fat accumulation. This also induces the release of inflammatory cytokines, such as TNF- α and IL-6 from hepatocytes and resident immune cells, leading to activation of inflammation signaling via NF- κ B and STAT3 transcription factors in hepatocytes (38, 46).

Genetic predispositions

Family and twin studies suggest that NAFLD development can be inheritable (47). Moreover, epidemiologic studies found an ethnic susceptibility to NAFLD and NASH. Based on these observations, multiple genome-wide associated studies were designed to identify genetic factors including single nucleotide polymorphisms (SNP). The strongest single nucleotide polymorphism to correlate with NAFLD is the *PNPLA3* I148M variant, which encodes patatin-like phospholipase domain containing 3 protein, involved in triglyceride remodeling. Furthermore, the transmembrane 6 superfamily member 2 (*TM6SF2*) E167K variant is also associated with NAFLD, as well as NASH and fibrosis development (48). This gene encodes a protein involved in triglyceride secretion through very low-density lipoproteins (VLDL). The glucokinase regulator (*GCKR*) P446L variant is also one of the most known genetic factors for NAFLD, NASH and fibrosis. This protein regulates *de novo* lipogenesis and glucose uptake.

As NAFLD development in humans is often associated with mitochondrial dysfunction, multiple studies have investigated the association between genetic variants of the peroxisome proliferator-activated receptor γ coactivator-1a gene (*PPARGC1A*) and NAFLD. Briefly,

PPARGC1A encodes the PGC-1 α protein, a transcriptional coactivator that induces genetic pathways involved in hepatic gluconeogenesis, mitochondrial function and detoxication of reactive oxygen species (49). It is reported that the PGC-1 α Gly482Ser variant (*PPARGC1A* rs8192678 1444G>A) is associated with diabetes, obesity and NAFLD (50, 51).

Peroxisome proliferator-activated receptor gamma coactivator-1

PGC-1 family members

The peroxisome proliferator-activated receptor gamma coactivators-1 is a family of three members, PGC-1 α , PGC-1 β and the PGC-1-related coactivator (PRC) (52). The PGC-1 proteins are known as transcriptional coactivators, binding and coactivating transcription factors or nuclear receptors for regulation of gene expression (53). PRC is expressed ubiquitously, while PGC-1 α and PGC-1 β are expressed mainly in tissues with high energy demands such as the brain, kidney, muscle, liver, brown adipose tissue and pancreas (52). PGC-1 α and PGC-1 β are well known to coactivate nuclear respiratory factor 1 (NRF-1) and 2 (NRF-2), peroxisome proliferator-activated receptor alpha (PPAR α) and gamma (PPAR γ), estrogen-related receptor alpha (ERR α) and thyroid hormone receptor (TR). These transcription factors are involved among others, in regulating energy metabolism and mitochondrial genes (54). Even though PGC-1 α and PGC-1 β are structurally similar, regulate many of the same biological pathways, and are expressed in many of the same tissues, they can have distinct functions (52, 54, 55). Some studies suggest PGC-1 β is involved in the basal mitochondrial activity, while PGC-1 α is involved in the adaptive response and increasing mitochondrial biogenesis when needed (55). Moreover, PGC-1 α is involved in adaptive thermogenesis in the brown adipose tissue, hepatic gluconeogenesis, lipid catabolism and muscle development, while PGC-1 β is reported to regulate hepatic lipid synthesis and lipoprotein production (54, 56, 57).

Tissue-specific functions of PGC-1 α

PGC-1 α is a key modulator of metabolic adaptation, and its capacity to coactivate multiple transcription factors allows it to have a variety of functions in different tissues (58). A whole-

body PGC-1 α knock-out mouse presented abnormalities in the brain, with decreased expression of genes involved mitochondrial activity and ROS detoxification (59). It is reported that PGC-1 α gene levels decrease in the brains of Alzheimer and Parkinson's patients (60-62). In the muscle, PGC-1 α is best known to be induced by exercise and is involved in development of specific type of muscle fibers. As muscle cells contain a great number of mitochondria that are dynamically regulated, PGC-1 α -mediated regulation of mitochondrial biogenesis is crucial (63). PGC-1 α also promotes angiogenesis around skeletal muscle cells, to facilitate mitochondrial oxygen exchange (64). In adipose tissue, PGC-1 α is involved in the transformation of white adipose cells into brown adipocytes. This mechanism consists in PGC-1 α coactivating the transcription factor PPAR γ , leading to an increase in *Ucp1* expression, a thermogenesis activator (65). In the liver, PGC-1 α is normally expressed at low levels but is induced by glucagon during the fasting state. It is involved in an array of biological pathways, but is mainly involved in hepatic glucose metabolism, mitochondrial biogenesis, oxidative and lipid metabolism (58, 60, 66). Since this project focuses on non-alcoholic fatty liver disease, here are described the main biological pathway in which PGC-1 α is involved in the liver.

Gluconeogenesis

Hepatic expression of PGC-1 α is induced by CREB and cAMP during fasting-mediated glucagon signaling, leading to coactivation of hepatic nuclear factor-4a (HNF4 α), glucocorticoid receptor (GR) and forkhead box o1 (FoxO1). These transcription factors are responsible for regulating expression of phosphoenolpyruvate carboxykinase (*Pepck*) and glucose-6-phosphatase (*G6pc*), involved in gluconeogenesis under the fasting state (53, 67).

Mitochondrial biogenesis and oxidative metabolism

As mentioned above, PGC-1 α is involved in mitochondrial biogenesis and coactivates nuclear respiratory factors (NRF-1 and 2), transcription factor A, mitochondrial (TFAM) and transcription factor B (TFB). These proteins regulate mitochondrial genes involved in mitochondrial fusion/fission process and mitochondrial DNA transcription (60, 68). During the fasting state, the liver accumulates lipids due to an influx of free fatty acids from adipose tissue

lipolysis. This hepatic lipid accumulation leads to activation of fatty acid β -oxidation, the TCA cycle and the electron transport chain pathway to process sugars and lipids to produce energy (69). Increased mitochondrial metabolism is induced by PGC-1 α , well known to promote the expression of genes involved in mitochondrial function in high energy demands such as fasting (68, 69).

Lipid metabolism

Moreover, a few studies investigated the impact of PGC-1 α levels on lipoprotein transport. Overexpression of PGC-1 α in rats increase the mitochondrial fatty acid β -oxidation which followed a decrease in triglycerides storage and transport (69). This is accompanied with decreasing levels of apolipoprotein B (*ApoB*) and microsomal triglyceride transfer protein (*Mttp*), proteins involved in triglyceride secretion (69). In contrast, it is shown that overexpression of PGC-1 α using adenoviral injection in mice leads to increase expression of apolipoprotein A4 (*ApoA4*), apolipoprotein C2 (*ApoC2*) and apolipoprotein C3 (*ApoC3*) and increases the circulating very low-density lipoprotein complex in the blood (70). These results show that PGC-1 α not only influences the lipid catabolism, but also the lipid transport.

ROS detoxification

Finally, increase expression of PGC-1 α promotes the fatty acid β -oxidation. However, the increase in β -oxidation, followed by energy production leads to increasing amounts of reactive oxygen species (ROS) that can be toxic to the cell. Interestingly, it is reported that PGC-1 α regulates the gene expression of ROS detoxification enzymes such as *Sod2* and *Gpx1*, while still inducing fatty acid β -oxidation (71). These multiple tissue-specific functions highlight the importance and the complexity of PGC-1 α regulation in the whole-body energy metabolism.

PGC-1 α and metabolic diseases

Genetic disruption of PGC-1 α can have serious repercussions on whole body. An array of studies reports a link between PGC-1 α deficiency in different tissues and metabolic diseases development. Genome wide association studies have identified PGC-1 α variants associated

with T2D and NAFLD development as mentioned above. The SNP *PPARGC1A* Gly482Ser induces a glycine to serine substitution that impacts the PGC-1 α function and conformation (50, 57). The PGC-1 α 482Ser allele has higher risks to develop type 2 diabetes (T2D) and insulin resistance compared to the subjects with the 482Gly allele as observed in the Danish and Japanese population (50, 51). Based on these observations, a study from our lab showed that the Ser482 of PGC-1 α has a shorter half-life than the glycine variant (58). Epigenetic modifications of *PPARGC1A* gene are associated with NAFLD hallmarks (47). DNA methylation of *PPARGC1A* correlate with insulin resistance and negatively correlate with the mRNA levels of *PPARGC1A*, suggesting a decrease in transcription. In human patients, increased DNA methylation of *PPARGC1A* gene is associated with NAFLD development (47). Taking all these data, this suggest that reduced hepatic PGC-1 α expression may be associated NAFLD and type 2 diabetes development.

Indeed, PGC-1 α protein levels are decreased in tissues of diabetic patients and subjects with familial history of diabetes (43). Other studies show that decreased PGC-1 α protein levels in diabetic muscle correlate with a decrease in the oxidative phosphorylation genes (44). This decrease in oxidative phosphorylation and mitochondrial-related pathways are also observed in livers of NAFLD patients (72). Many studies investigate the link between mitochondrial dysfunction, PGC-1 α expression and NAFLD development. Interestingly, the decrease in mitochondrial-related genes reported in NAFLD subjects, such as the fatty acid β -oxidation and the electron transport chain programs, is concomitant with decreased mRNA and proteins levels of PGC-1 α (42, 73). Moreover, PGC-1 α mRNA levels are decreased in livers of NAFLD patients and further decreased in livers of obese subjects with NAFLD or NASH (74, 75). These observations suggest that PGC-1 α may be important in the adaptive response to metabolic challenges, in promoting the fatty acid β -oxidation and lipid metabolism. Disruption of hepatic PGC-1 α levels may increase the susceptibility to develop metabolic diseases such as NAFLD (76).

PGC-1 α loss-of-function murine models

Multiple studies have investigated whether PGC-1 α is involved or has a causative role in the development of metabolic diseases. A loss-of-function model of PGC-1 α was generated, the PGC-1 α ^{-/-} null mice, to study the importance of PGC-1 α function in metabolic adaptation and mitochondrial function as mentioned (59). These PGC-1 α ^{-/-} null mice have decreased muscle performance and exercise capacity, decreased hepatic glucose production; however, there is evidence of improved insulin sensitivity compared to wildtype mice (66, 77). The complete loss of PGC-1 α also affects body temperature regulation of these mice (78). Moreover, short-term starvation induced hepatic steatosis with decrease in mitochondrial activity and increase in lipogenesis (59). However, this mouse model has its limitations and caveat. The PGC-1 α ^{-/-} null mice are protected from the diet-induced insulin resistance and obesity since they are lean. This is likely due to the mice hyperactivity and increased energy expenditure (77). This hyperactivity is the result of lesions observed in the brain, more precisely in the striatum region involved in the motor coordination (78). This mouse model not only shows how PGC-1 α is important and plays different functions in different tissues, but also how a whole-body knock-out of PGC-1 α mice may not be the most informative model. A tissue-specific approach is suggested to better study the tissue-specific function of PGC-1 α (78, 79).

To study the impact of PGC-1 α levels in liver metabolism, a liver-specific PGC-1 α heterozygous mice (LHet) exists. This is achieved by crossing mice harbouring a floxed PGC-1 α allele (PGC-1 α ^{fl/+}) with a transgenic mouse expressing Cre-recombinase under the albumin promoter. This generated a mouse with only one allele expressing PGC-1 α with the other allele disrupted only in hepatocytes (80). The liver-specific reduction of PGC-1 α by 50% reflects best the human data, since only decrease hepatic PGC-1 α levels and not complete absence is observed in NAFLD. After 24 hours of fasting, LHet mice develop steatohepatitis, with increased hepatic triglycerides and cholesterol levels (59). Moreover, these mice developed early hallmarks of NAFLD, such as decreased expression of fatty acid β -oxidation genes and altered insulin sensitivity in liver (80). These findings provide novel potentially causative role for hepatic PGC-1 α levels in the development of fatty liver disease.

A study from our lab shows that levels of PGC-1 α in the liver negatively correlates with NAFLD severity in mice (58). This is consistent with human studies reporting a decrease in PGC-1 α levels in livers of NAFLD and NASH patients (75). Previous work in our lab investigated the long-term impact of reduced PGC-1 α on the fatty liver disease progression mediated by prolonged diet-induced obesity (58). When fed a long-term obesogenic diet, mice have accelerated NAFLD progression with evidence of increased oxidative stress, inflammation and weight gain. This study reveals PGC-1 α as an important actor in the ROS detoxification process. They show that PGC-1 α is expressed more in females than male mice because in females, it is essential for the estrogen-dependent antioxidant gene expression. These findings explain why liver heterozygote for PGC-1 α female mice have higher ALT levels, more oxidative liver damage and lipid content than wildtype female littermates. Female mice are more sensitive to the hepatic loss of PGC-1 α because of its role in the estrogen-mediated ROS detoxification. These findings show how PGC-1 α is crucial in modulating the metabolic adaptation in a context of NAFLD development (58).

PGC-1 α impacts the cell secretome

Decreased hepatic PGC-1 α levels reported to improve the whole-body glucose metabolism and insulin sensitivity in mice compared to control littermates (81). Interestingly, when PGC-1 α reduction is liver-specific, mice have increased glucose uptake in the white adipose tissue (WAT) and the muscle. Thus, the authors analyzed the liver secretome to find a secreted factor involved in the peripheral glucose metabolism. They noticed increased expression and circulating levels of the fibroblast growth factor-21 (FGF-21), a known secreted hormone from the liver involved in improving the peripheral insulin sensitivity. Levels of FGF-21 are increased in a dose-dependent manner in livers of liver-specific PGC-1 α knock-out (LKO) and LHet mice, potentially explaining why these mice have better glucose tolerance. These results provide a novel role for PGC-1 α in regulating the liver secretome and thereby influencing the whole-body energy metabolism.

This novel role for PGC-1 α in regulating organ secretome correlates with another study demonstrating how PGC-1 α levels influences the expression of multiple secreted proteins in different cell lines (82). Using loss-of-function of PGC-1 α using knock-out cells and gain-of-function adenoviral overexpression, the expression of known secreted proteins were measured in brown adipocytes, fibroblast, myocytes and hepatocellular carcinoma cell lines. In general, overexpression of PGC-1 α in cell lines decreased the expression of secreted factors, while knock-out of PGC-1 α increased their expression compared to wildtype littermates (82). This study concludes that PGC-1 α levels highly influence the cell secretome by altering expression of secreted and extracellular matrix (ECM) proteins.

Preliminary results

Previous work from a former student in our lab investigated how low hepatic PGC-1 α levels combined with western diet-mediated NAFLD in mice altered the liver secretome (83). Mice with hepatic-specific 50% reduction of PGC-1 α (liver-specific heterozygote LHet) and hepatic-specific knock-out of PGC-1 α (liver-specific knock-out LKO) using the cre/lox recombinase technology under the control of the albumin promoter (80, 81). This ensured that the genetic disruption of PGC-1 α is hepatocyte-specific and contamination of signals from other cell types was minimized. Primary hepatocytes isolated from wildtype (WT), LHet and LKO mice fed chow or high-fat, high-fructose (HFHF) diet for six weeks were maintained in cellular culture. After 16 hours of incubation *in vitro*, the cells maintained their metabolic profiles and the cell culture conditions were optimal to reflect the physiological liver cell secretome. The conditioned media containing all secreted factors was collected and analyzed by mass spectrometry using isobaric tags for relative and absolute quantification (iTRAQ). This hepatocyte-specific secretome analysis reduced the contamination of abundant blood proteins and ensured these secreted factors were liver specific (83). This work identified hepatokines whose expression were altered by hepatic PGC-1 α levels, the high-fat, high-fructose diet or the combination of both conditions.

From the quantitative proteomic analysis, they identified SERPINA3N, whose secreted levels were strongly influenced by the diet, the hepatic disruption of PGC-1 α and the combination of

both conditions (Figure 2) (83). SERPINA3N secreted levels increased in wildtype mice fed high-fat, high-fructose diet (HF-WT) compared to chow fed mice (CH-WT). SERPINA3N secreted levels were also basally increased in LHet and LKO mice fed chow diet compared to wildtype littermates. However, LHet and LKO mice fed HFHF diet had decreased SERPINA3N levels compared to wildtype fed HFHF diet; thus, mice lacking PGC-1 α lacked the adaptive upregulation of SERPINA3N that occurs in wild-type mice in response to western diet. Thus, we chose SERPINA3N for further investigation based on the robust changes of its expression between experimental conditions, and it was significantly influenced by the interaction of the HFHF diet and the genotype. SERPINA3N is a serine protease inhibitor and an acute phase protein induced by multiple cytokines. It is involved in wound healing, secreted in glucocorticoid-mediated muscle atrophy and highly expressed in various cancers (84-88). Based on these preliminary *in vitro* results and the robustness of SERPINA3N changes and how the interaction of diet and decreased PGC-1 α levels influences SERPINA3N secreted levels, we further investigated on this protein. The link between SERPINA3N circulating levels and hepatic PGC-1 α in a context of NAFLD is still yet to discover.

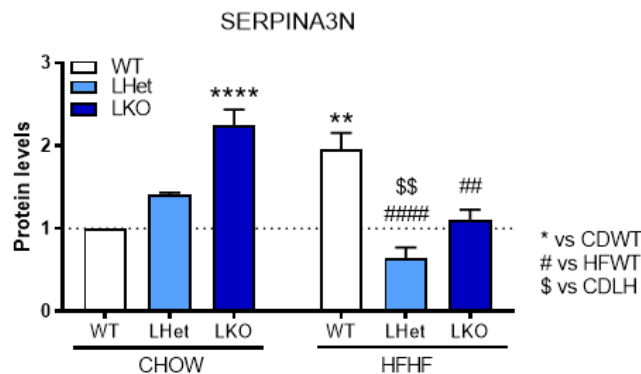


Figure 2. - SERPINA3N secreted levels in conditioned media.

(A) SERPINA3N levels in conditioned media measured with isobaric tags for relative and absolute quantification by mass spectrometry (ITRAQ-LC-MS/MS). CD: Chow diet, HF: High-fat, high-fructose diet, WT: wildtype, LHet: liver-specific PGC-1 α heterozygote, LKO: liver-specific PGC-1 α knock-out. **** $P < 0.0001$, ** $P < 0.01$, \$\$ $P < 0.01$, ##### $P < 0.0001$, ## $P < 0.01$, &&&& $P < 0.0001$. (Figure adapted from Besse-Patin, A. (2018), Université de Montréal) (83).

Research objectives

Based on previous findings showing that low hepatic PGC-1 α levels are associated with NAFLD development and influences the peripheral metabolism through alteration of the liver secretome, we are interested in investigating how hepatic PGC-1 α is involved in NAFLD development through modulation of hepatokine expression. This project will allow to better understand how hepatic PGC-1 α levels impacts the whole-body metabolism and determine whether SERPINA3N circulating levels are responsible for this impact. Moreover, since SERPINA3N is not well-known in metabolic disease, this may help to reveal if PGC-1 α -mediated SERPINA3N secretion is protective or contributing to NAFLD progression. But first, we need to determine if hepatic PGC-1 α levels is can induce significant changes in SERPINA3N circulating levels in mice serum, as observed in the preliminary *in vitro* results.

Based on these results, we hypothesize that circulating SERPINA3N levels in mouse serum will correlate with hepatic PGC-1 α levels and be influenced by the western diet.

In the present study, we provided evidence that hepatic PGC-1 α influences the secreted levels of SERPINA3N differently in response to metabolic challenges, revealing a potentially novel function for the transcriptional co-activator in the adaptive and acute phase response. We reported that circulating SERPINA3N levels in mouse serum reflected the hepatocyte-specific secretome previously observed *in vitro*, and that its levels correlated with hepatic PGC-1 α expression in a diet and sex-dependent manner *in vivo*. Moreover, *Serpina3n* hepatic expression was greatly influenced by PGC-1 α levels. SERPINA3N secretion was independent of NF- κ B and in contrast, induced by glucocorticoids. This glucocorticoids-mediated regulation of SERPINA3N was influenced by PGC-1 α levels, revealing a novel interaction between glucocorticoids and PGC-1 α in hepatic protein regulation. This study reveals SERPINA3N as a potential blood indicator of low hepatic PGC-1 α in context of NAFLD susceptibility and PGC-1 α possibly involved in regulating the acute phase response to high-fat, high fructose feeding.

Material and methods

Animal models

Previously generated conditional floxed *Ppargc1a* knockout (KO) mice (B6.129-Ppargc1a^{tm2.1Brsp/J} or Ppargc1a^{f/f}) bred with Tg(Alb-cre)^{21Mgn/J} (Alb-cre⁺⁰) mice expressing Cre recombinase under hepatocyte-specific albumin promoter were used (Estall, JL, 2009). From this mouse model, we generated Alb-cre⁺⁰ control mice (Ppargc1a^{+/+}), floxed control (Ppargc1a^{f/+} Alb-cre^{0/0}), liver-specific heterozygote mice (LHet: Ppargc1a^{f/+}Alb-cre⁺⁰) and liver-specific knockout mice (LKO: Ppargc1a^{f/f}Alb-cre⁺⁰).

Mouse housing and diets

Male and female mice were fed regular chow (18% fat, 24% proteins and 58% carbohydrates, #2918, Teklad Global diet 2018, Envigo) or high fat, high sucrose diet (45% fat, 20% protein and 35% carbohydrates, D12451I; Research diets Inc, New Brunswick, NJ) supplemented with 30% D-fructose in drinking water (HFHF) for 6 weeks (n=7 per condition) or 25 weeks (n=4 per condition) starting from 10 weeks of age. Animals were allowed *ad libitum* access to food and were maintained at 22°C (12-hour light to 12-hour dark cycle). Mice were weighed at 9 weeks of age to normalize weight distributions between groups before starting the diets. Sub-mandibular bleeds were performed every two weeks (week 0, 2, 4 and 6) after start of diets. All blood samples coagulated at 4°C for 1 hour before centrifugation at 3000 rpm for 5 minutes at 4°C. Serum was collected and conserved at -80°C. All experiments were performed in accordance with the Institut de Recherches Cliniques de Montreal (IRCM) Animal Facility Institutional Animal Care and Use Committee regulations.

Primary hepatocytes isolation

Primary mouse hepatocytes were isolated by a 2-step liberase perfusion (Liberase TL; Roche, Indianapolis, IN) and 50% Percoll gradient purification. Cells were plated in monolayers and cultured in Dulbecco's modified Eagle's low glucose medium (5.5 mM glucose) supplemented with 2 mM sodium pyruvate, 0.1 μM dexamethasone, 1% penicillin/streptomycin and 1 nM insulin for up to 48 hours. The non-adherent cells were removed by changing the media after 2 hours of incubation post-isolation. Before treatments, primary cells were maintained at least 2

hours in media with no bovine serum albumin (BSA) (maintenance media) containing low glucose (5.5 mM), 2.5 mM insulin and 100 nM dexamethasone. Before treatments, all cells were starved in starvation media containing low glucose (5.5 mM), without dexamethasone, BSA or insulin.

Cell culture and treatments

Primary hepatocytes (n=3) were infected with adenovirus expressing either control vector encoding for the green fluorescent protein (GFP) (1 uL/mL) or PGC-1 α (3 uL/mL) as previously explained (Estall, JL, 2009). To assess the impact of glucocorticoid on *Serpina3n* expression, primary hepatocytes were treated with 1 uM of dexamethasone for 8 hours or vehicle (starvation media). For assessment of the NF- κ B activity, we used JSH-23, an inhibitor of the p65 subunit, blocking its translocation to the nucleus. Primary hepatocytes were treated with 10 uM of JSH-23 or the corresponding volume of DMSO (vehicle) as control for a duration of 6 hours. For all treatments, the concentration of reagents and time of incubation were chosen from previous optimized experiments (Supplementary figure 1 and 2).

Conditioned media

Conditioned media from primary hepatocytes was harvested, centrifuged full speed for 20 minutes to remove debris. Clean conditioned media was then concentrated using Amicon Ultra-2 mL kit (Centrifugal Filters, #UFC200324 Millipore). Conditioned media (2 mL) was added to filter device with filtrate collection tube and centrifuged at 4000 x g for 3 minutes. The concentrated conditioned media was recovered by centrifuging upside down the filter device in concentrate collection tube at 1000 x g for 2 minutes. For optimal concentration, samples were then evaporated in SpeedVac evaporation concentrator for 90 minutes or until pellet formation. Samples were resolubilized with radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors.

Protein isolation and immunoblotting

Hepatic and serum proteins were solubilized in RIPA buffer with protease (100X) and phosphatase inhibitors. Samples are added to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-page electrophoresis) or TGX Stain-free FastCast Acrylamide gels (10%, Bio-Rad, #1610183) for protein separation, then transferred on nitrocellulose membranes and probed with primary and secondary antibodies. Quantification of images was performed using ImageJ software and ImageLab (Bio-rad) (Supplementary Table 1 for antibodies).

Histology

Liver tissues were fixed with 10% neutral buffered formaldehyde for 24 h prior to being embedded in paraffin and sectioned onto 5 μ m slides. The histology slides were then stained with hematoxylin and eosin (H&E).

Hepatic lipids quantification

Frozen liver tissue was solubilized in 2:1 chloroform:methanol, dried overnight and purified lipids resuspended in 60% butanol: 40% TritonX114: methanol (2:1). Total triglycerides were measured colorimetrically with the Total triglyceride reagent kit (Sigma-Aldrich, St-Louis, MO). The glycerol values were subtracted from the triglyceride value to obtain the acyl-glycerol content.

RNA isolation and RT-qPCR

RNA was isolated from frozen liver tissue or cultured cells using TRIzol reagent. DNase treatment was performed on 1 μ g of RNA with DNase I to be then reverse transcribed using a High Capacity Reverse Transcription kit (Applied Biosystems, Carlsbad, CA). Following generation of complementary DNA (cDNA), expression of mRNAs was quantified using the Life Technologies ViiA7 real-time PCR system using SYBR green PCR master mix and the $\Delta\Delta$ CT threshold cycle method of normalization. Gene expression levels were normalized to

hypoxanthine-guanine phosphoribosyltransferase (Hprt) messenger RNA (mRNA) and expressed relative to control (Supplementary Table 2 for primer sequences).

Serum depletion

Serum samples were depleted of the 7 most abundant serum proteins (Albumin, α 1-Antitrypsin, Transferrin, Fibrinogen, IgG, IgM, Haptoglobin) using the Seppro Mouse Spin Columns kit (#SEP110, Sigma-Aldrich). 150 μ g of serum proteins were depleted using 50 mM Triethylammonium bicarbonate (TEAB) (#T7408, Sigma-Aldrich) with 150 mM NaCl as buffer to elute proteins and wash columns as described in the kit protocol. The elution fraction was evaporated using the SpeedVac evaporation concentrator until pellet formation. Protein concentrates were reconstituted in radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors for SDS-page electrophoresis.

Statistical analysis

Data were analyzed using Graphpad Prism 8.0.1 (San Diego, CA) software. Two-way ANOVA was used for mean comparisons followed by Tukey post-hoc tests corrected for multiple comparisons. We used multiple t-tests corrected for multiple comparison using the Holm-Sidak method. Threshold for statistical significance was $P < 0,05$. Data are expressed as mean \pm SEM.

Results

NAFLD mouse model validation and simple steatosis characterization.

Low hepatic levels of PGC-1 α correlate with NAFLD severity and decreased expression of PGC-1 α in the liver influences whole-body metabolism by altering the expression of hepatic secreted factors (81). Our preliminary data *in vitro* experiment revealed that secreted SERPINA3N levels correlated with PGC-1 α levels and that this was influenced by a 6-week HFHF diet in cultured cells. We wanted to determine if hepatic disruption of PGC-1 α levels induces significant changes in SERPINA3N circulating levels in mouse serum *in vivo* and if diet still influences the secreted levels of this hepatokine in blood.

To test this, we used the cre/lox technology driven by the hepatocyte-specific albumin promoter to generate liver-specific heterozygote and liver-specific PGC-1 α knock-out mice (80, 81). Our mouse cohort was composed of albumin-cre expressing mice as wildtype controls (WT), liver-specific heterozygote of PGC-1 α (LHet) and liver-specific PGC-1 α knock-out mice (LKO). Both male and female mice were fed chow diet or high-fat, high-fructose (HFHF) diet for six weeks. First, we measured *Ppargc1a*, the gene encoding the PGC-1 α protein, to assure the efficiency of the knock down in liver (Figure 3A). Hepatic *Ppargc1a* mRNA levels were not affected by the diet in both males and females. As previously shown, *Ppargc1a* basal mRNA levels in the liver was higher in females compared to males (58). As expected, hepatic *Ppargc1a* mRNA levels decreased by approximately 50% in LHet and were undetected in LKO to the same extent in both sexes.

Next, we characterized the liver phenotype of our mice in order to identify at which stage of the NAFLD spectrum these mice had developed after 6 weeks of HFHF diet. We first measured hepatic acylglycerol content. Livers from male mice fed HFHF diet had a mild but significant increase in hepatic lipid content compared to chow diet fed wildtype mice and this increase was independent of the loss of PGC-1 α (Figure 3B). Livers of females fed HFHF diet also showed a mild but significant accumulation of hepatic acylglycerol as observed in males. In addition, liver-heterozygote (LH) females fed HFHF diet showed significantly higher hepatic lipid content compared to wildtype and liver knock-out littermates, as previously observed in published

results (58). We next stained liver sections with hematoxylin and eosin (H&E) (Figure 3C and D). We observed no visible difference in lipid content between genotypes in mice fed chow diet. Livers from wildtype male mice fed HFHF diet presented with microvesicular steatosis (white arrows) with slight evidence of cell ballooning and inflammatory cells infiltration (black arrows), but this was not observed in wildtype female livers. Livers from LHet and LKO male and female mice fed HFHF diet presented with macrovesicular and microvesicular steatosis (white arrows) across the liver sections, with more pronounced phenotype in males (89). We also noticed cell ballooning and the beginning of lobular inflammation with inflammatory cells lining on the wall of few blood vessels.

Altogether, these observations revealed that male mice appeared more affected by the diet, based on hepatic histopathological phenotype after 6 weeks. Even if females accumulated as much hepatic acylglycerol as males, their livers presented less histopathological NAFLD characteristics, suggesting that females may be protected from the effects of obesogenic diet and that the higher levels of PGC-1 α may be responsible for this protection. This correlate with human data showing that men are more affected and develop NAFLD earlier than women (90). The steatosis development was more remarkable in males with decreased hepatic PGC-1 α levels compared to wildtype males and females.

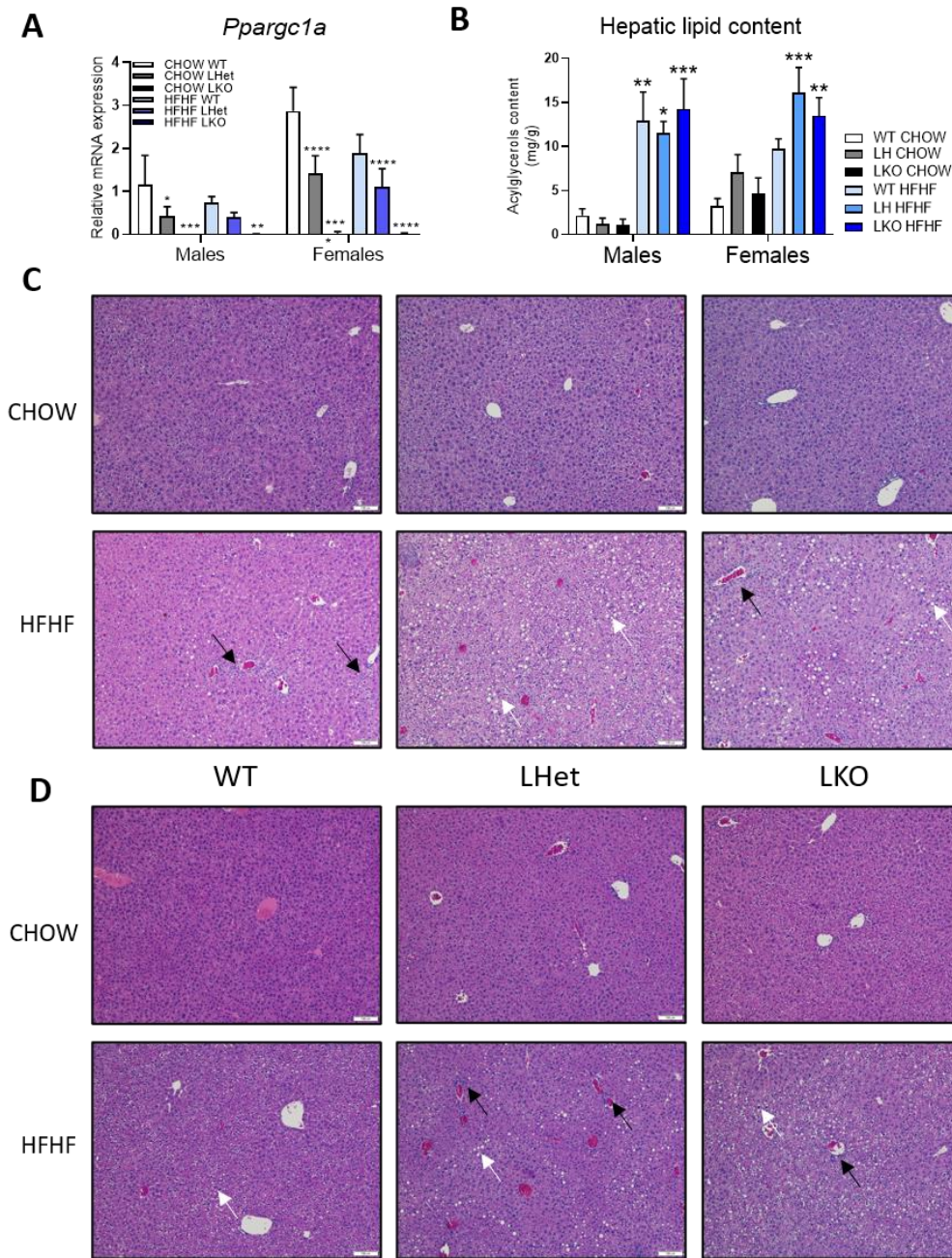


Figure 3. - Histological characterization of male and female mice livers.

(A) Measurement of hepatic *Pparg1a* gene expression by qPCR, n=7 (B) Intrahepatic total acylglycerol content at 6 weeks, n=7. (C) Hematoxylin and eosin (H&E) staining of liver sections from male mice, n=3 and (D) female mice, n=3. WT: wildtype, LHet: liver-specific PGC-1 α heterozygote, LKO: liver-specific PGC-1 α knock-out and HFHF: high-fat, high-fructose diet. Macro and microvesicular steatosis = white arrows, immune cell infiltration = black arrows. **** P <0.0001, *** P <0.001, ** P <0.01, * P <0.05. Histology magnification = 10X.

The hepatic expression of genes involved in NAFLD development.

We measured mRNA expression levels from different pathways previously identified as hallmarks of NAFLD progression in order to determine if the fat accumulation previously observed in the livers induced activation of pathways involved in NAFLD progression. We looked at five different pathways reported to be involved in the pathogenesis of NAFLD development: fibrosis formation, immune cell infiltration markers, inflammatory cytokines and chemokines, endoplasmic reticulum (ER) stress and ROS detoxification (38). For each of these five pathways, we measured key genes such as *Col1a1*, *Acta2* and *Tgfβ1* for fibrosis formation, *Cd4*, *Cd8* and *B220* for immune cell infiltration markers, *Tnfa*, *Il-1b*, *Il-6*, *Ccl2* and *Ccl3* for inflammatory cytokines and chemokines, *Bip*, *Chop*, *Xbp1* and *Xbp1* spliced for ER stress and finally *Prx5*, *Cat* and *Sod2* for the ROS detoxification pathway.

Investigating the impact of diet first, we compared hepatic gene expression of wildtype mice fed CHOW or HFHF diet for 6 weeks. In male mice, we noticed a significant general diet impact on fibrosis, inflammation, cytokines and chemokines and ER stress markers, however we observed no significant changes in individual gene expression (Figure 4A). In females, we noticed a trend toward increase in *Cd68* and *B220* inflammatory cell infiltration markers and in *Ccl3* cytokine gene expression. However, no genes changed significantly in response to HFHF diet (Figure 4B). Moreover, gene expression of antioxidant enzymes were not affected by the HFHF diet at 6 weeks in both males and females (58). We did not observe any changes in expression of any of these genes in LHet or LKO, male and female mice fed CHOW compared to their wildtype littermates (Supplementary figure 3).

We next investigated the impact of the liver-specific disruption of PGC-1 α and diet on hepatic gene expression, since it was reported that low PGC-1 α levels in the liver combined with a western diet contributes to accelerated NAFLD progression (58). In males fed HFHF diet, we noticed a significant genotype impact on fibrosis, cell infiltration markers, inflammatory cytokines and ER stress markers (Figure 5A). Livers from LHet and LKO mice had a trend toward decreased gene expression compared to wildtype fed HFHF diet. This decreased gene

expression was similar to the gene expression observed in livers from CHOW fed control mice (see dotted line). In females, the hepatic gene expression is not affected by the genetic disruption of PGC-1 α . We only noticed a non-significant diet impact on cell infiltration markers, *Cd68* and *B220*, inflammation marker *Tnf- α* and *Ccl3*, regardless of genotype. (Figure 5B).

These results revealed that the hepatic gene expression of wildtype male mice was influenced by the HFHF diet. Interestingly, livers of LHet and LKO male mice fed HFHF diet had opposite expression of key genes of fibrosis, inflammatory cytokines and ER stress pathways than what observed in livers of wildtype mice. The expression of these genes decreased to basal levels. This suggests that male livers with decreased expression of PGC-1 α may not adapt properly to the hepatic metabolic challenge induced by western diet after 6 weeks. In contrast, livers from wildtype females had no significant changes in key genes of the fibrosis, immune cell infiltration, inflammatory cytokines, ER stress and antioxidation pathways in response to hepatic disruption of PGC-1 α and diet. This suggests that female mice may be more resistant to hepatic loss of PGC-1 α and diet-induced steatosis at 6 weeks.

Finally, based on the hepatic lipid content, the histological characterization and measurements of genes involved in NAFLD development and progression, we were able to define which part of the NAFLD spectrum of liver pathologies these mice have developed. There was no evidence of significant activation of inflammation and fibrosis formation at the gene level, but livers of male mice showed more histopathological NAFLD characteristics compared to females, even if both showed similar acylglycerol accumulation. This suggests that both males and females were starting to develop early NAFLD, the fatty liver after 6 weeks of HFHF diet.

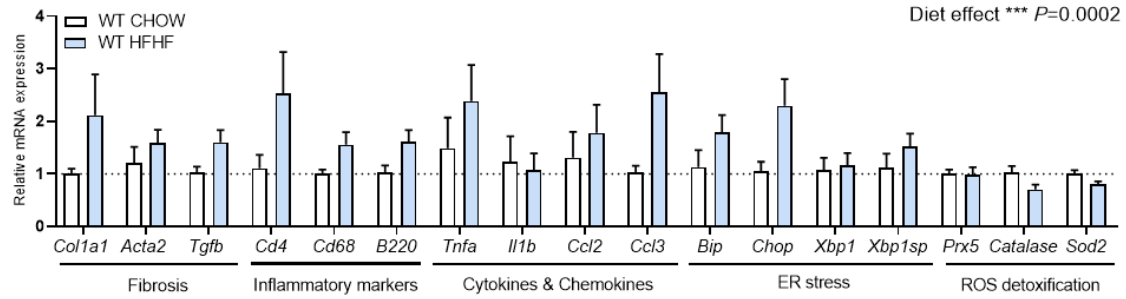
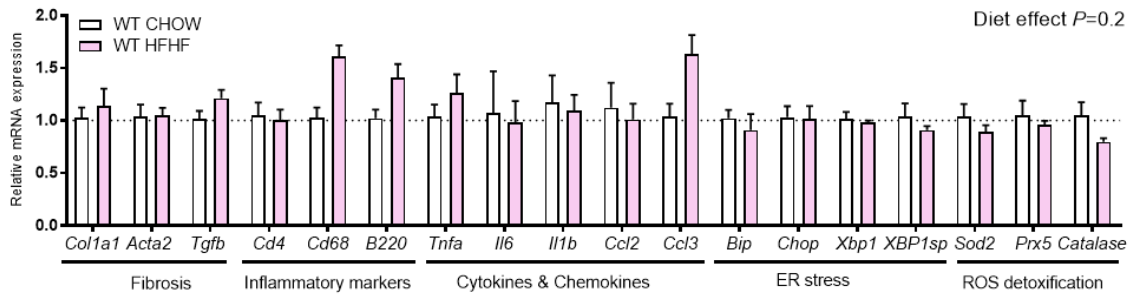
A**B**

Figure 4. - Impact of diet on hepatic expression of genes involved in NAFLD development.

(A) Hepatic gene expression by qPCR in wildtype males fed chow or high-fat, high-fructose diet for 6 weeks. (B) Hepatic gene expression by qPCR in wildtype females fed chow or high-fat, high-fructose diet for 6 weeks. WT: wildtype, LHet: liver-specific PGC-1 α heterozygote and LKO: liver-specific PGC-1 α knock-out. Normalized to housekeeping gene *HPRT*. Diet overall impact on pathways, males= *** $P=0.0002$, females = $P=0.02$. Mice per condition, $n=7$.

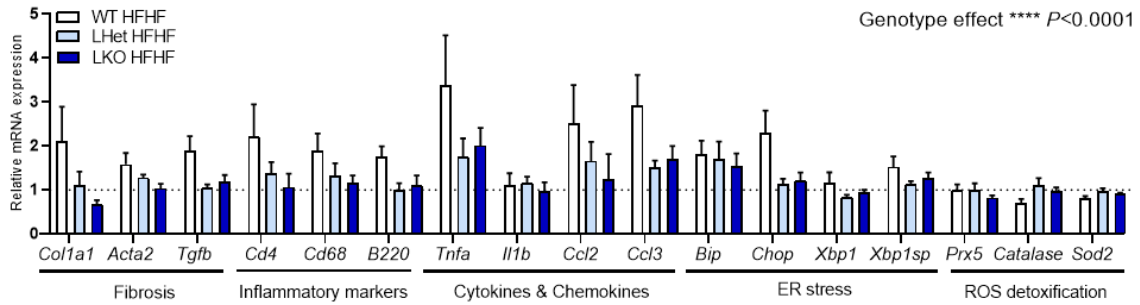
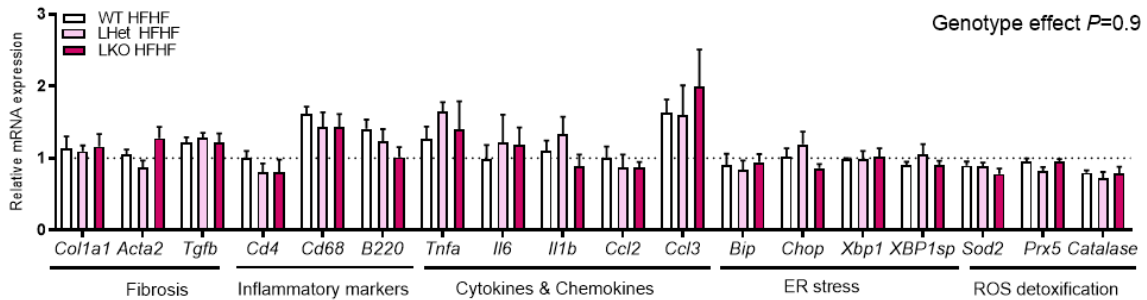
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Figure 5. - Impact of hepatic disruption of PGC-1 α and diet on gene expression at 6 weeks.

(A) Hepatic gene expression by qPCR in WT, LHet and LKO male mice fed high-fat, high-fructose diet for 6 weeks. (B) Hepatic gene expression by qPCR in WT, LH and LKO female mice fed high-fat, high-fructose diet for 6 weeks. WT: wildtype, LHet: liver-specific PGC-1 α heterozygote and LKO: liver-specific PGC-1 α knock-out. Normalized to housekeeping gene *HPRT*. PGC-1 α overall impact on pathways males = **** $P < 0.0001$, females = $P = 0.9$. Mice per condition, $n = 7$.

SERPINA3N circulating levels in male and female mice serum.

Preliminary results showed levels of SERPINA3N secreted from primary hepatocytes correlated with hepatic PGC-1 α levels differently under basal and obesogenic conditions (Figure 2). Furthermore, results report an increase of hepatic proteins levels and circulating levels of SERPINA3N in mice fed high-fat, high-fructose diet for 6 weeks (83). These preliminary findings led us to investigate whether the hepatic disruption of PGC-1 α *in vivo* causes significant changes in circulating SERPINA3N levels in mice serum.

We measured SERPINA3N levels in serum from wildtype, liver-specific PGC-1 α heterozygote (LHet) and liver-specific PGC-1 α knock-out (LKO) male mice fed chow control diet or HFHF diet for a duration of six weeks. For optimal immunoblot resolution, serum samples were depleted of the 7 most abundant proteins using antibody-containing columns. Comparing the impact of diet per genotype, secreted SERPINA3N levels increased with HFHF diet in wildtype mice compared to chow fed wildtype mice. This increase with the HFHF diet was not observed in LHet or LKO mice (Figure 6A). When comparing the impact of genotype per diet on SERPINA3N serum levels, LHet and LKO mice fed chow had increased SERPINA3N circulating levels compared to wildtype. However, SERPINA3N secreted levels decreased in LHet and LKO mice fed HFHF diet compared to wildtype mice fed HFHF diet (Figure 6B). Wildtype mice fed HFHF diet had higher SERPINA3N circulating levels than wildtype mice fed chow diet. These results show that SERPINA3N serum levels are induced by obesogenic diet and the hepatic disruption of PGC-1 α increases its secreted levels in chow fed mice and decreases its secreted levels in high-fat high-fructose fed mice. We observed two different patterns of SERPINA3N circulating levels in chow and HFHF diet, but both dependent on hepatic PGC-1 α levels as shown in preliminary experiments (Figure 2). These findings show how hepatic PGC-1 α regulates hepatokines differently under metabolic challenges such as a western diet, suggesting a novel function for PGC-1 α in the metabolic and acute adaptation in response to diet.

When investigating SERPINA3N in female mouse serum, we noticed that SERPINA3N circulating levels were not affected by HFHF diet in WT, LHet nor LKO mice (Figure 7A). Furthermore, there

was no difference in circulating SERPINA3N levels between genotypes on either diet (Figure 7B). From these results, we observed unchanged SERPINA3N serum levels in female mice regardless of hepatic disruption of PGC-1 α levels nor diet at 6 weeks.

In summary, SERPINA3N circulating levels negatively correlated with hepatic PGC-1 α levels in male mice fed chow for 6 weeks. In contrast, SERPINA3N circulating levels positively correlated with hepatic PGC-1 α levels in male mice fed HFHF diet for 6 weeks. Female mice had unchanged SERPINA3N circulating levels regardless of hepatic PGC-1 α levels or diet at 6 weeks. Interestingly, various blood proteome profiling studies report gender differences in bloodstream protein composition and moreover, metabolic diseases such as NAFLD are well known to be sexual dimorphic diseases (90, 91). These results suggest that male and female livers adapt differently in response to genetic disruption of PGC-1 α and high-fat, high-fructose diet at 6 weeks and present different liver secretome profiles.

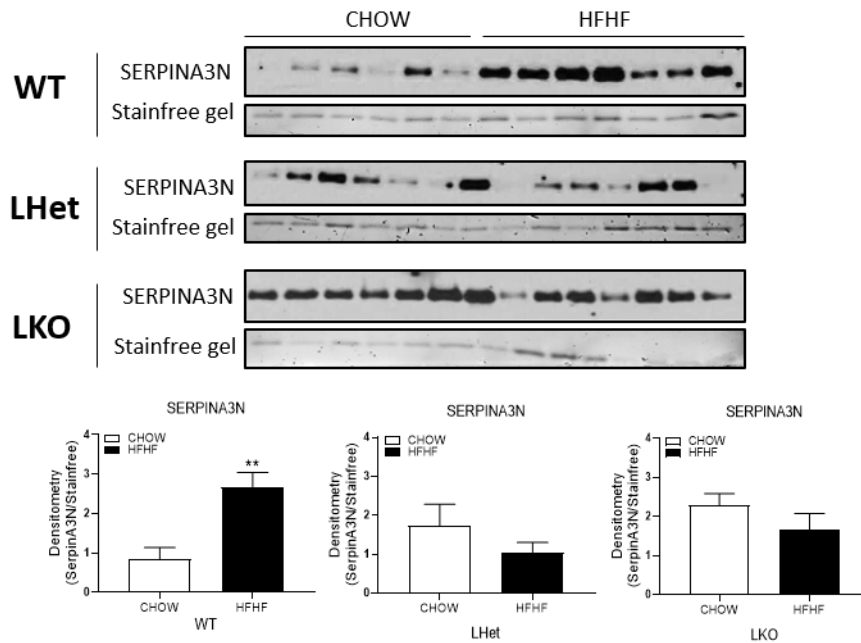
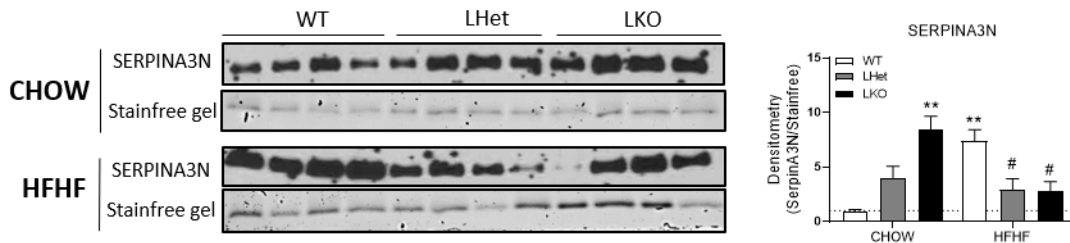
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Figure 6. - Diet and hepatic PGC-1 α levels influence SERPINA3N serum levels in male mice.

(A) Western blot showing the effect of diet on SERPINA3N serum levels in WT, LHet and LKO mice after 6 weeks of CHOW and HFHF diet. Quantification of blots using ImageJ below (n=7). (B) Western blot showing the effect of hepatic PGC-1 α levels per diet on SERPINA3N serum levels in WT, LH and LKO mice after 6 weeks of CHOW or HFHF diet. Right panel: Quantification of SERPINA3N immunoblots normalized to stain-free gels as loading controls with ImageJ software (n=4). WT: wildtype, LHet: liver-specific PGC-1 α heterozygote and LKO: liver-specific PGC-1 α knockout. ** P <0.01 indicated group vs control, # P <0.05 LHet and LKO HFHF vs WT HFHF.

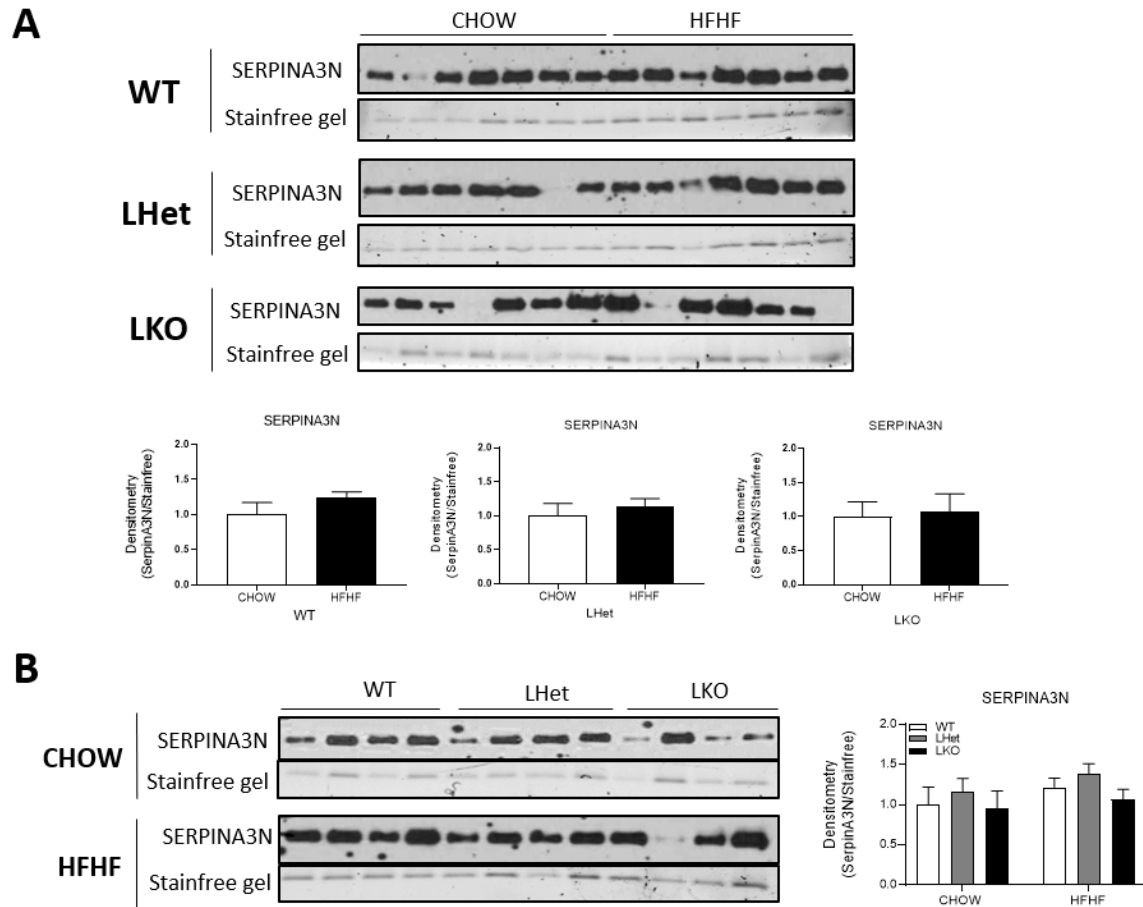


Figure 7. - Unchanged serum levels of SERPINA3N in female mice after 6 weeks.

(A) Effect of diet on SERPINA3N serum levels in WT, LHet and LKO mice after 6 weeks of CHOW and HFHF diet. Quantification of blots using ImageJ below (n=7). (B) Effect of hepatic PGC-1 α levels per diet on SERPINA3N serum levels in WT, LHet and LKO mice after 6 weeks of CHOW or HFHF diet. Right panel: Quantification of SERPINA3N immunoblots normalized to stain-free gels as loading controls with ImageLab software (n=4). WT: wildtype, LHet: liver-specific PGC-1 α heterozygote and LKO: liver-specific PGC-1 α knockout.

SERPINA3N circulating levels decrease with NAFLD progression.

A previous study from our lab demonstrated how hepatic reduction of PGC-1 α combined with a prolonged western diet can accelerate NAFLD progression to NASH (58). However, SERPINA3N is an acute phase protein and is expressed in early manifestations of inflammation. SERPINA3N serum levels were previously shown to decrease with time, from 8, 16 to 24 weeks in the Long-Evans Agouti rat, a well-established animal model of type 2 diabetes mellitus (92). We therefore hypothesized that in our mouse model, SERPINA3N circulating levels might decrease with NAFLD progression. For this part of the project, as our mice were sacrificed after 6 weeks of diet, we used serum samples collected at 6 and 25 weeks from a previous characterized mouse cohort fed for 25 weeks. This mouse cohort had only wildtype mice and LHet mice serum available.

In line with published literature, SERPINA3N circulating levels decreased significantly with NAFLD progression in both male and female mice (Figure 8). In males, SERPINA3N circulating levels were lower in LHet than WT mice at both 6 and 25 weeks (Figure 8A). This data validates what we previously observed, a positive correlation between SERPINA3N serum levels and hepatic PGC-1 α levels in male mice fed HFHF diet for 6 weeks. In females, SERPINA3N serum levels were not changed at 6 weeks between wildtype and liver-heterozygotes, as presented earlier. However, we noticed that female mice had lower serum levels of SERPINA3N at 25 weeks like in males, but were now further reduced by low hepatic PGC-1 α levels (Figure 8B), suggesting that a longer exposure to the diet is needed to reveal the importance of PGC-1 α to the female liver.

Mice fed high-fat, high-fructose diet for 25 weeks present important hepatic lipid accumulation with histopathological evidence of inflammation and fibrosis, weight gain and increasing circulating ALT levels (58). In this previous study, they also note female mice are generally more protected than male mice from NAFLD, but females are more significantly impacted by the hepatic disruption of PGC-1 α in terms of oxidative liver damage after 25-33 weeks on the diet.

They report that female mice are particularly more affected likely due to an important role for PGC-1 α in regulating estrogen-mediated ROS detoxification (58).

Based on these observations and in line with many previously published studies, females developed NAFLD slower than males. Therefore, our observations correspond well with human data, reporting that men tend to develop metabolic diseases such as NAFLD earlier in life than women (93, 94). In the time when we could observe early signs of NAFLD in male mice (steatosis, disruption of inflammation, fibrosis and ER stress pathways) reduction in PGC-1 α had significant impact on the pathobiology and secretome from the liver. Interestingly, SERPINA3N serum levels correlated positively with low hepatic PGC-1 α levels during a time when male mouse livers were inefficiently adapting to the metabolic challenge. At 6 weeks, females livers showed no evidence of metabolic stress. However, circulating SERPINA3N levels in females eventually correlated with hepatic PGC-1 α levels in a similar manner to males, but after longer exposure to the western diet, when damage to female livers is beginning to become evident (58).

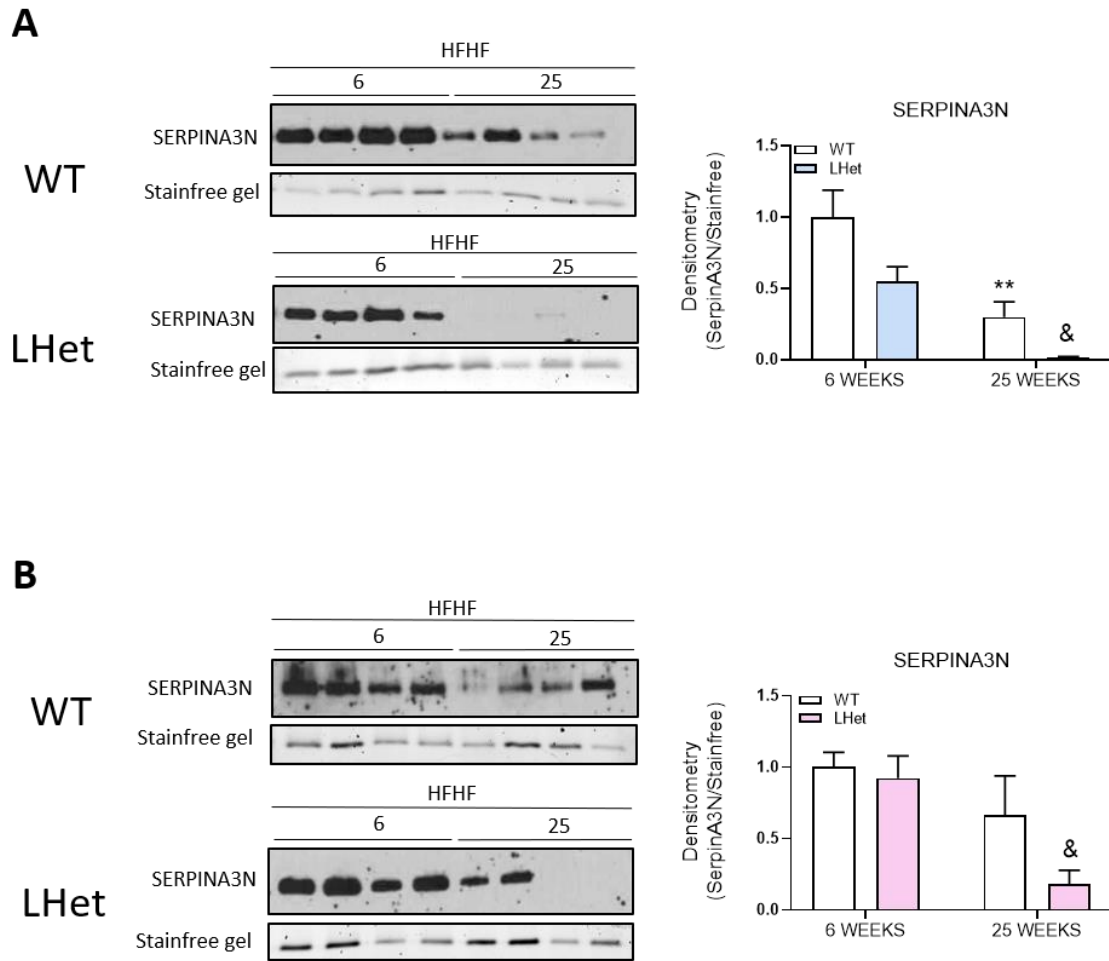


Figure 8. - SERPINA3N circulating levels decrease with NAFLD progression.

(A) Immunoblot and densitometry of SERPINA3N circulating levels in serum of WT and LHet males fed HFHF diet for 6 weeks or 25 weeks. (B) Immunoblot and densitometry of SERPINA3N circulating levels in serum of WT and LHet females fed HFHF diet for 6 weeks or 25 weeks. WT: wildtype, LHet: liver-specific PGC-1 α heterozygote. Quantification of SERPINA3N immunoblots normalized to stain-free gels using ImageJ. Mice per condition, n=4. ** $P=0.063$ vs WT 6 weeks, & $P<0.05$ vs LHet 6 weeks.

The hepatic regulation of *Serpina3n* gene expression.

Next, we investigated how hepatic *Serpina3n* is regulated differently in mice fed chow and high-fat, high-fructose diet and if PGC-1 α is directly regulating its gene expression. Interestingly, secreted SERPINA3N levels increased with the hepatic loss of PGC-1 α in chow fed mice, while in mice fed HFHF diet, SERPINA3N circulating levels decreased in LHet and LKO mice compared to wildtype (Figure 1 and Figure 4B). We therefore hypothesized that SERPINA3N is regulated by PGC-1 α through two different biological pathways in basal versus metabolically challenging conditions. Multiple cytokines known to be involved in the inflammatory signaling, such as TNF- α , IL-6 and IL-1 β regulate *Serpina3n* expression in different tissues. Glucocorticoids also increase *Serpina3n* expression in muscle cells and a combination of glucocorticoid and TNF- α or IL-6 can have a synergistic impact on *Serpina3n* expression (95, 96). Multiple studies have revealed that the human *Serpina3* contains binding sites for STAT and NF- κ B in its regulatory region (97). It is shown that inhibition of NF- κ B activity by chemical inhibitor blunts the palmitate and cytokine-mediated increase of *Serpina3* expression (98). The next part of this project will investigate two hypotheses by which *Serpina3n* gene may be regulated in the liver and determine if there is a link between hepatic PGC-1 α and *Serpina3n* gene expression.

Hepatic *serpina3n* expression is independent of NF- κ B activity.

The previous data showed an increase in serum levels of SERPINA3N in LH and LKO male chow-fed mice, compared to wildtype littermates. These results suggest that in the basal, healthy state, there is a negative regulation of *Serpina3n* expression by PGC-1 α . PGC-1 α is mainly known to co-activate transcription factors, leading to increased expression of nuclear-encoded target genes (60). However, a few studies demonstrate that PGC-1 can act as a repressor of the transcriptional activity of NF- κ B in skeletal muscle cells, by preventing the phosphorylation of its subunit p (99). From this, we hypothesized that hepatic PGC-1 α basally represses NF- κ B transcriptional activity to regulate gene expression of *Serpina3n*. We predicted that when hepatic PGC-1 α is disrupted, the NF- κ B is activated and induces the expression of *Serpina3n* (Figure 9A).

To test our hypothesis, we did a PGC-1 α gain- and loss-of-function experiment to investigate if *serpina3n* hepatic expression was controlled by PGC-1 α regulation. For loss-of-function, we isolated primary hepatocytes from wildtype and liver-specific PGC-1 α knock-out (LKO) male mice fed chow for 6 weeks (Figure 9B). For gain-of-function, wildtype primary hepatocytes were infected with either adenovirus overexpressing vector control (GFP) or PGC-1 α (Figure 9C). All cells were treated with DMSO as control or 10 μ M JSH-23 for 6 hours, an established inhibitor of NF- κ B activity. The inhibitor concentration and time of incubation used were optimized and tested beforehand, however this was performed with TNF- α -mediated activation of NF- κ B activity (Supplementary figure 1). SERPINA3N is known to be induced by multiple cytokines like TNF- α , IL-6 and IL-1B, therefore we did not use a TNF- α -mediated activation of NF- κ B in the final experiment in order to not interfere the NF- κ B direct impact on *Serpina3n* expression.

NF- κ B is known to regulate the basal levels of inhibitors of apoptosis *Bcl2* and *Xiap*, as well as *Cyclind1* (100-103). When investigating positive controls for inhibition of NF- κ B activity, *Bcl2* and *Xiap* gene expression decreased but only *Cyclind1* gene expression decreased significantly with JSH-23 treatment, suspecting that the JSH-23 compound did not work efficiently in this experiment (Figure 9D). *Serpina3n* gene expression decreased with the overexpression of PGC-1 α and decreased even more significantly in primary hepatocytes overexpressing PGC-1 α and treated with JSH-23 (Figure 9E). However, we observed no JSH-23 effect on *Serpina3n* expression in the AdCTL cells. In the loss-of-function experiment, which consists of primary hepatocytes from wildtype (WT) and liver-specific PGC-1 α knock-out (LKO) mice, we noticed significant increases of basal *Serpina3n* expression in LKO cells (Figure 9E). The JSH-23 treatment did not have any effect on *Serpina3n* gene expression in the loss-of-function experiment. Since we did not observe significant impact of JSH-23 treatment on *Serpina3n* gene levels, we investigated whether inhibition of NF- κ B could influence SERPINA3N protein or secreted levels in the cell lysate and the conditioned media respectively. We observed no significant changes in SERPINA3N at the protein levels in the cell lysates nor at the level of secretion in response to JSH-23 treatment (Figure 9F and G). Moreover, we did not observe any impact of JSH-23 on SERPINA3N protein levels in the adCTL or WT cells, suggesting SERPINA3N

may not be regulated basally by NF- κ B. However, we can still witness that PGC-1 α overexpression suppresses SERPINA3N protein levels and PGC-1 α knock-out cell had increased SERPINA3N protein levels, as observed at the gene level (Figure 9E, F, G). However, one caveat of this experiment was that we could not determine if NF- κ B is involved in *Serpina3n* expression, since the JSH-23 inhibitor did not work efficiently.

These preliminary experiments to explore the potential mechanisms involved in regulating SERPINA3N revealed that *Serpina3n* gene, SERPINA3N protein and its secreted levels are indeed strongly influenced by hepatic PGC-1 α levels. However, its hepatic expression did not seem to be regulated by the NF- κ B transcription factor. All together, hepatic PGC-1 α seemed to regulate SERPINA3N expression independently of NF- κ B.

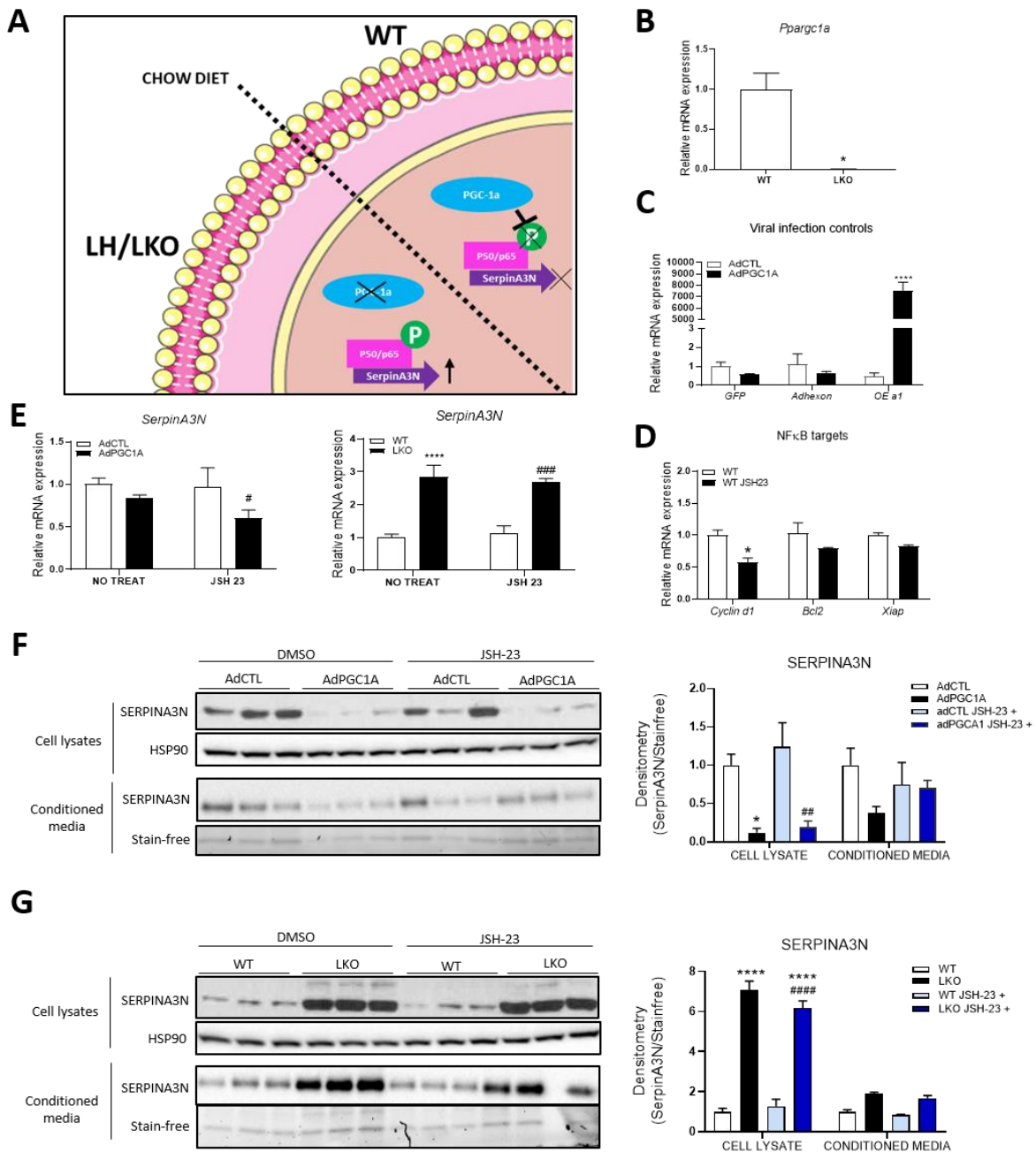


Figure 9. - SERPINA3N expression is influenced by PGC-1α levels independently of NF-κB.

(A) Schematic of the hypothesis. (B) Gene expression by qPCR from (B) WT and LKO cells, (C) GFP or PGC-1α overexpressing cells and (D) DMSO or JSH-23 (10uM 6 hours) treated cells. (E) Gene expression by qPCR, (F) protein and (G) secreted levels of SERPINA3N by immunoblotting. n=3. *P<0.05, **P<0.01, ****P<0.0001 vs control, #P<0.05, ##P<0.01, ####P<0.001 vs wildtype JSH-23 treated, &P<0.05 vs LKO no treatment. Quantification of SERPINA3N immunoblots normalized to stain-free gels using ImageLab software. Technical replicates = 1.

Effect of glucocorticoids and hepatic PGC-1 α on SERPINA3N proteins levels.

We observed increased secretion of SERPINA3N in wildtype mice fed HFHF diet. However, loss of hepatic PGC-1 α blunted SERPINA3N secretion in response to the HFHF diet. This suggests PGC-1 α may be essential in the adaptive and acute phase response in response to the diet. Glucocorticoids are anti-inflammatory hormones known to induce expression of acute phase proteins and especially the expression of SERPINA3N in muscle, lung and liver (104). The glucocorticoid receptor (GR) is co-activated by PGC-1 α and facilitates DNA binding of the transcription factor (105). From these findings, we hypothesize that high-fat, high-fructose diet increases glucocorticoid circulating levels, inducing translocation of the hepatic glucocorticoid receptor to the nucleus to be co-activated by PGC-1 α , leading to increased expression of *Serpina3n*. In the previous figure, we noticed that PGC-1 α repressed *Serpina3n* expression under basal conditions. However, we hypothesize that PGC-1 α is involved in the adaptive response to the HFHF diet and its regulation of *Serpina3n* will be adapted to the metabolic challenge. This would imply that when hepatic PGC-1 α levels are disrupted, the glucocorticoid receptor alone is not efficient to fully regulate SERPINA3N expression, as an anti-inflammatory signal (Figure 10A).

To test our hypothesis, in preliminary experiments we again used PGC-1 α gain and loss-of-function to investigate how SERPINA3N is regulated in the liver by hepatic PGC-1 α in response to glucocorticoid activity (Figure 10A). For the loss-of-function, we isolated primary hepatocytes from wildtype and liver-specific PGC-1 α knock-out (LKO) male mice fed chow for 6 weeks (Figure 10B). We isolated primary hepatocytes from mice fed chow because we wanted to investigate if glucocorticoids were the HFHF diet-mediated factor involved in *Serpina3n* expression. For the gain of function, wildtype primary hepatocytes were infected with either adenovirus overexpressing vector control (GFP) or PGC-1 α (Figure 10C). All cells were either untreated or treated with 1 μ M dexamethasone, a potent glucocorticoid agonist, for 8 hours.

We first investigated glucocorticoid receptor targets such as *HNF4 α* , *Ppara* and *Tnf- α* to determine if the dexamethasone treatment worked. All three genes responded to the

dexamethasone treatment as positive controls (Figure 10D). *HNF4α* and *Ppara* are known to be induced by dexamethasone and glucocorticoid receptor transcription activity, while *Tnf-α* is repressed by glucocorticoids (106-108). *Serpina3n* gene expression decreased in primary hepatocytes overexpressing PGC-1α, as shown in Figure 9. The dexamethasone treatment did not have any impact on *Serpina3n* gene expression (Figure 10E). Comparing the protein and secreted levels in the cell lysate and the conditioned media respectively, we noticed that dexamethasone treatment alone increased SERPINA3N levels in vector only (GFP)-infected cells (Figure 10F). Cells overexpressing PGC-1α and treated with glucocorticoids secreted more SERPINA3N protein than cells only overexpressing PGC-1α. This suggests that the glucocorticoids impact overcomes the repressive regulation of PGC-1α on SERPINA3N.

Overexpression of PGC-1α decreases *Serpina3n* expression, and in consistency, LKO primary hepatocytes expressed more *Serpina3n* than wildtype cells. We observed increased SERPINA3N protein and secreted levels with glucocorticoids treatment in wildtype primary hepatocytes, but the glucocorticoid-mediated increase in SERPINA3N protein levels were not observed in LKO cells (Figure 10G). However, the impact of glucocorticoids on LKO cells might not be measurable since SERPINA3N proteins levels were already very high and possibly saturated. For future perspectives, using a 50% reduction of PGC-1α (LHet mice) could be more informative and better demonstrate whether the inhibition of *Serpina3n* expression is PGC-1α dose-dependent. Since glucocorticoids induce the expression of SERPINA3N protein levels and its secretion from the liver, these results suggested that glucocorticoids may be the factor in the high-fat high-fructose diet inducing SERPINA3N secreted levels. In addition, hepatic PGC-1α levels suppresses *Serpina3n* expression in chow fed mice. However, we need to assess how hepatic PGC-1α regulates *Serpina3n* expression in high-fat high-fructose diet fed mice. When combining PGC-1α levels and glucocorticoids, we provide novel insight on how hepatic PGC-1α influences the glucocorticoid-mediated regulation of SERPINA3N protein and secreted levels.

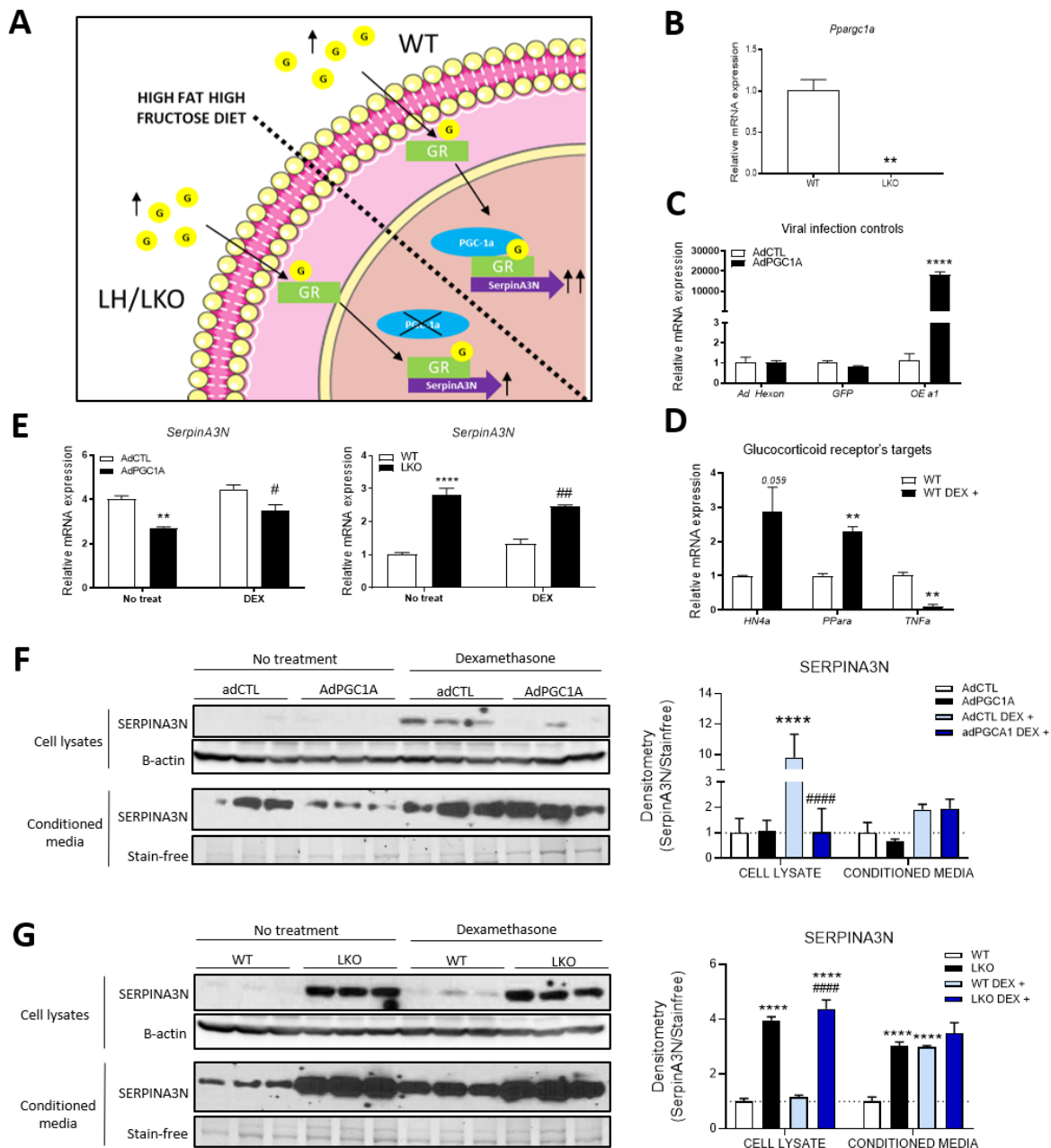


Figure 10. - SERPINA3N expression influenced by PGC-1 α through the glucocorticoid receptor. (A) Schematic of the hypothesis. (B) Gene expression by qPCR from (B) WT and LKO cells, (C) GFP or PGC-1 α overexpressing cells and (D) untreated or dexamethasone (1 μ M 8hours) treated cells. (E) Gene expression by qPCR, (F) protein and (G) secreted levels of SERPINA3N by immunoblotting. n=3. *P<0.05, **P<0.01, ****P<0.0001 vs control, #P<0.05, ##P<0.01, ####P<0.001 vs wildtype DEX treated, &P<0.05 vs LKO no treatment. Quantification of SERPINA3N immunoblots normalized to stain-free gels using ImageLab software. Technical replicates=1.

Discussion

In summary, we identify SERPINA3N as a hepatic secreted protein whose expression correlates with hepatic PGC-1 α levels in a diet and sex-dependent manner. We showed that hepatic PGC-1 α influences secreted SERPINA3N levels differently in healthy chow fed mice versus mice that are metabolically challenged with a western diet. In chow fed mice, SERPINA3N levels increased with the loss of PGC-1 α , while HFHF fed mice had decreased secreted SERPINA3N levels with the loss of PGC-1 α . These results revealed the potential importance of hepatic PGC-1 α in the acute adaptative response to metabolic challenges. We showed that the impact of PGC-1 α on SERPINA3N serum levels is different between males and females. We revealed that reduction of hepatic PGC-1 α levels may impact males and females at different times during the development of NAFLD or be dependent of the precise metabolic status of the liver, which differs greatly between the sexes compared at similar times. Finally, we showed that in basal conditions *in vitro*, overexpression of PGC-1 α repressed *Serpina3n* expression, while PGC-1 α knocked-out cells had increased *Serpina3n* expression. Furthermore, we showed that glucocorticoids increased SERPINA3N protein and secreted levels. This glucocorticoid-mediated increase in SERPINA3N secretion was greatly influenced by PGC-1 α levels.

As briefly mentioned, SERPINA3N is part of the serpin family, which is the largest family of protease inhibitors having a great diversity in function. Serpin stands for serine protease inhibitor and as their name suggests, they generally inhibit proteases (84). SERPINA3N is the closest mouse ortholog of the human alpha-1-antichymotrypsin (ACT), also known as SERPINA3, whose protein shares 61% similarity with mice (109). *Serpina3* gene was duplicated and diversified during evolution to give 13 different proteins in the murine SERPINA3 cluster (110). These proteins in mice are distributed in different tissues and are specific to different proteases. Mouse SERPINA3N is a positive acute-phase protein known to inhibit chymotrypsin, cathepsin G, granzyme B and elastases (84, 109). SERPINA3N is highly expressed in the liver but can also be expressed in multiple tissues such as the kidney, lungs and brain. SERPINA3N is secreted mainly by the liver but studies have reported it can be secreted from muscle cells and neurons (87, 98). SERPINA3N is secreted through the classical secretory pathway, where it can inhibit proteases in the extracellular compartment. It is shown that SERPINA3N is associated

with wound healing and muscle dystrophy by promoting tissue regeneration and preventing degradation (85, 87, 111). Human SERPINA3 is associated with Alzheimer and Parkinson's disease (97). A few studies show increased SERPINA3N expression in the hypothalamus in response to high fat diet (98, 112). High SERPINA3N levels also correlate with hepatocellular carcinoma cell proliferation, but it is suggested to be a cancer suppressor because it regulates chromatin condensation and represses the mTOR pathway (86, 88). However, there is no evidence of SERPINA3N implication in NAFLD development. Interestingly, SERPINA3N is associated with type 2 diabetes and other related comorbidities (92, 113). Various studies investigated the role of circulating SERPINA3N in type 2 diabetes and notice increased circulating SERPINA3N levels in diabetic rats as well as in diabetic humans (92, 113). Moreover, SERPINA3N circulating levels are high in human with hepatocellular carcinoma (HCC) and mice with muscle atrophy (86, 87, 111, 114). These findings suggest that SERPINA3N may be highly secreted for cellular proliferation and migration in HCC (86). In muscle atrophy, SERPINA3N is highly secreted to exert a protective activity by inhibiting extracellular proteases (111). Based on the literature and our results, we hypothesized that hepatic PGC-1 α is involved in regulating the expression of SERPINA3N as an adaptive response to metabolic challenges and protect the liver from damaging proteases. Whether SERPINA3N is involved or not in NAFLD pathogenesis, it remains that SERPINA3N secreted levels could be a potential biomarker of the liver state, as an indicator of disrupted hepatic PGC-1 α levels.

In this study, we reproduced the *in vitro* preliminary data measuring SERPINA3N levels in conditioned media *in vivo* in mouse serum. We showed that serum levels of SERPINA3N strongly reflected the hepatocyte-specific SERPINA3N secretion. We observed lower circulating levels in LHet male mice in early NAFLD-promoting diet. We also show that circulating SERPINA3N levels decreased with NAFLD progression (or age) in both male and female serum and that serum levels of SERPINA3N remained lower in LHet male mice even after 25 weeks of HFHF diet when compare to mice on diet for 6 weeks. While in females, circulating levels of SERPINA3N were unaffected by the diet or genotype after 6 weeks. After 25 weeks, female LHet mice had lower circulating levels of SERPINA3N compared to wildtypes. The difference in

females between 6 weeks and 25 weeks of diet, could be the level of steatosis development. After 6 weeks, female mice presented mild hepatic lipid accumulation with no evident activation of inflammatory, fibrosis or ER stress signaling by qPCR or histology. Prolonged western diet feeding induces exacerbated NAFLD progression, with evidence of hepatic oxidative damage, weight gain and increase ALT levels (58). After 25 weeks of diet, LHet female mice have more hepatic oxidative damage compared to control females, as loss of hepatic PGC-1 α decreases estrogen-mediated ROS detoxification capacity (58). This pathway may not be active at earlier points in the diet, since we did not see evidence of oxidative stress, and thus low PGC-1 α levels in female livers may not be biologically relevant until the damaging stimuli become active. Altogether, SERPINA3N circulating levels appear to correlate with hepatic PGC-1 α levels when mice are starting to show signs of NAFLD development, which occurs later in female mice.

Since we observed a decrease expression of *Serpina3n* with disruption of PGC-1 α in murine livers combined with an obesogenic diet, can we expect the same outcome if mice are changed to CHOW control diet after 6 weeks? This interesting question highlights how metabolic challenges may trigger new functions for PGC-1 α in the liver and influences the hepatic secretome differently from basal conditions. As mentioned before, wildtype mice fed a high-fat, high-fructose diet for 6 weeks have high circulating SERPINA3N, while LH mice fed the same diet have lower SERPINA3N than their wildtype littermates. After 6 weeks, if we changed the high-fat, high-fructose diet for the CHOW control diet, we could speculate that the hepatocytes are less subjected to metabolic challenge-related stress, therefore restore their phenotype and retroactively process the excessive fat accumulation. In addition, we speculate that PGC-1 α will now suppress *Serpina3n* expression, as observed in CHOW fed wildtype mice (Figure 2), since our hypothesis proposes that under metabolic stress, PGC-1 α may play an adaptive and protective role and promote the expression of the acute phase protein *Serpina3n*.

In this project we focused on NAFLD; non-alcoholic fatty liver disease, a well-known disease studied for steatosis development, however alcoholic fatty liver disease also induces a steatotic

liver. Studies have shown that PGC-1 α can be suppressed by the hepatic oxidative stress caused by acute alcohol consumption, with evidence of suppressed fatty acid β -oxidation and Krebs cycle due to excessive NADH production from the process of ethanol oxidation in the ADH pathway (115, 116). Therefore, it would be interesting to investigate if *Serpina3n* expression shows similar pattern in hepatic PGC-1 α -related AFLD and NAFLD development.

However, how SERPINA3N is regulated in the liver and how PGC-1 α levels affect its regulation in the context of NAFLD is still not clear. Whether the release of SERPINA3N in the blood within the context of NAFLD development promotes or protects from disease progression is also unknown. Given the strong correlation between SERPINA3N secretion and PGC-1 α expression, we were interested in studying molecular mechanisms involved in regulating SERPINA3N expression in livers from mice fed chow and HFHF diet, and if hepatic PGC-1 α was involved in its regulation. As mentioned before, SERPINA3N is an acute phase protein synthesized and secreted by the liver in response to acute inflammation (109). As mentioned previously, multiple factors influence *Serpina3n* expression, however in the context of metabolic disease and PGC-1 α -related, the molecular mechanisms contributing to *Serpina3n* expression are still uncertain.

Palmitate-mediated increases of *Serpina3n* expression in the hypothalamus can be blunted by inhibition of NF- κ B (98). Since PGC-1 α is reported to inhibit the activity of NF- κ B in muscle cells, and we observed increased SERPINA3N expression with the loss of hepatic PGC-1 α in mice fed chow diet, we next investigated whether the transcription factor NF- κ B could be the intermediate between hepatic PGC-1 α and SERPINA3N expression (99). However, we observed no impact of NF- κ B inhibition using JSH-23 inhibitor on *Serpina3n* gene, protein and secreted levels. We therefore suggest that the correlation between SERPINA3N and PGC-1 α levels in chow fed mice is independent of NF- κ B activity.

The inflammatory environment generated by the HFHF diet is key to increased SERPINA3N secretion in wildtype male mice fed HFHF diet (Figure 6A). However, the diet-mediated

increases in SERPINA3N secretion in wildtype male mice were not observed in LHet and LKO mice. This is concomitant with the decreased expression of key genes of the inflammation pathway in LHet and LKO mice fed HFHF diet, suggesting that the loss of PGC-1 α may interrupt the general inflammation response to the diet. Based on the unaffected genes of inflammatory cytokines and immune cell infiltration markers in response to the diet, we investigated whether there were other diet-related factors involved in regulating SERPINA3N in the liver. Glucocorticoids are steroid hormones synthesized by the adrenal glands stimulated by the hypothalamic-pituitary axis and are involved in the anti-inflammatory responses (117, 118). High circulating glucocorticoids levels induce muscle atrophy, insulin resistance, visceral adiposity, diabetes and are associated with obesity (117). In addition to increasing circulating levels of acute phase proteins, glucocorticoids are well known to induce the expression of *Serpina3n* in muscle, lung and liver (87, 95). Interestingly, PGC-1 α co-activates the glucocorticoid receptor and enhances its binding to DNA (105). Based on these observations, we tested if glucocorticoids were the diet-related factor involved in the regulation of SERPINA3N as part of the anti-inflammatory response. Long-term glucocorticoid administration is often associated with the development of metabolic disease such as liver steatosis and obesity (119). However, short-term diet-mediated increases in glucocorticoid circulation mainly exerts an anti-inflammatory activity.

We hypothesized that glucocorticoids may be key in switching the repressive regulation of PGC-1 α on *Serpina3n* expression observed in chow fed mice, to an adaptive response when cells are metabolically challenged. We found that in line with published literature, glucocorticoids induced SERPINA3N protein and secreted levels. Interestingly, the hepatic PGC-1 α levels influenced glucocorticoid-mediated regulation of SERPINA3N secretion. In cells overexpressing PGC-1 α , glucocorticoid induction of SERPINA3N was blunted in the cell lysates and conditioned media. In line with PGC-1 α having a repressive role on *Serpina3n* expression, SERPINA3N was basally very high in LKO cells; however glucocorticoid induction of SERPINA3N protein levels was not observed in LKO cells. This may be due to already saturated SERPINA3N levels, suggesting using primary hepatocytes from LHet mice for future validation. These findings

reveal a novel role of PGC-1 α in influencing the glucocorticoid receptor regulation of SERPINA3N secretion from the liver. To further validate this hypothesis, it would be helpful to measure the circulating levels of glucocorticoids in our murine model to determine whether they are also affected indirectly by low PGC-1 α in the liver.

Further validation needs to be done to better understand the implication of hepatic PGC-1 α in the regulation of acute phase proteins. Furthermore, we would like to investigate whether hepatic PGC-1 α levels induces significant changes in SERPINA3 circulating levels in humans. As mentioned in the introduction, a specific PGC-1 α single nucleotide polymorphism (SNP) is associated with NAFLD and type 2 diabetes incidence in humans (120). This PGC-1 α Gly482Ser variant leads to decreased levels of PGC-1 α protein. Previous work in mouse models, showed that a PGC-1 α protein with a serine at position 482 instead of a glycine has a shorter half-life in the liver cells, lower gene expression and protein activity in muscle cells (58, 120). Based on these results, we could measure the human SERPINA3 in blood of human cohorts to see whether it correlates with the PGC-1 α Gly482Ser SNP. However, there are many caveats to measuring circulating markers in human cohorts since they often do not develop only one disease and live in different environmental conditions. Specific patients would be chosen based on NAFLD stage, diet, obesity, presence of other diseases known to have altered circulating levels of SERPINA3. Moreover, we could not ensure that circulating SERPINA3 levels are from the liver since in humans we can not control low PGC-1 α levels specifically and only in the liver.

Next, we would like to investigate how low hepatic PGC-1 α impacts the whole-body metabolism through alterations of hepatokines. More specifically, investigate the PGC-1 α -mediated alteration of circulating SERPINA3N levels impact peripheral tissues in context of low PGC-1 α and diet-promoting NAFLD. To do so, overexpression of SERPINA3N in mice to investigate peripheral tissue metabolism could be interesting. Since SERPINA3N can be secreted by other tissues such as the muscle and the brain, we want to investigate the liver-specific secretion of SERPINA3N. As part of my project, I generated a plasmid for adeno-associated virus (AAV), expressing floxed-*Serpina3n* with a peptide signal for optimal secretion and V5 tag for

detection (Supplementary figure 4). We chose to overexpress *Serpina3n* using AAV because it will allow to express it over longer period of time and therefore assess circulating SERPINA3N impact on peripheral tissue. Since mice express the Cre-recombinase only in the liver in the ALB-cre transgenic mice, SERPINA3N will be expressed liver-specifically using the Cre-lox technology by injecting the AAV expressing floxed-*Serpina3n*. This will allow to better understand the impact of circulating SERPINA3N and determine if it is involved in protecting or promoting NAFLD progression.

So far, the impact of PGC-1 α on the whole cell secretome has only been studied in basal conditions (82). The effect of high fat diet on PGC-1 α 's influence on the liver secretome has never been studied. Therefore, we showed for the first time that hepatic PGC-1 α levels influence the liver secretome differently in mice fed high-fat, high-fructose diet compared to chow fed mice. We identified SERPINA3N, a secreted protein from the liver released into the bloodstream, whose circulating levels correlate with the hepatic PGC-1 α levels in basal conditions as well as in context of fatty liver disease. However, to date there is no studies showing a link between PGC-1 α and *Serpina3n* expression. Interestingly, males and females showed similar PGC-1 α -dependent patterns in circulating SERPINA3N levels, however this pattern was dependent on the development of the disease, which occurs earlier in males and later in females. The molecular mechanisms contributing to SERPINA3N expression and secretion are still unclear and more experiments are needed to determine the precise pathways linking PGC-1 α to SERPINA3N secretion. Moreover, to assess that SERPINA3N is solely secreted from the liver, a liver-specific knock-out of *Serpina3n* is needed. However, we observed a strong impact of hepatic PGC-1 α levels on *serpina3n* gene, protein and secreted levels, therefore we hypothesize that SERPINA3N in the blood is mainly secreted from the liver. Revealing how SERPINA3N is regulated in the liver could provide insight on how PGC-1 α adapts and influences the anti-inflammatory signaling in context of metabolic challenges. In this project, we provide SERPINA3N a circulating protein whose expression is influenced by hepatic PGC-1 α levels. Whether this circulating protein is regulated by PGC-1 α or involved in NAFLD pathogenesis still needs to be investigated.

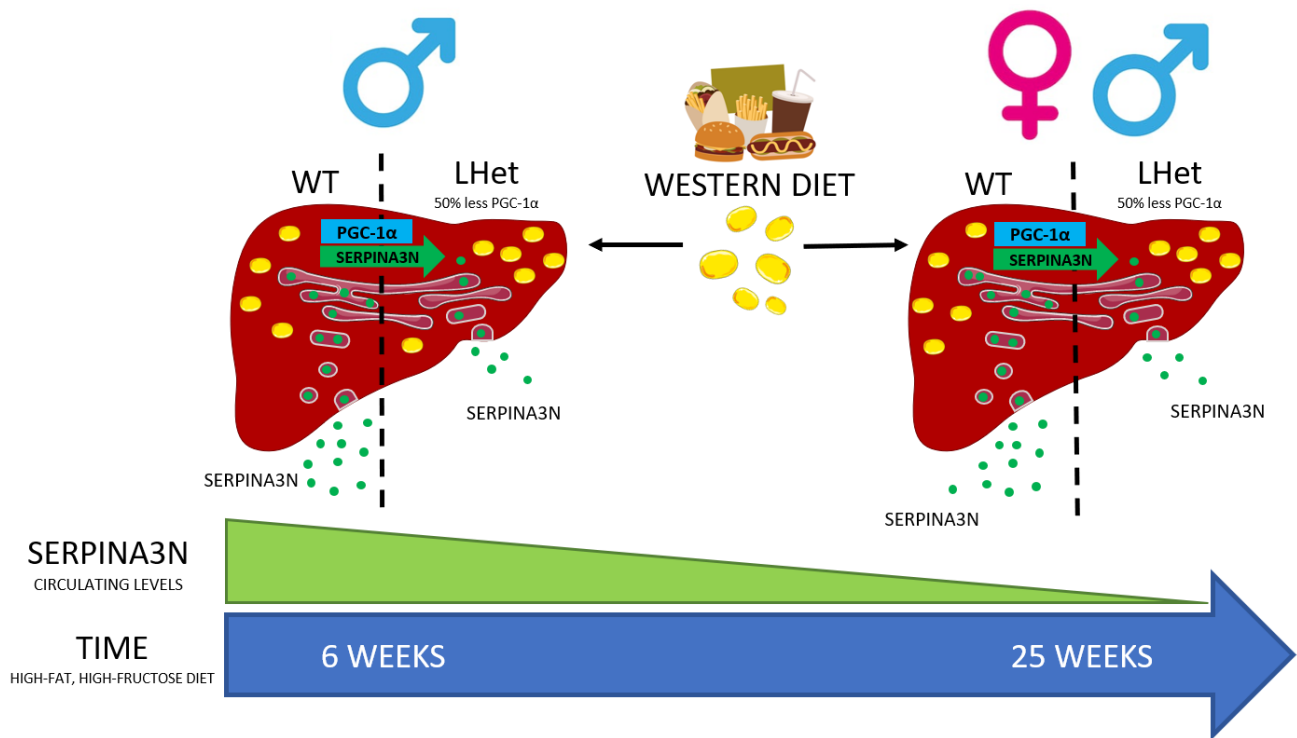


Figure 11. – SERPINA3N secretion in murine model of PGC-1 α -related NAFLD progression.

SERPINA3N expression and secretion from murine livers. SERPINA3N is highly secreted into the circulation in wildtype (WT) male mice fed high-fat, high-fructose diet for 6 weeks and its circulating levels decrease in liver-specific PGC-1 α heterozygote (LHet) male mice littermates. This pattern is also observed later after prolonged western diet feeding, but in both male and female mice this time. However, SERPINA3N circulating levels decrease after prolonged western diet feeding compared to early timepoints.

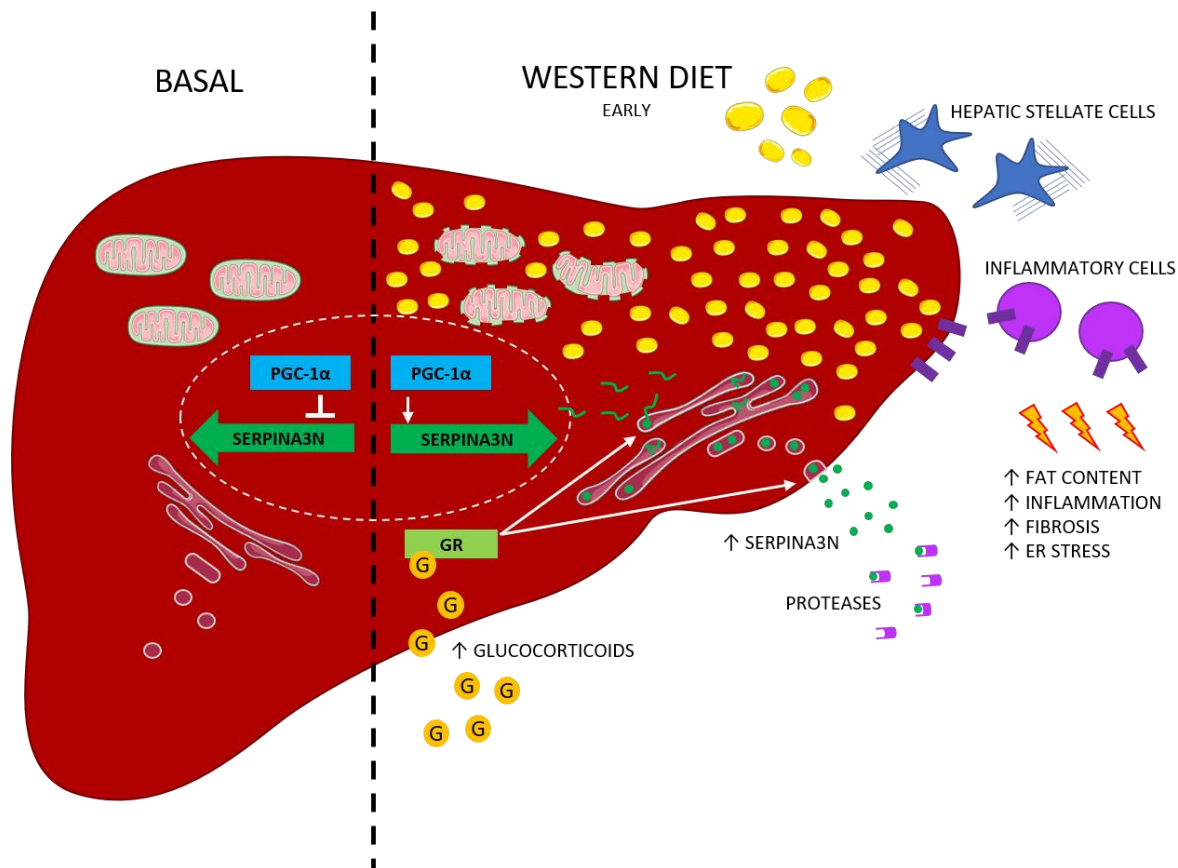
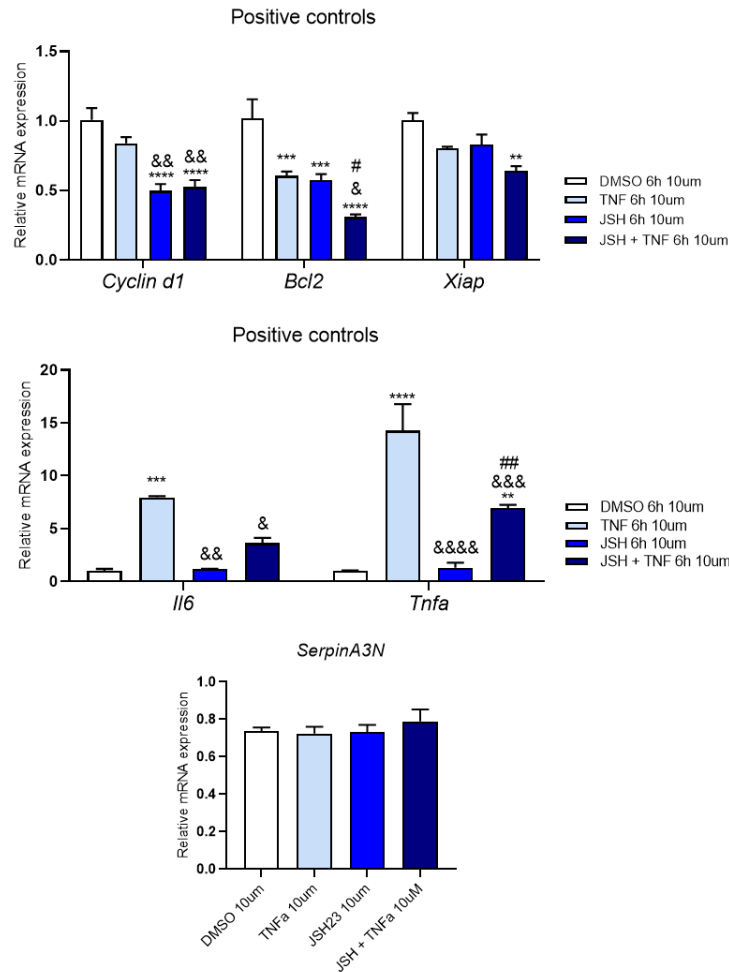


Figure 12. – PGC-1 α regulation of hepatic *Serpina3n* expression in basal and western diet conditions.

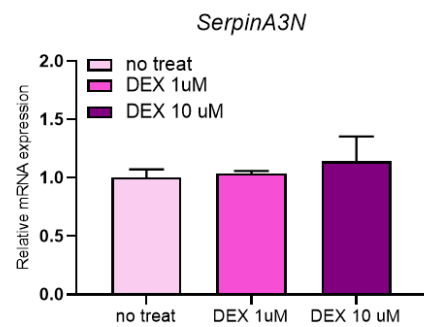
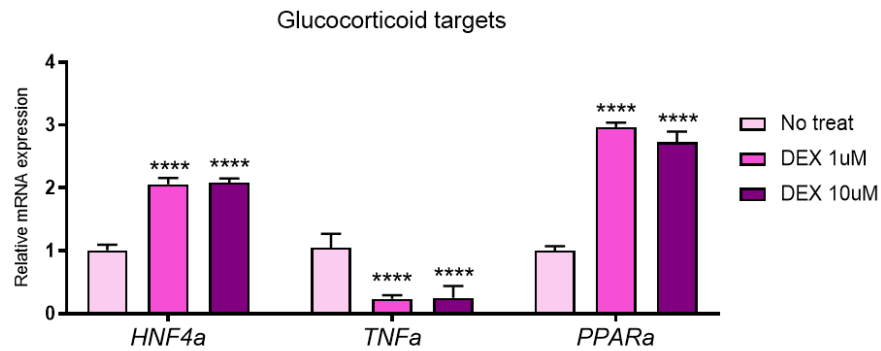
PGC-1 α regulation of hepatic *Serpina3n* expression in basal and western diet feeding conditions. In basal condition, there is no liver damage or metabolic stress, therefore we found that PGC-1 α suppresses *Serpina3n* expression. When mice are fed a western diet for 6 weeks, we found that PGC-1 α induces *Serpina3n* expression, which may lead to inhibition of extracellular proteases according to the literature. Moreover, we found that that glucocorticoids induce SERPINA3N protein and secreted levels in mice livers.

Annexe



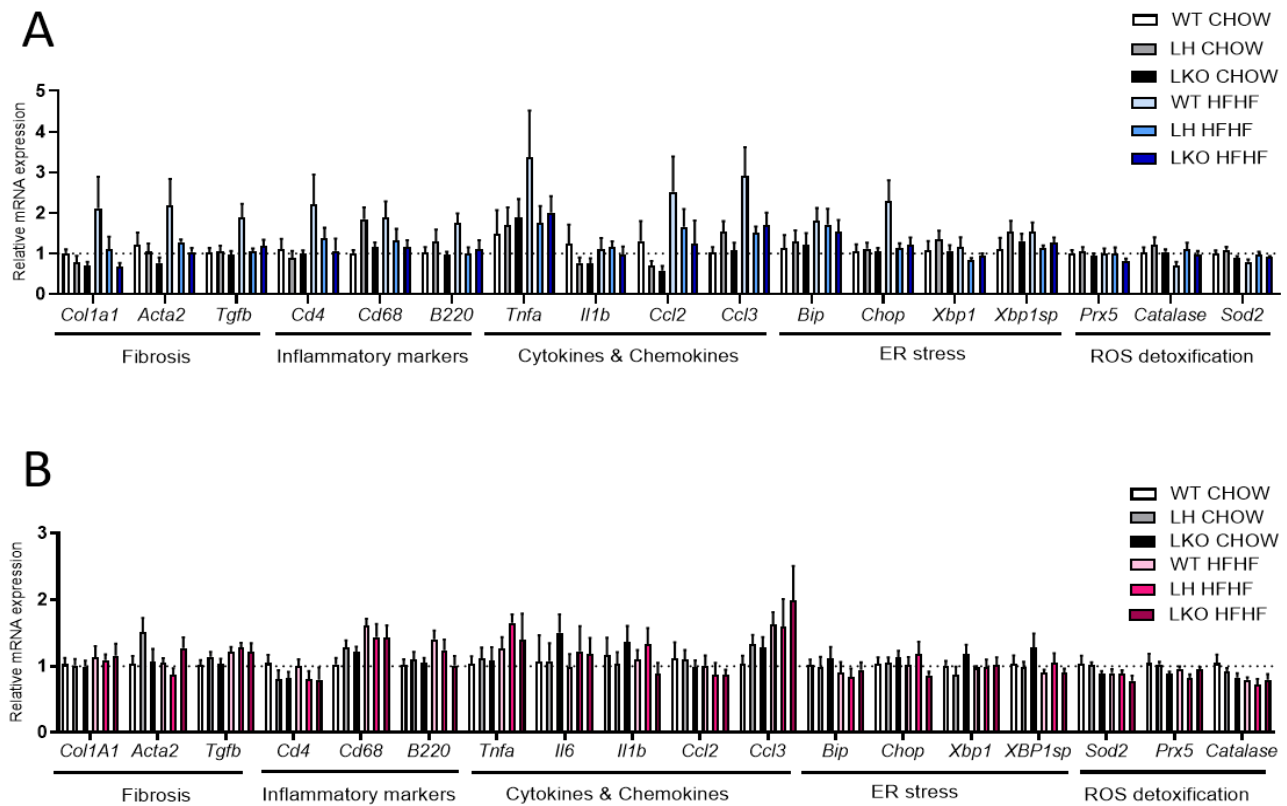
Supplementary figure 1. - Expression of NF- κ B targets in TNF- α -mediated activation of NF- κ B and inhibition by JSH-23 inhibitor.

Gene expression in primary hepatocytes from wildtype mice treated with DMSO, 20 ng/ml of TNF- α , 10 uM of JSH-23 inhibitor or 20 ng/ml of TNF- α combined with 10 uM of JSH-23 inhibitor for 6 hours. n=2. *** P<0.001, ****P<0.0001 vs DMSO treated cells, &P<0.05, &&P<0.001, &&&P<0.001, &&&&P<0.0001 vs TNF- α treated cells. #P<0.05, #P<0.01 vs JSH-23 treated cells.



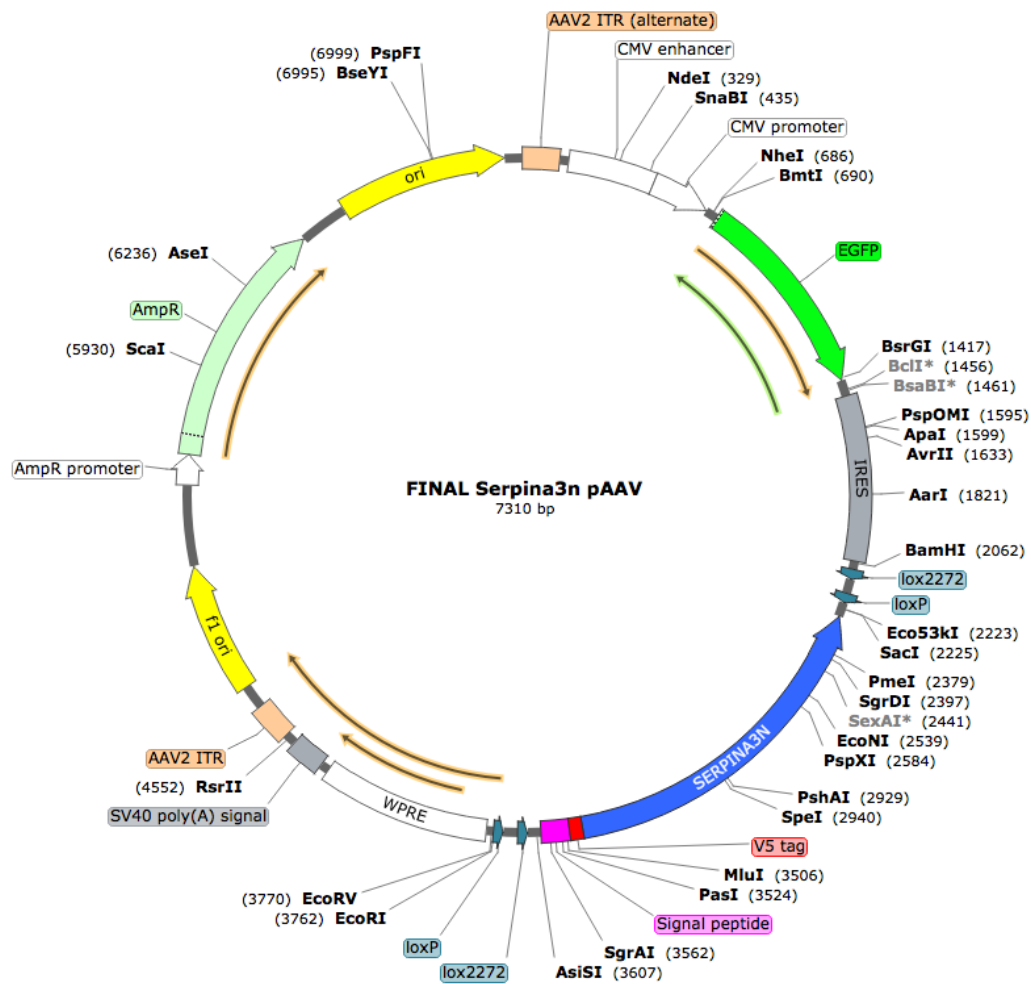
Supplementary figure 2. - Dexamethasone concentration and incubation time optimization

Gene expression in primary hepatocytes from wildtype mice treated with 1 uM or 10 uM dexamethasone for 8 hours. N=2. Normalized to housekeeping gene *HPRT*. **** P<0.0001 vs no treat samples.



Supplementary figure 3. - Hepatic gene expression in WT, LHet and LKO mice fed chow or HFHF diet.

(A) Gene expression in livers from WT, LHet and LKO male mice fed chow (grey bars) or HFHF diet (blue bars). (B) Gene expression in livers from WT, LHet and LKO female mice fed chow (grey bars) or HFHF diet (blue bars). Normalized to housekeeping gene *HPRT*. N=7.



Supplementary figure 4. - Plasmid construct for adeno-associated virus (AAV) expressing *serpina3n*.

Plasmid construct expressing floxed-*serpina3n* with the cytomegalovirus (CMV) promoter and enhanced green fluorescent protein (eGFP). The *serpina3n* gene sequence contains signal peptide sequence at the N-terminal for secretory pathway. We also added a V5 tag in *serpina3n* gene sequence for alternative detection and measurement method. Plasmid construct generated in Snapgene software.

Supplementary Table 1.1 Antibodies		
Protein	Company	Dilution
SerpinA3N	R&D systems	1/2000
ATCB (b-actin)	Sigma #A1978	1/2000
HSP90	Cell signaling technologies #4874	1/1000

Table 1. - Antibodies for immunoblot.

Gene	Forward	Reverse
Mouse Primers		
<i>Acta2</i>	CCA TCA TGC GTC TGG ACT T	GGC AGT AGT CAC GAA GGA ATA G
<i>Ad PGC-1α1 OE</i>	GGA CTA CAA AGA CGA TGA CG	CTC TAT GTC ACT CCA TAC AGA GTC
<i>Ad5 Hexon</i>	TAA GGG TTG ACG GAG CCA GCA TTA	TAA AGG ACT GGT CGT TGG TGT CGT
<i>B220</i>	AAT GGA GAC CAG GAA GTC TGT GCT	TGT AGG CTG AGG CTC TGT TTG TGT
<i>Bcl2</i>	GGA GGA TTG TGG CCT TCT TT	GTT CAG GTA CTC AGT CAT CCA
<i>Bip</i>	GAGACTGCTGAGGCGTATTT	CAGCATCTTTGGTTGCTTGTC
<i>cat</i>	GCGGATTCTGAGAGAGTGGTAC	GCCTGACTCTCCAGCGACTGTGGAG
<i>Ccl2</i>	TCA CCT GCT GCT ACT CAT TCA CCA	TAC AGC TTC TTT GGG ACA CCT GCT
<i>Ccl3</i>	TTT GAA ACC AGC AGC CTT TGC TCC	TCA GGC ATT CAG TTC CAG GTC AGT
<i>Cd4</i>	ACC TCA AGC TCC AGC TGA AGG AAA	GGT TGC CAG AAC CAG CAA ACT GAA
<i>Cd68</i>	TTC TCC AGC TGT TCA CCT TGA CCT	GTT GCA AGA GAA ACA TGG CCC GAA
<i>Chop</i>	CAACAGAGGTCACACGCACAT	CCTGGGCCATAGAACTCTGACT
<i>Col1a1</i>	GAA ACC CGA GGT ATG CTT GA	GTT GGG ACA GTC CAG TTC TT
<i>cyclin d1</i>	CAG AGG CGG ATG AGA ACA AG	GAG GGT GGG TTG GAA ATG AA
<i>Flag</i>	GGA CTA CAA AGA CGA TGA CG	
<i>Gfp</i>	AGCAAAGACCCCAACGAGAA	GCGGCGGTACGAACTC
<i>HNF4αa</i>	GCTGGCATGAAGAAGGAAGCT	GACCTCCGCGTGCTGATC
<i>hprt</i>	GGCCAGACTTTGTTGGATTTG	TGCGCTCATCTTAGGCTTTGT
<i>Il-6</i>	ATC CAG TTG CCT TCT TGG GAC TGA	TAA GCC TCC GAC TTG TGA AGT GGT
<i>Il-1b</i>	GGA GCT ATC ACT TGA CCA CAT C	GGA AGC AGC CCT TCA TCT T
<i>PGC-1α exon 3-5</i>	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG
<i>Ppara</i>	ACAAGGCCTCAGGGTACCA	GCCGAAAGAAGCCCTTACAG
<i>ppargc1a</i>	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG
<i>Prx5</i>	GGGAAGGCGACAGACTTATTATT	CCTTCACTATGCCGTTGTCTATC
<i>Serpina3n</i>	CCT GAC AGA ATT CCA GGA GAA G	CTG GGT CTG TTT CCT CAC ATA G
<i>sod2</i>	GGCCAAGGGAGATGTTACAAC	GCAACTCTCCTTTGGGTTCTC
<i>Tgfβ1</i>	GTG CGG CAG CTG TAC ATT GAC TTT	TGT ACT GTG TGT CCA GGC TCC AAA
<i>Tnf-α</i>	CCC TCA CAC TCA GAT CAT CTT CT	GCT ACG ACG TGG GCT ACA G
<i>Xbp1</i>	ATCAGCTTTTACGGGAGAAACTC	CCATCCCAAGCGTGTCTT
<i>Xbp1 sp</i>	CTTTCATCCAGCCATTGTCTGA	GCCCTCATATCCACAGTCACTGT
<i>Xiap</i>	CCA GCC ATG GCA GAA TAT GA	TCG CCT TCA CCT AAA GCA TAA A
Genotyping Primers		
alb-cre	TTAGAGGGGAACAGCTCCAGATGG	GTGAAACAGCATTGCTGTCACTT
PGC-1αloxP	TCCAGTAGGCAGAGATTTATGAC	TGCTGTTTGACAATCTGCTAGGTC

Table 2. - Primers used for quantitative real-time PCR: listed in 5'-3' direction.

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