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Antiobesity and antidiabetic activity of *P. balsamifera*, its active Salicortin, and *L. laricina*, medicinal plants from the traditional pharmacopoeia of the James Bay Cree

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Cette thèse intitulée:

Antiobesity and antidiabetic activity of *P. balsamifera*, its active Salicortin, and *L. laricina*, medicinal plants from the traditional pharmacopoeia of the James Bay Cree

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Résumé

La prévalence de l'obésité, du diabète de type 2, et du syndrome métabolique, sont à la hausse chez les Cris d'Eeyou Istchee (CEI-Nord du Québec). Ces problèmes sont aggravés par leur diète non traditionnelle, leur sédentarité, ainsi que par une résistance culturelle aux produits pharmaceutiques. Afin de développer des traitements antidiabétiques culturellement adaptés, notre équipe a effectué une enquête ethnobotanique qui a identifié 17 plantes provenant de la pharmacopée traditionnelle des CEI. À partir des études de criblage effectuées *in vitro*, deux plantes parmi les 17 ont attiré notre attention. *Populus balsamifera* L. (Salicaceae) pour ses propriétés anti-obésité et *Larix laricina* K. Koch (Pinaceae) pour ses propriétés antidiabétiques. *P. balsamifera* et son composé actif salicortin ont inhibé l'accumulation de triglycérides durant l'adipogénèse dans les adipocytes 3T3-L1. *L. laricina* a augmenté le transport de glucose et l'activation de l'AMPK dans les cellules musculaires C2C12, l'adipogénèse dans les 3T3-L1 et a démontré un fort potentiel découpleur (propriété anti-obésité).

Les objectifs de cette thèse sont d'évaluer les potentiels anti-obésité et antidiabétique et d'élucider les mécanismes d'action de *P. balsamifera*, salicortin, et *L. laricina* chez la souris C57BL/6 rendue obèse par une diète riche en gras (HFD). Les souris ont été soumises pendant huit (étude préventive) ou seize semaines (étude traitement) à une HFD, ou à une HFD dans laquelle *P. balsamifera*, salicortin, ou *L. laricina* a été incorporé soit dès le départ (prévention), ou dans les 8 dernières des 16 semaines d'administration de HFD (traitement).

Les résultats démontrent que *P. balsamifera* (dans les deux études) et salicortin (évalué dans l'étude traitement) diminuent: le poids corporel, le gras rétropéritonéal, la sévérité de la stéatose et l'accumulation de triglycérides hépatique (ERK impliqué), les niveaux de glycémie et d'insuline, et le ratio leptine/adiponectine. Dans les deux études, *P. balsamifera* a significativement réduit la consommation de nourriture mais cet effet coupe-faim nécessite d'être approfondi. Dans l'étude préventive, *P. balsamifera* a augmenté la dépense énergétique (hausse de la température à la surface de la peau et de l'activation de la protéine découplante-1; UCP-1). Les voies de signalisation activées par *P. balsamifera* et par salicortin (de façon plus modeste) sont impliquées dans: la production de glucose hépatique (Akt), l'expression de Glut4 dans le muscle squelettique, la captation du glucose et du métabolisme des lipides (Akt dans le tissu adipeux), la différenciation des adipocytes (ERK et PPARγ), l'inflammation dans le foie (IKKαβ), et l'oxydation des acides gras dans le muscle, le foie, ou le tissu adipeux (PPARα et CPT-1). D'autre part, *L. laricina* a également diminué les niveaux de glycémie et d'insuline, le ratio leptine/adiponectine, le gras rétropéritonéal et le poids corporel. Ces effets ont été observés en conjonction avec une augmentation de la dépense énergétique: hausse de température à la surface de la peau (prévention) et amélioration de la fonction mitochondriale et de la synthèse d'ATP (traitement).

En conclusion, l'utilisation de *P. balsamifera*, salicortin et *L. laricina* comme des traitements alternatifs et culturellement adaptés aux CEI représente une contribution importante dans la prévention et le traitement de l'obésité et du diabète.

Mots-clés : anti-obésité, antidiabétique, adipokines, souris C57BL/6, *Populus balsamifera* L. (Salicaceae), *Larix laricina* K. Koch (Pinaceae), médecine traditionnelle des autochtones, température, respiration mitochondriale, Akt , p44/42 MAPk, PPARα, IKKαβ.

Abstract

The prevalence of obesity, insulin resistance, and the metabolic syndrome is increasing among the Cree of Eeyou Istchee (CEI - Northern Quebec). Non-traditional diet and sedentary lifestyle along with cultural disconnect of modern type 2 diabetes (T2D) therapies are involved. In order to establish culturally adapted antidiabetic treatments, our research team conducted an ethnobotanical survey, where 17 plants were identified from the CEI traditional pharmacopoeia. Based on data obtained from *in vitro* screening studies, two plant species out of 17 were of particular interest for their properties as antiobesity, namely *Populus balsamifera* L. (Salicaceae), and antidiabetic agents, namely *Larix laricina* K. Koch (Pinaceae). *P. balsamifera* and its active salicortin inhibited triglyceride accumulation during adipogenesis in 3T3-L1 adipocytes. *L. laricina* increased glucose uptake and AMPK activation in C2C12 myotubes, adipogenesis in the 3T3-L1 adipocyte cell line, and was observed as one of the strongest uncouplers, severely disrupting mitochondrial function (increasing fuel consumption/metabolic rate; antiobesity property).

The purpose of this PhD thesis is to evaluate the antiobesity and antidiabetic potential of *P. balsamifera*, salicortin, and *L. laricina*, in an *in vivo* model of diet-induced obese (DIO) C57BL/6 mice, as well as to investigate their possible mechanisms of action. Mice were subjected for eight (prevention study) or sixteen weeks (treatment study) to a high fat diet (HFD), or HFD to which *P. balsamifera*, salicortin, or L. *laricina* were incorporated either at onset (prevention), or in the last 8 of the 16 weeks of administration of the HFD (treatment). The results showed that *P. balsamifera* (in either study) and salicortin (incorporated in HFD only in treatment study) decreased the weight of whole

body, retroperitoneal fat pad, reduced the severity of hepatic macrovesicular steatosis and triglyceride accumulation (ERK pathway implicated). They also decreased glycemia and improved insulin sensitivity by diminishing insulin levels, and altering adipokine secretion whereby reducing the leptin/adiponectin ratio. In both studies, *P. balsamifera* significantly reduced food intake. This appetite-reducing effect needs to be investigated further. In the prevention study this was accompanied by an increase in energy expenditure (increase in skin temperature and tends to increase expression of uncoupling protein-1; UCP-1). The signaling pathways activated by *P. balsamifera* and slightly by salicortin are implicated in either controlling hepatic glucose output (Akt), skeletal muscle Glut4 expression, glucose uptake and lipid metabolism in adipose tissue (Akt), adipocyte differentiation (ERK pathway and PPARγ), decreasing the hepatic inflammatory state (IKKαβ), and increasing muscular, hepatic, or adipose tissue fatty acid oxidation (PPARα, CPT-1). As for *L. laricina*, it effectively decreased glycemia levels, insulin levels and the leptin/adiponectin ratio, improved insulin sensitivity and slightly decreased abdominal fat pad and body weights. This occurred in conjunction with increased energy expenditure as demonstrated by elevated skin temperature in the prevention study, and tendency to improve mitochondrial function and ATP synthesis in the treatment protocol.

In conclusion, these results represent a major contribution, identifying *P. balsamifera*, salicortin, and *L. laricina*, as promising alternative, and culturally adapted therapies for the prevention and treatment care of obesity and diabetes among the CEI.

Keywords : antiobesity, antidiabetic, adipokines, C57BL/6 mice, *Populus balsamifera* L. (Salicaceae), *Larix laricina* K. Koch (Pinaceae), aboriginal traditional medicine, skin temperature, mitochondrial respiration, Akt , p44/42 MAPK, PPARα, IKKαβ.

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List of abbreviations

AA: amino acids

ABCA1: ATP-binding cassette transporter

ACC: acetyl-CoA carboxylase

ADP: adenosine diphosphate

AGEP: advanced glycation end product formation

AgRP: agouti-related protein

AICAR: 5-amino-1-β-D-ribufuranosyl-imidazole-4-carboxamide

AMP: adenosine monophosphate

AMPk: adenosine monophosphate-activated protein kinase

AMPKK: AMPK kinase

ANGPTL: angiopoietin related protein

ANOVA: one-way analysis of variance

aP2/FABP4: adipocyte protein 2 or fatty acid binding protein 4

APS: adaptor protein

a-MSH: a-melanocyte stimulating hormone

ATP: adenosine triphosphate

AUC: area under the curve

AUC_T: total area under the curve

AUC_{F1}: area under the curve between weeks 0 to 4 of plant extract administration

AUC_{F2}: area under the curve between weeks 4 to 8 of plant extract administration

BAT: brown adipose tissue

BCAA: branched-chain amino acids

BMI: body mass index

BP: balsam poplar

CAC: citric acid cycle

CAP: Cbl-associated protein

CART: cocaine and amphetamine regulated transcript

CB1: cannabinoid receptor

CBS: cystathionine beta synthase domains

CCBW: cumulative change in body weight

CCEI: cumulative change in energy intake

C/EBP: CCAAT-enhancer binding proteins

CEI: Cree of Eeyou Istchee

ChREBP: carbohydrate responsive element-binding protein

CIP4/2: Cdc42-interacting protein 4/2

CO_2: carbon dioxide

CPT-1: carnitine palmitoyltransferase-I

CREB: cAMP-response element binding protein

CYP: cytochrome P450

CXCL5: C-X-C motif chemokine 5

DAG: diacylglycerol

DHAP: dihyroxyacetonephosphate

DIO: diet-induce obese

DPP-4: dipeptidyl peptidase-4

EIF4E: eukaryotic initiation factor 4E-binding protein 1

EIF4EBP: eukaryotic translation initiation factor 4E binding protein

ERK: extracellular signal-regulated kinase

ER: endoplasmic reticulum

$FADH_2$: flavin adenine dinucleotide plus 2 hydrogens (hydroquinone form)

FAS: fatty acid synthase

FATP: fatty acid transport protein

FFA: free fatty acids

FOXO-1: forkhead box protein O1

GA3P: glyceraldehyde-3-phosphate

GABA: gamma-aminobutyric acid

GAD: glutamic acid decarboxylase

G6P: glucose-6-phosphate

GEF: Glut4-enhancer factor

GIP: glucose-dependent insulinotropic peptide

GLP-1: glucagon like peptide-1

Glut: glucose transporter

Glut4: glucose transporter 4

Grb2: growth factor binding protein 2

GS: glycogen synthase

GSK-3: glycogen synthase kinase 3

HbA1C: glycated hemoglobin

HDL: high-density lipoprotein

HFD: high-fat diet

HIF: hypoxia inducible factor

HLA: human leukocyte antigen

HMG-CoA: beta-hydroxy-beta-methylglutaryl-CoA

HMGR: 3-hydroxy-methyl-glutaryl-CoA-reductase

HMIT: H+/myoinositol transporter

HNF: hepatocyte nuclear factor

HSL: hormone-sensitive lipase

IAPP: or amylin/ islet amyloid polypeptide

IDDM: insulin-dependent diabetes mellitus

IDL: intermediate density lipoprotein

IFG: impaired fasting glucose

IGT: impaired glucose tolerance

IKK: IkB kinase

IL-: interleukin

IP3: inositol 1,4,5-triphosphate

IPF-1: insulin promoter factor 1

IRS: insulin receptor substrate

JAK/STAT: Janus kinase/signal transducer and activator of transcription

JNK: c-Jun-N-terminal kinase

KATP: ATP-sensitive potassium channels

LADA: latent autoimmune diabetes of adults

LCFA: long-chain fatty acids

LDL: low density lipoprotein

L. laricina: *Larix laricina* K. Koch (Pinaceae) or tamarack

LKB1/MO25/STRAD: liver kinase B1

LPC: lysophosphatidylcholine

LPL: lipoprotein lipase

MACP: mitochondrial anion carrier proteins

MAPk: mitogen-activated protein kinase

MARCKS: myristoylated alanine rich PKC substrate

MCP: monocyte chemotactic protein

MEF2: myocyte enhancer factor-2

MEK or MAPKK: Map kinase kinase

METAP2: methionine aminopeptidase

MIF: migration inhibitory factor

MIP: macrophage inflammatory protein

MMP: matrix metalloproteinases

MODY: maturity onset diabetes of the young

mTOR: mammalian target rapamycin

NADH: nicotinamide adenine dinucleotide plus hydrogen

NADPH: nicotinamide adenine dinucleotide phosphate

NAFLD: non-alcoholic fatty liver disease

NAMPT: nicotinamide phosphoribosyltransferase

NASH: non-alcoholic steatohepatitis

NF-κB: nuclear factor kappa light chain enhancer of activated B cells

NIDDM: non-insulin dependent diabetes mellitus

NPY: neuropeptide Y

OAA: oxaloacetate

OGTT: oral glucose tolerance test

PAF : platelet-activating factor

PAI-1: plasminogen activator inhibitor-1

P. balsamifera: *Populus balsamifera* L. (Salicaceae) or balsam poplar

PC1 or PC2: prohormone convertase 1 or 2

PDK-1: protein kinase 3-phosphoinositide dependent protein kinase-1

PEP: phosphoenolpyruvate

PEPCK: phosphoenolpyruvate carboxykinase

PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PH: pleckstrin homology domain

PIP2: phosphatidyl inositol 4,5-biphosphate

PI3K: phosphoinositol 3 kinase

PI(3,4,5)P3: phosphatidylinositol

PI(4,5)P2: phosphatidylinositol (4,5) biphosphate

PKA: cAMP-dependent protein kinase A

PKC: protein kinase C

POMC: proopiomelanocortin

PPAR: peroxisome proliferator-activated receptor

PP1: phosphoprotein phosphatase-1

PPRE: peroxisome proliferator response elements

PTEN: 3-phosphoinositide phosphatase

PTP: protein tyrosine phosphatase

RBP4: retinol binding protein 4

RER: rough endoplasmic reticulum

RIP140: or NRIP1, nuclear receptor-interacting protein 1

RNAi: ribonucleic interference

ROS: reactive oxygen species

RXR: retinoid x receptor

SCD-1: stearoyl-CoA desaturase-1

SD: standard diet or Chow

Shc: src homologous and collagen protein

SGLT: sodium-dependent glucose co-transporter

SHARP: SMRT and histone deacetylase associated repressor

SHP: small heterodimer partner

SIRT-1: sirtuin-1

SMRT: silencing mediator for retinoid or thyroid hormone receptors

SNAP25: synaptosomal-associated protein 25

SNARE: soluble NSF attachment protein receptor

SOCS: suppressor of cytokine signaling

SoHo: sorbin homology family

SOS: son of sevenless

SOX: Sry-related HMG box

SRB1: scavenger receptor B1

SREBP-1c: sterol regulatory element binding protein

SSRIs: selective serotonin reuptake inhibitors

SUR: sulfonylurea receptor

TG: triglycerides

T1D: type 1 diabetes

T2D: type 2 diabetes

TNF-α : tumor necrosis factor alpha

TSC : tuberus sclerosis complex

Tzds : thiazolidinediones

UCP-1: uncoupling protein-1

UTP: uridine triphosphate

VAMP: vesicle-associated membrane protein

VEGF: vascular endothelial growth factor

VLDL: very-low density lipoprotein

WAT: white adipose tissue

WHO: World Health Organization

To my wonderful parents and
to my amazing husband

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growing epidemic of obesity and diabetes, and the traditional medicines that are available to overcome them.

Last but not least, I would like to thank my parents and my husband for all their love and support, for always believing in me and encouraging me every step of the way. I love you all more than words can express, and I consider myself extremely blessed for being surrounded by truly amazing individuals. You reinforced in me the importance of family, respect, hard work, discipline, perseverance, teamwork, and how important it is to stay humble in life. I dedicate this thesis to them.

Chapter 1: Introduction

1. Introduction

Over the last few decades, obesity and type 2 diabetes (T2D) have been increasing at an alarming rate, becoming pandemic health problems. The prevalence of obesity has more than tripled and currently affects more than 350 million people worldwide (WHO 2007a). As for T2D, it is projected that by 2030, there will be 366 million people affected on a worldwide scale, among which 3 million Canadians (WHO 2004a; Wild et al. 2004; WHO 2007b).

Both these conditions are widespread among Canadian aboriginals, with obesity being present among 36% of the population as compared to 25% in non-aboriginal Canadians (PHAC 2009; PHAC 2011b), and the incidence of T2D being three to five times higher than the rest of the Canadian population (Brassard et al. 1993; Légaré 2004a; Légaré 2004b; Kuzmina 2004). The incidence and prevalence rates of these diseases, as well as the complications that arise from them, are consistently higher in aboriginal women than men (Kuzmina 2009).

The Cree of Eeyou Istchee (CEI) of the eastern James Bay area of Quebec, are a subpopulation (13 500) of the greater Cree Nation of Canada (72 000). They are spread out into 9 communities across northern Quebec (Brassard et al. 1993). The CEI are particularly affected by T2D with an age-adjusted prevalence of 29% in 2009 among adults (>20 years) (Dannenbaum 2008; Kuzmina 2009). The reasons behind these alarming incidences are their sedentary lifestyle, non-traditional diet, their difficulty of compliance to modern type 2 diabetes treatments, and genetic predisposition (Boston 1997; Young 2000; Hegele 2001). The Cree women also suffer from gestational diabetes with a rate that is twice as high as the rest of the North American population (Rodrigues 1999). This may be explained by excessive weight gain observed in young Cree mothers during pregnancy, and also by more aggressive screening for T2D during pregnancy (Kuzmina 2009). It is noteworthy that women with pre-existing T2D or gestational

diabetes have a much higher risk of developing maternal and neonatal complications (Kuzmina 2009). Their children also have an increased predisposition to developing obesity and diabetes at a young age (Kuzmina 2009).

In Canada, the increase in prevalence of obesity and T2D will create a major clinical and economical burden in the future. The estimated cost of obesity is $4.3 billion and this figure is said to be greatly underestimated since it does not include the cost of people who are overweight but not obese, and includes only adult obesity (PHAC 2009; PHAC 2011b). Concerning T2D, its economic burden has nearly doubled since 2000, reaching approximately $12.2 billion in 2010, and it is expected to rise by another $4.7 billion by 2020 (CDA 2009). These numbers highlight the need to implement effective programs targeting the prevention of obesity and T2D, such as reinforcing existing clinical prevention practices involving, for instance, dietary intervention, reinforcing educational programs, and targeting physical inactivity (Young 2000; Kuzmina 2009).

Despite the availability of conventional T2D treatments and intervention programs, western medicine is failing to improve the health of aboriginal communities such as the CEI (NWAC 2007), and this may be due to cultural inappropriateness. Wherefrom emanates the need to find effective prevention and treatment options, that such populations can identify to in terms of culture and lifestyle (Boston 1997; Gray-Donald 2000; Young 2000; Légaré 2004b; Légaré 2004a).

For centuries prior to European colonization, the aboriginal population relied on traditional healing, a knowledge that was verbally taught and passed on from generation to generation. Although with time some of this knowledge was lost, anthropologists nevertheless were able to document the use and administration of more than 500 plant species belonging to aboriginal medicine (Turner 1985; Blouin 2003). The plants were administered either alone or in

combination, and varied from herbal teas, to preparations to be chewed and swallowed, to poultices, or to inhaled vapors (Turner 1985).

In order to respond to their primary health care needs, the CEI are now turning to their Elders and healers for treatments stemming from their vast traditional pharmacopoeia, reinforcing the importance to make these treatments more accessible and acceptable by proving their healing properties. Due to the high prevalence of T2D present among the CEI, our research team in collaboration with six CEI communities: Mistissini, Whapmagoostui, Nemaska, Waskaganish, Wemindji, and Oujé-Bougoumou conducted ethnobotanical surveys, based on 15 symptoms associated with T2D (Leduc et al. 2006; Fraser et al. 2007)(Figure 1).

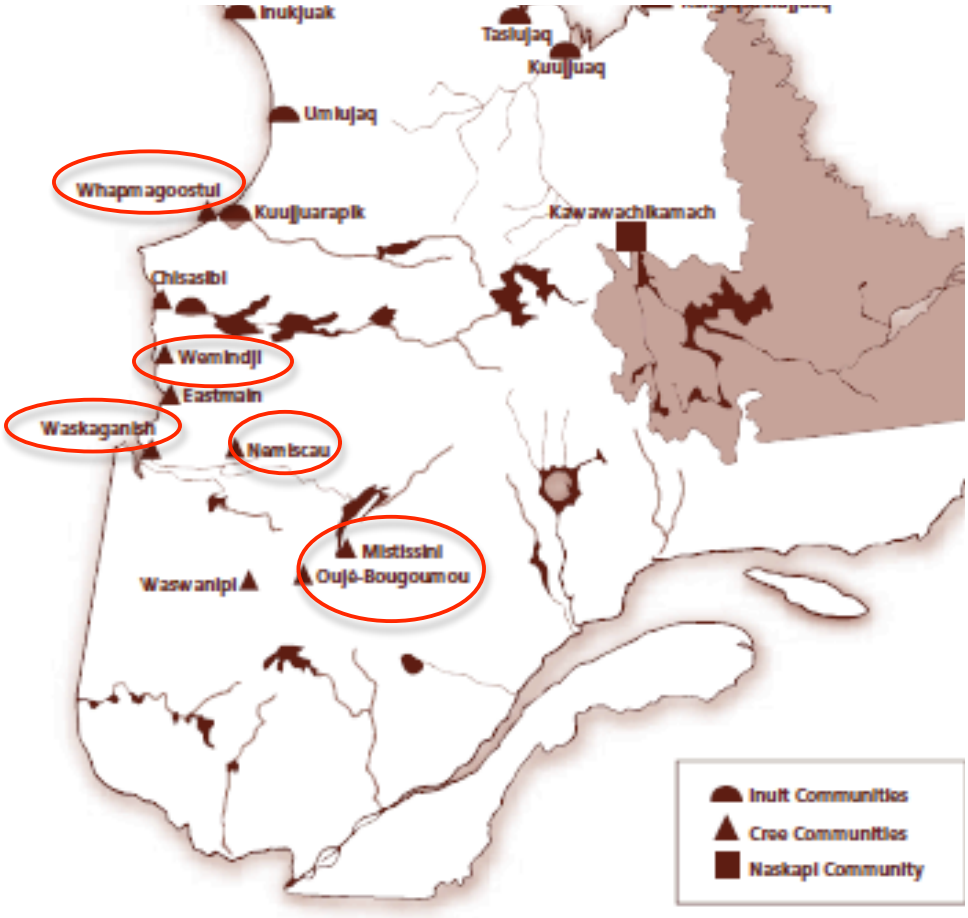


Figure 1: Map of Quebec showing the Cree communities that participated in the ethnobotanical study (adapted from: (AINC 2009)).

Over 30 plant species were identified. They were then classified and ranked by frequency of citation, number of symptoms, and the quality of symptoms (Leduc et al. 2006). A subset of 17 of the more significant plants were then screened *in vitro* for their antidiabetic activity, revealing that roughly half of them possessed significant and varied antidiabetic activities (Spoor et al. 2006; Harbilas et al. 2009). These studies also suggested, based on the *in vitro* mechanisms of action, that *Populus balsamifera* and its identified active salicortin (Harbilas et al. 2009; Martineau 2010a; Martineau 2010b), along with *Larix laricina* (Spoor et al. 2006; Martineau et al. 2010) possess antiobesity and antidiabetic potential.

The purpose of this PhD thesis is to evaluate the antiobesity and antidiabetic potential of *Populus balsamifera*, its active salicortin, as well as that of *Larix laricina* in an *in vivo* model of diet-induced obesity, as well as to investigate their possible mechanisms of action.

1.1 Energy homeostasis

Energy homeostasis is maintained when energy intake equates energy output. Food consumption provides the body with all of the necessary macronutrients, such as carbohydrates, lipids, and proteins, and with most micronutrients such as minerals and vitamins (Woods 1998). Carbohydrates and lipids are the main source of energy/fuel for the body (Da Poian 2010). They are metabolized through anabolic (gluconeogenesis, glycogenesis, lipogenesis) and catabolic processes (glycolysis, glycogenolysis, lipolysis) (Dean 2004). Lipids are considered a compact energy store (Ophardt 2003b), whereas proteins are mainly used as tissue building and maintenance, as well as building blocks for hormones and enzymes that regulate metabolism, support immune system, and other body functions (IOC 2010; Hermann). Proteins are used very

little for energy supply when caloric intake is sufficient (IOC 2010; Hermann). When metabolized, each one provides a different amount of food energy per gram of food consumed: lipids 9 kcal/g, carbohydrates 4 kcal/g, and proteins 4 kcal/g (Da Poian 2010; Insel 2011). Creating any positive imbalance between energy intake and output can eventually lead to obesity and T2D.

1.2 Glucose homeostasis

Glucose is metabolized through aerobic or anaerobic respiration yielding energy sources mainly in the form of adenosine triphosphate (ATP) (Widmaier 2004; Salway 2006). It is considered the key source of energy for the body, and especially for the brain (Fairclough and Houston 2004; Gailliot 2007; Gailliot and Baumeister 2007; Masicampo and Baumeister 2008). The body needs to maintain blood glucose within a narrow range, since either high (hyperglycemia) or low (hypoglycemia) levels of blood glucose lead to complications. The normal range of blood glucose is between 4 and 7 mmol/L fasting and below 11 mmol/L after having eaten. If a patient is consistently at a fasting blood glucose above 7 mmol/L, or above 11 mmol/L after a meal, they are considered hyperglycemic (ADA 2006). In the long run hyperglycemia leads to the development of insulin resistance and T2D, and to the many secondary complications arising from T2D such as retinopathy, nephropathy, and peripheral nerve damage, just to name a few (Kumar 2003). On the other hand, if blood glucose levels are consistently below 4 mmol/L, they are considered hypoglycemic. Symptoms of hypoglycemia include lethargy, impaired mental functioning, irritability, shaking, weakness, loss of consciousness, and may even lead to brain damage (de Courten-Myers et al. 2000; NIDDK 2008a; NIDDK 2008b). Tight regulation between intestinal absorption of glucose, disposal into

peripheral tissues, and glucose production by the liver, as well as catabolic and anabolic hormones are involved in glucose homeostasis. Catabolic hormones such as glucagon, cortisol and catecholamine are involved in increasing blood glucose levels, whereas insulin (discussed in detail in section 1.4), an anabolic hormone, is involved in decreasing blood glucose. Glucagon is a pancreatic hormone released from alpha-cells of the islets of Langerhans in response to low blood glucose levels, or increased levels of catecholamines (epinephrine and norepinephrine). Its biological functions are antagonistic to those initiated by insulin. For example, insulin promotes glycolysis or glycogenesis, whereas glucagon once it binds to its receptors in the liver initiates gluconeogenic and glycogenolytic pathways, in order to promote glucose release into the bloodstream.

1.2.1 Glycolysis

This metabolic pathway consists in the conversion of glucose into pyruvate, in the process forming high-energy compounds such as ATP and nicotinamide adenine dinucleotide plus hydrogen (NADH) (Figure 2). It consists of two phases: a preparatory phase, where ATP is consumed and a pay-off phase where ATP is formed (Berg 2002; Nelson 2004). Three key enzymes regulate it: hexokinase or glucokinase, phosphofructokinase, and pyruvate kinase. Hexokinase converts glucose into glucose-6-phosphate (G6P). This step is the first step in glycolysis or glycogenesis. Glucokinase belongs to the family of hexokinases, and is found primarily in the liver, but also in the pancreas, gut and brain (Efanov et al. 2005). Glucokinase has a lower affinity to glucose as compared to other hexokinases, thus enabling it to respond to high concentrations of glucose present after a meal, and favoring glycogen formation (Voet 2004). At low glucose concentrations, the liver (low affinity glucokinases) does not compete with

other tissues, such as the brain (high affinity hexokinases) for critically dependent glucose supply (Voet 2004). When there is ample glucose available, after it has been converted to glycogen, the remainder gets converted into triglycerides (TG), and is exported and stored in adipose tissue (Voet 2004). G6P is isomerized to fructose-6-phosphate (F6P) by phosphoglucose isomerase, whereas phosphofructokinase converts F6P into fructose-1,6-biphosphate, which eventually leads to the formation of phosphoenolpyruvate (PEP)(Voet 2004). Pyruvate kinase then converts PEP into pyruvate (Voet 2004).

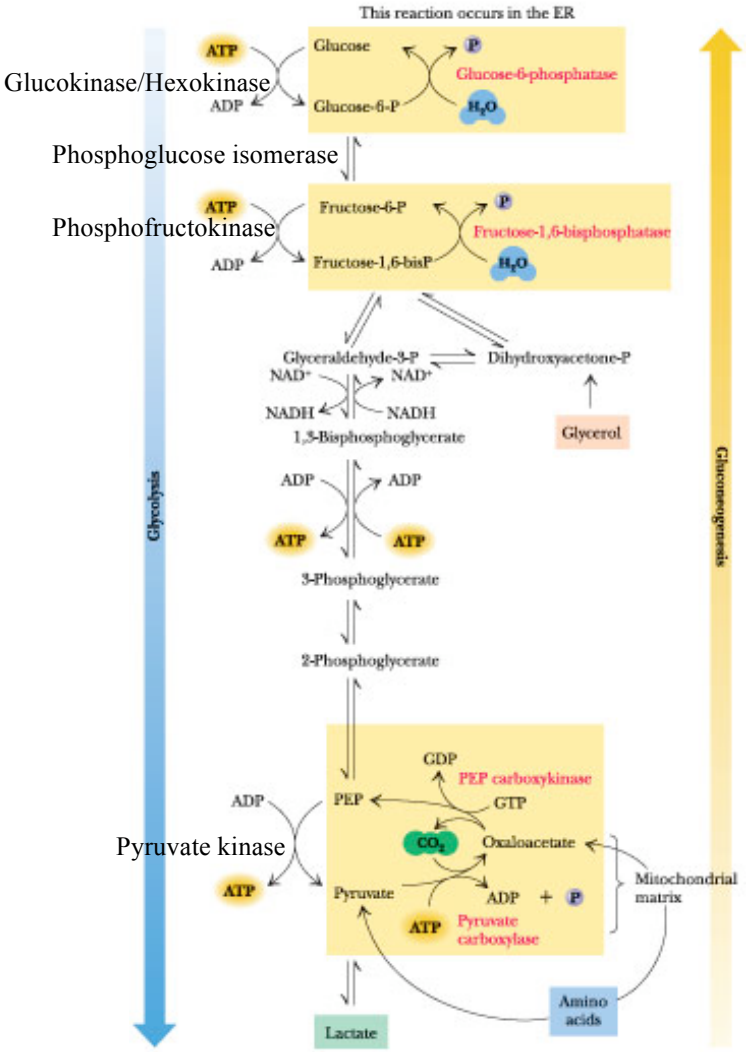


Figure 2: Summary of the glycolytic pathway described in section 1.2.1, and of the gluconeogenesis pathway described in section 1.2.4 (adapted from:(Garrett 2010)).

Once pyruvate is formed, and if oxygen is present, the process continues into aerobic respiration, where pyruvate is further metabolized to acetyl-CoA (common breakdown product of carbohydrate, fatty acid, and amino acid) and CO_2 within the mitochondria in a process called oxidative decarboxylation, facilitated by pyruvate dehydrogenase (Voet 2004) (Figure 3).

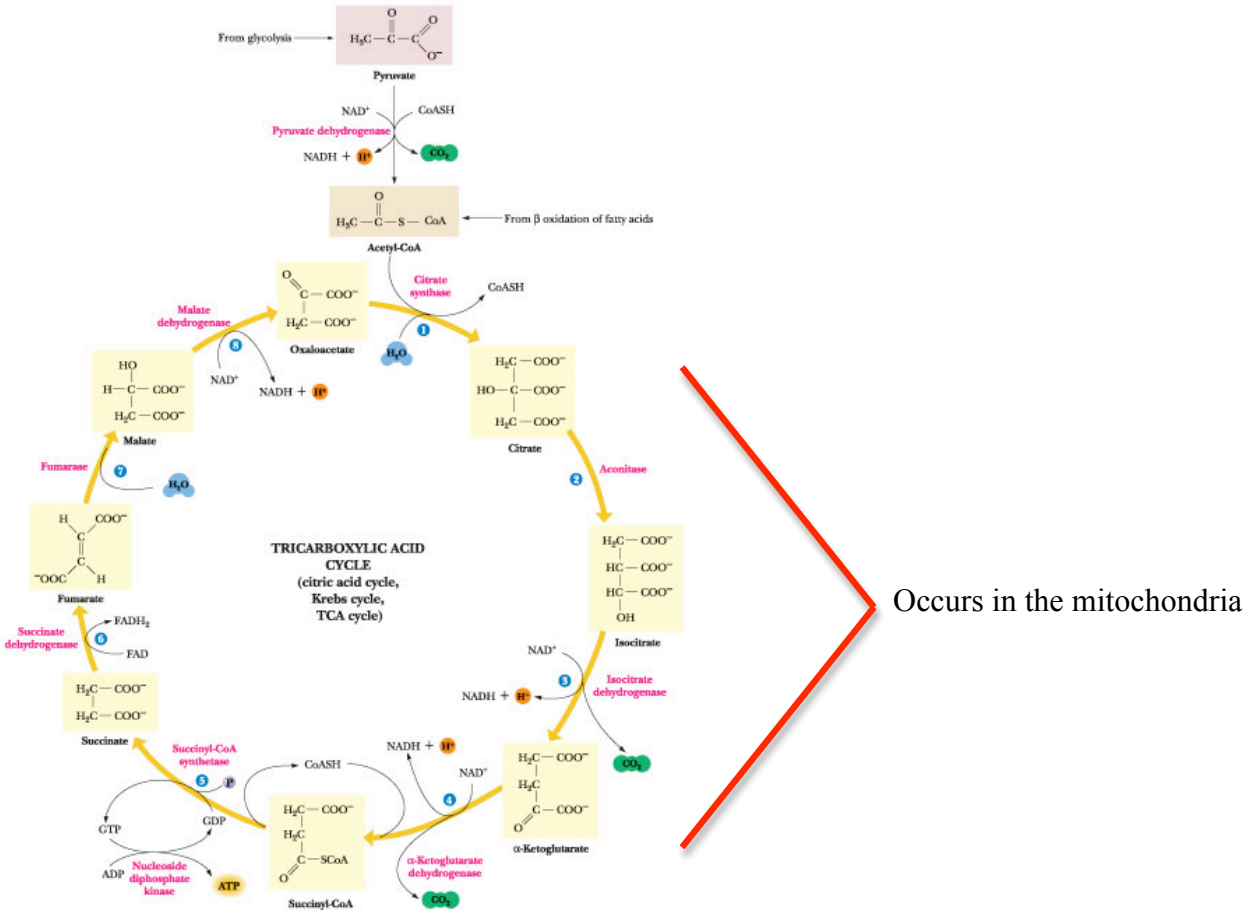


Figure 3: Pyruvate metabolized to acetyl CoA, which then enters CAC (adapted from:(Garrett 2010)).

Acetyl-CoA then enters the citric acid cycle (CAC), also known as the Krebs cycle, and gets fully oxidized into CO_2, water, NADH and $FADH_2$. These are vital products of the CAC that are reoxidized in the presence of oxygen through the coupling of electron transport chain with

oxidative phosphorylation, completing the breakdown process of metabolic fuel and driving ATP synthesis (Voet 2004; Miesfeld 2008). The final step is termed oxidative phosphorylation, where NADH and $FADH_2$ are oxidized to NAD+ and FAD by the electron transport chain, oxygen being the final electron acceptor. This creates a hydrogen ion gradient across the inner mitochondrial membrane, which is used to produce a large amount of ATP through the ATP synthase proton pump (Figure 4).

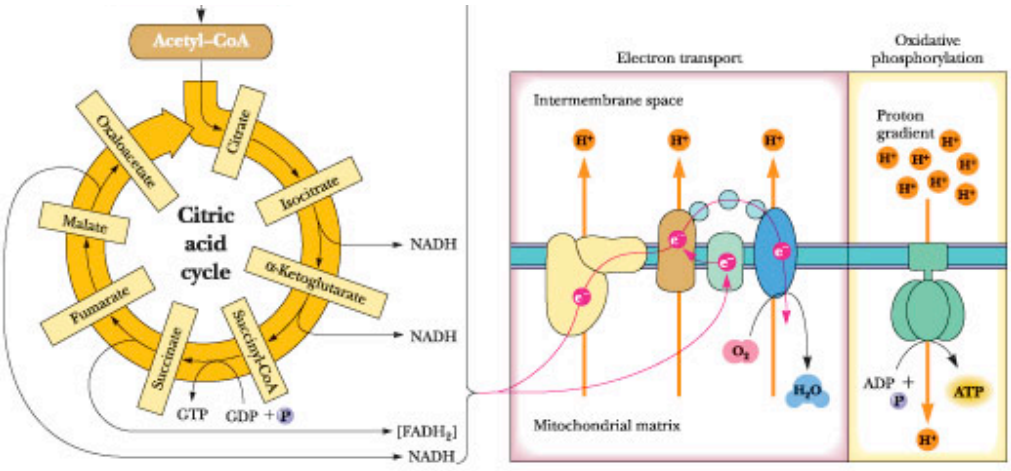


Figure 4: CAC products oxidized through coupling of electron transport chain with oxidative phosphorylation (in the presence of oxygen) driving ATP synthesis (adapted from:(Garrett 2010)).

If oxygen is not present, or when ATP demands are too high, such as during intense muscle activity when ATP is needed in a short time frame, then anaerobic glycolysis takes place. This leads to lactate formation, where pyruvate is converted to lactate, by lactate dehydrogenase. The Cori cycle shifts the metabolic burden from the muscle to the liver: lactate produced in muscle is taken up by the liver, initiating the Cori cycle, and is converted to pyruvate, then into glucose, which goes back into the bloodstream and returns to the muscle, as fuel for glycolysis, or to

replenish glycogen stores, if activity has decreased (Ophardt 2003a; Nelson 2004; Voet 2004)(Figure 5).

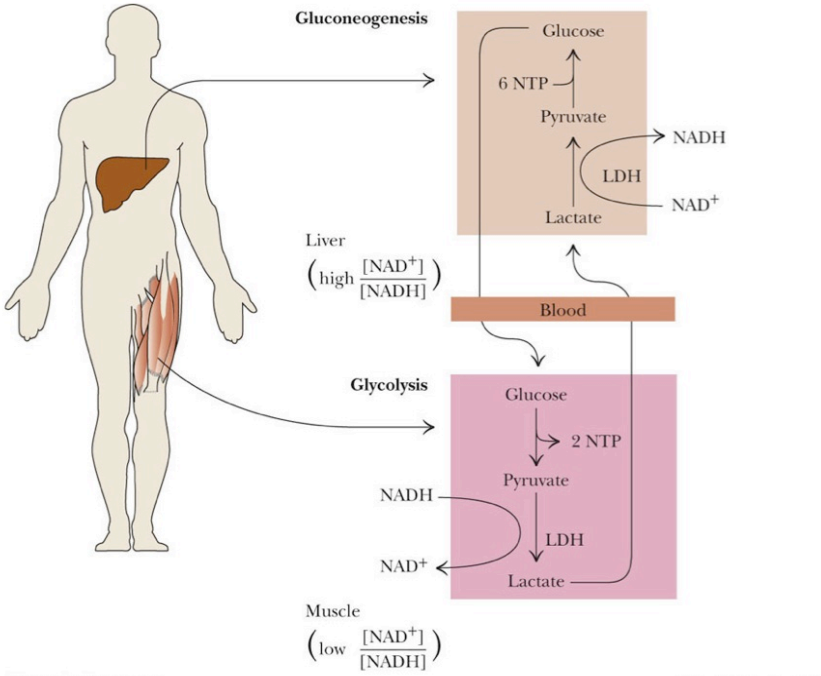


Figure 5: The Cori cycle occurring in situations such as intense exercise, where oxygen concentrations are low and anaerobic glycolysis takes place, producing lactate (adapted from:(Garrett 2010)).

1.2.2 Glycogenesis

Glucose is metabolized to glucose-6-phosphate, and then converted by phosphoglucomutase to glucose-1-phosphate. There are then three important enzymes involved in catalyzing the glycogen synthesis pathway: UDP-glucose pyrophosphorylase, glycogen synthase, and glycogen branching enzyme. UDP-glucose pyrophosphorylase catalyzes the reaction of glucose-1-phosphate with UTP (uridine triphosphate) forming UDP-glucose. Glycogen synthase (GS) then catalyzes the reaction between UDP-glucose and an already existing glucan chain

(initiated by self-catalyzed attachment of a glucose residue to glycogenin, forming a primer), elongating it in the process. Then follows the action of glycogen branching enzyme that transforms the glucan chains into glycogen (Voet 2004)(Figure 6).

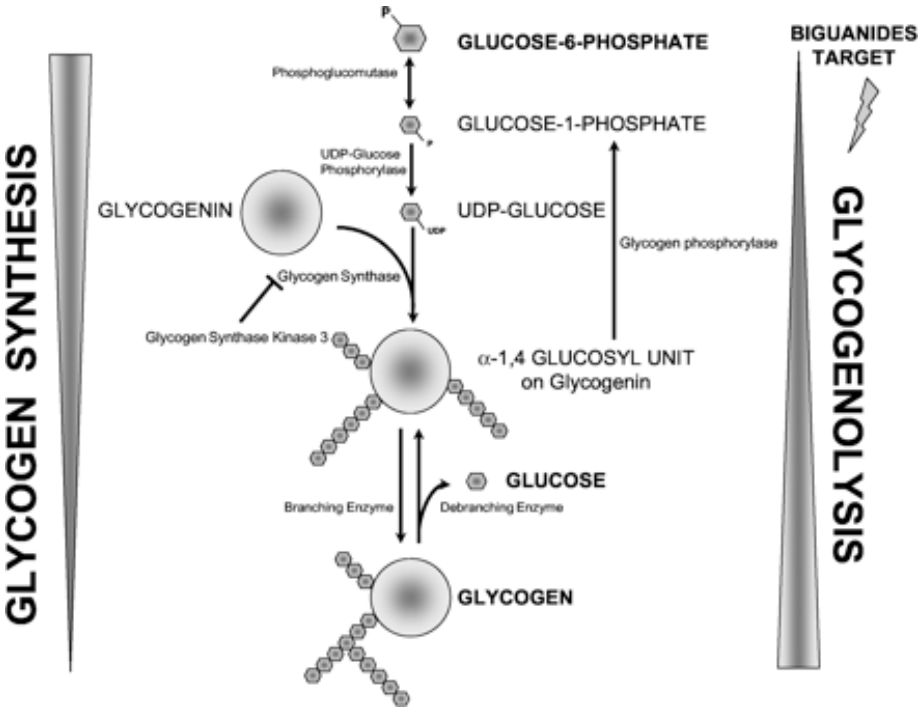


Figure 6: Summary diagram representing glycogenolysis versus glycogen synthesis (taken from:(Bouche et al. 2004)).

Glycogen synthase (GS) activity is modulated by phosphorylation (decrease in activity) and dephosphorylation, as well as by allosteric regulation by substrates such glucose-6-phosphate (Bouche et al. 2004). It can be phosphorylated at 9 different sites by protein kinases such as PKA (cAMP-dependent protein kinase A), calmodulin-dependent kinases, glycogen synthase kinase 3 (GSK-3), protein kinase C (PKC) and others (Bouche et al. 2004) (Figure 7).

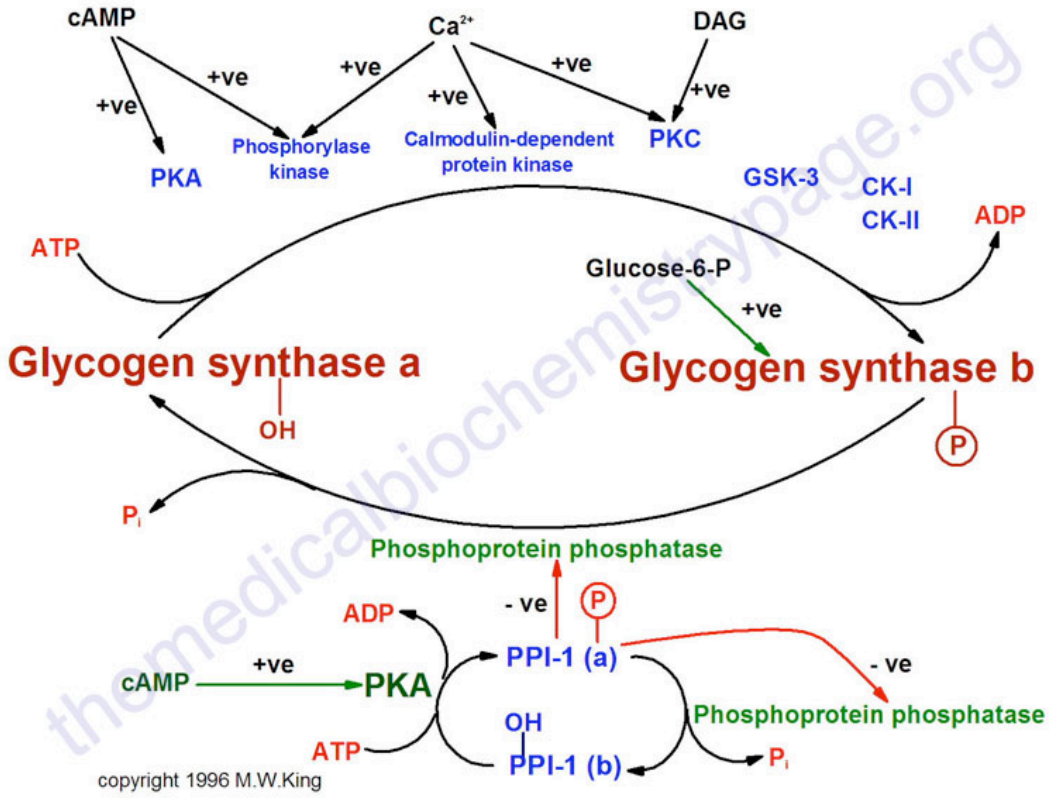


Figure 7: Examples of protein kinases regulating glycogen synthase activity (taken from:(King 2012)).

Glycogen synthesis is stimulated by insulin, glucose and depleting glycogen levels, and downregulated by hormones such as glucagon and adrenaline, which promote glycogen breakdown. Insulin activates the PI3K/Akt pathway, which phosphorylates GSK-3 whereby inhibiting its protein kinase activity. This enhances GS and promotes glycogen synthesis in the presence of ample amount of glucose in the blood (Bouche et al. 2004). GSK-3 is a multifunctional serine/threonine kinase that is regulated, as mentioned, by insulin, as well as by PKC-mediated, or cAMP-dependent PKA phosphorylation. GSK-3 phosphorylates and inhibits glycogen synthase and the glycogen-associated form of phosphoprotein phosphatase-1 (PP1β) (Figure 8).

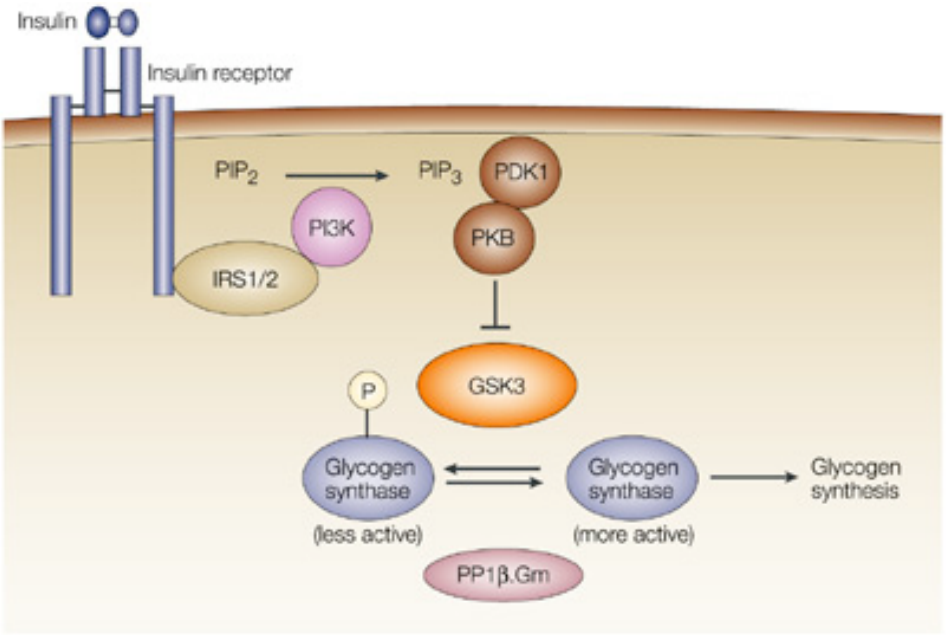


Figure 8: Insulin inhibits GSK-3 mediated phosphorylation and enhances PP1 mediated dephosphorylation of GS thereby promoting glycogen synthesis (taken from:(Cohen and Goedert 2004)).

PP1 is also implicated in the control of glycogen metabolism, by catalyzing the dephosphorylation of GS, rendering it active. PP1 also dephosphorylates glycogen phosphorylase, rendering it inactive. Glycogen phosphorylase is an enzyme involved in glycogen breakdown (Bouche et al. 2004). Concerning the AMPK pathway, it enhances catabolic pathways in order to increase ATP synthesis, and inhibits anabolic pathways, such as glycogen synthesis, thereby reducing ATP utilization. AMPK activates GSK-3, which phosphorylates and inactivates GS (Saltiel and Kahn 2001; King et al. 2006; Cammisotto et al. 2008)(Figure 9).

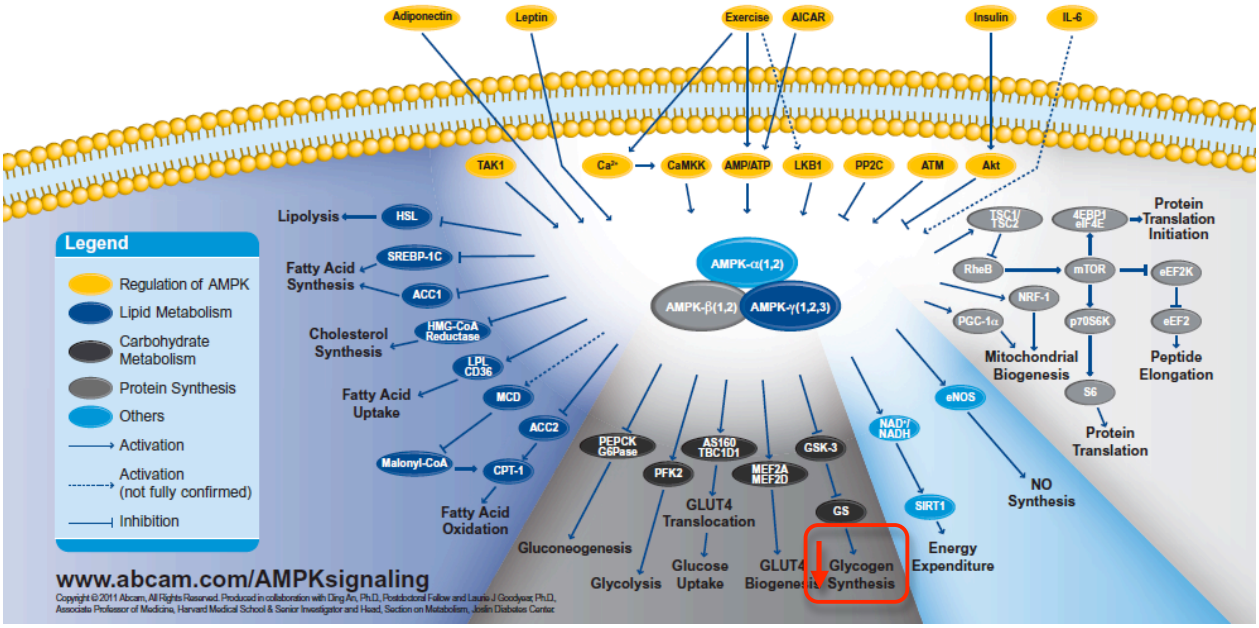


Figure 9: AMPK signaling pathway and its downstream effectors (taken from: www.abcam.com/AMPKsignaling).

1.2.3 Glycogenolysis

This is the process by which glycogen is converted to glucose. This occurs in the presence of hormones such as glucagon or epinephrine, and in situations of low blood glucose concentrations. It can take place in the muscle or the liver. The first step is the conversion from glycogen to glucose-1-phosphate by glycogen phosphorylase, which is regulated/activated by phosphorylation (Johnson 1992; Voet 2004). Glucose-1-phosphate is then converted to glucose-6-phosphate by phosphoglucomutase (Voet 2004) (see Figure 6).

At this stage, glucose-6-phosphate can either go through the process of glycolysis, or with the action of glucose-6-phosphatase release glucose into the blood for uptake by other tissues such as the muscle (Voet 2004). Glucose-6-phosphatase is regulated by transcription factors FOXO-1 and PGC-1α (Puigserver et al. 2003). It is to note that, in the muscle, there is very little presence of

glucose-6-phosphatase and therefore once glucose-6-phosphate is formed it is used in glycolysis (Berg 2002; Tischler 2008).

1.2.4 Gluconeogenesis

Gluconeogenesis is another pathway besides glycogenolysis that aims to maintain blood glucose levels (Voet 2004). This process occurs mainly in the liver but can also take place to a smaller extent in the kidney (Voet 2004). It is activated in situations of fasting or starvation, in situations where the person is on a low-carbohydrate diet, or is undergoing intense physical exercise. Many substrates can be used in the process of gluconeogenesis in the liver, such as lactate originating from the muscle (Voet 2004). Lactate dehydrogenase converts lactate to pyruvate in the Cori cycle (see Figure 5). Other non-carbohydrate precursors include pyruvate, citric acid cycle (CAC) intermediates, and amino acids formed during protein breakdown (Voet 2004). Amino acids either enter the cycle as pyruvate, or oxaloacetate (OAA) or enter the CAC directly (as CAC intermediates), forming OAA, which is then converted to phosphoenolpyruvate (PEP) by phosphoenolpyruvate carboxykinase (PEPCK) (Voet 2004)(Figure 10).

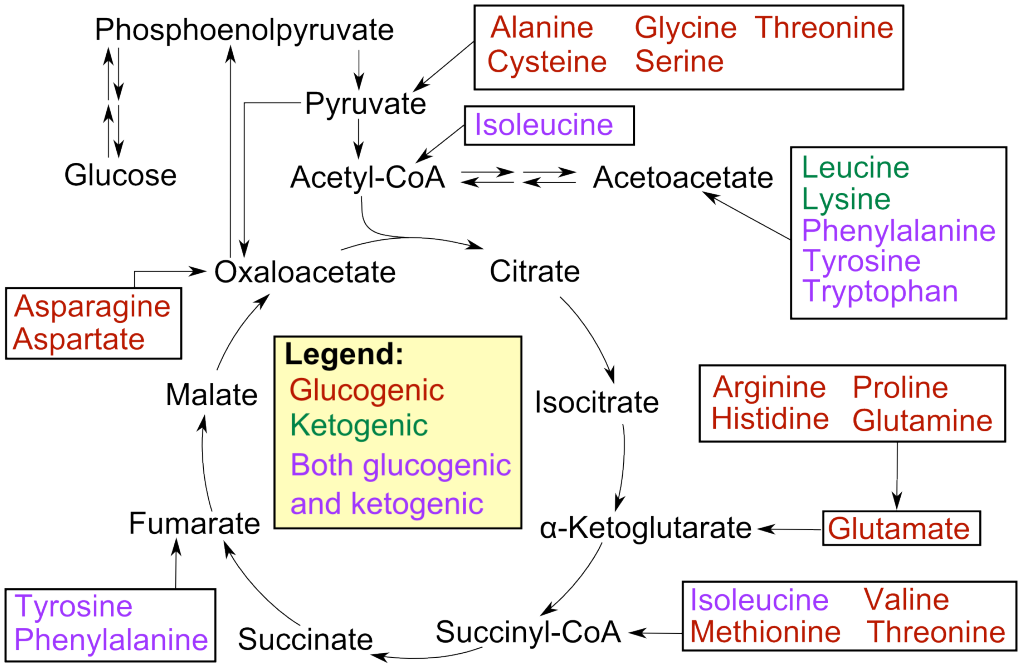


Figure 10: Amino acids used in the process of gluconeogenesis (Champe 2008).

PEP eventually leads to the formation of glucose-6-phosphate, which is transported to the endoplasmic reticulum (ER) by glucose-6-phosphate transport protein T1, and then converted to glucose by glucose-6-phosphatase (located in ER of liver, kidney, intestine, but not in muscle or adipose tissue) (Bhagavan 2002). Glucose is released from the ER into the cytoplasm. However, the exact mechanism by which it does so remains unknown. It was thought to occur through the presence of glucose transporter 7 (Glut7) present on the ER, however this hypothesis turned out to be an experimental artifact (Waddell et al. 1992; Burchell 1998). Theories currently under investigation include direct vesicular transport of glucose from the ER to the plasma membrane, where Glut2 then releases it into circulation (Fehr et al. 2005).

Glycerol can also be used to form glucose de novo by being converted to glycerol-3-phosphate by glycerol kinase, then to dihydroxyacetone phosphate by glycerol-3-phosphate dehydrogenase, to fructose-6-phosphate, glucose-6-phosphate and finally releasing glucose (Voet

2004) (summarized in Figure 2). As mentioned, PEPCK and glucose-6-phosphatase are regulated by FOXO-1 and PGC-1α (Puigserver et al. 2003). In the presence of insulin the PI3K/Akt pathway is activated leading to phosphorylation of FOXO-1, which is no longer able to bind PGC-1α, resulting in inhibition of gluconeogenesis by inhibiting enzymes such as PEPCK and glucose-6-phosphatase (Puigserver et al. 2003)(Figure 11).

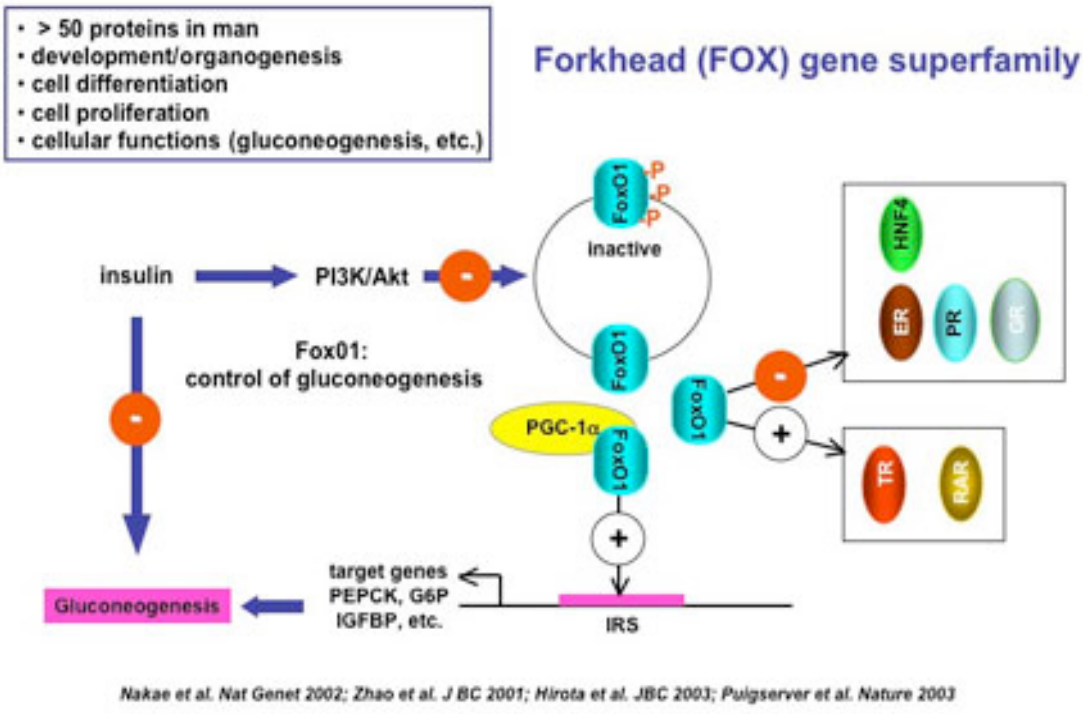


Figure 11: The effects of the insulin/PI3K/Akt pathway on FOXO-1 and gluconeogenesis enzymes (taken from:(Zhao et al. 2001; Nakae et al. 2002; Hirota et al. 2003; Puigserver et al. 2003)).

1.3 Glucose transporters

Glucose transporters allow the facilitated transport of glucose or related hexoses from the extracellular fluid into the cell (Zhao and Keating 2007) in order for it to be utilized in the production of cellular energy (glycolysis and ATP production) or as storage (glycogen or fatty

acids)(Roach 2002). There are two types of glucose transporters: facilitative glucose transporters (Glut), and sodium-dependent glucose co-transporters (SGLT).

1.3.1 Glut transporters

Glut transporters are composed of 12 membrane-spanning helices, where both the amino and carboxyl terminal are exposed to the cytoplasmic side of the plasma membrane (Olson and Pessin 1996; Wood and Trayhurn 2003; Thorens and Mueckler 2009; Augustin 2010). Once glucose binds to the transporter it induces a conformational change that allows the substrate to be transported into the cell and released into catabolic pathways, such as glycolysis or glycogenesis. There is an abundance of glucose transporters present, encoded by distinct genes, and their expression as well as regulation depends on tissue distribution. There have been 13 members of facilitated diffusion glucose transport (Glut) identified thus far, not including the 3 members belonging to the sodium-dependent glucose co-transporter (SGLT)(Zhao and Keating 2007)(Table 1).

Protein	Major isoform (aa)[1]	K_m[2] (mM)	Major sites of expression	Proposed function
Facilitative glucose transporters (GLUT)				
GLUT1	492	3-7	Ubiquitous distribution in tissues and culture cells	Basal glucose uptake; transport across blood tissue barriers
GLUT2	524	17	Liver, islets, kidney, small intestine	High-capacity low-affinity transport
GLUT3	496	1.4	Brain and nerves cells	Neuronal transport
GLUT4	509	6.6	Muscle, fat, heart	Insulin-regulated transport in muscle and fat
GLUT5	501		Intestine, kidney, testis	Transport of fructose
GLUT6	507	?[3]	Spleen, leukocytes, brain	
GLUT7	524	0.3	Small intestine, colon, testis	Transport of fructose
GLUT8	477	2	Testis, blastocyst, brain, muscle, adipocytes	Fuel supply of mature spermatozoa; Insulin-responsive transport in blastocyst
GLUT9	511/540	?	Liver, kidney	
GLUT10	541	0.3	Liver, pancreas	
GLUT11	496	?	Heart, muscle	Muscle-specific; fructose transporter
GLUT12	617	?	Heart, prostate, mammary gland	
HMIT	618/629	?	Brain	H^+/myo-inositol co-transporter
Na^+/glucose cotransporters (SGLT)				
SGLT1	664	0.2	Kidney, intestine	Glucose reabsorption in intestine and kidney
SGLT2	672	10	Kidney	Low affinity and high selectivity for glucose
SGLT3	660	2	Small intestine, skeletal muscle	Glucose activated Na^+ channel

[1]aa, amino acids. [2]Net influx for 2-Deoxyglucose or glucose; [3]?=unknown.

Table 1: Summary table of glucose transporters (Glut) and members of the sodium-dependent glucose co-transporter (SGLT)(taken from:(Zhao and Keating 2007)).

The glucose transporters are divided into 3 subclasses: class I, II, III depending on sequence or structure similarities. Class I contains Glut1, Glut2, Glut3, Glut4, class II with Glut5, Glut7, Glut9, Glut11, and class III with Glut6, Glut8, Glut10, Glut12, Glut13 also known as H+/myoinositol transporter (HMIT).

1.3.2 Insulin and non-insulin responsive regulation of Glut translocation

It is to note that among these only Glut4, 8 and 12 are responsive to insulin (Zhao and Keating 2007). Glut1 is ubiquitously distributed in all tissues. It is responsible for glucose uptake under basal conditions (Giorgino et al. 2000), and more modestly upon insulin stimulation (Pessin et al. 1999). The main insulin-responsive glucose transporter is Glut4, which is present in skeletal and cardiac muscle, as well as adipose tissue (James et al. 1989). In a resting state, Glut4 is stored in intracellular vesicles with less than 5% being present at the cell surface (Brewer et al. 2011). In the presence of insulin the PI3K/Akt or TC10 pathways are activated, which leads to increased translocation to the plasma membrane and activation of Glut4 (Funaki et al. 2004)(Figure 12).

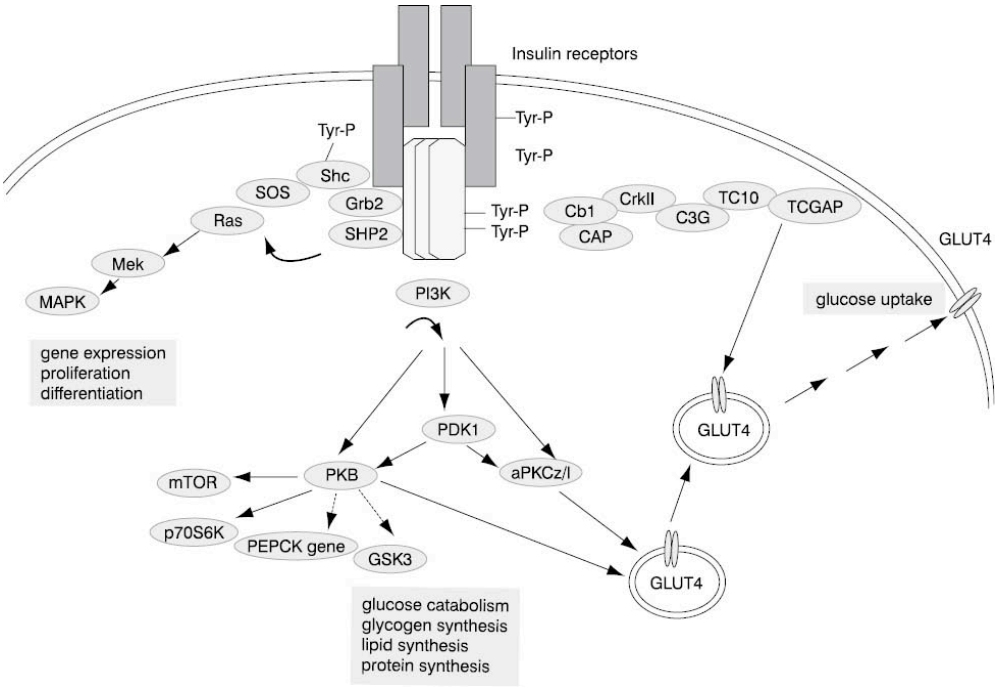


Figure 12: PI3K/Akt and TC10 mediated Glut4 translocation and glucose uptake (taken from:(Milnar 2006)).

On the other hand, activation of the AMPK pathway results in a non-insulin dependent translocation and activation of Glut4 and Glut1 (Barnes et al. 2002)(Figure 13). It is to note that in an insulin resistant state there is either a decreased ability of insulin signaling, which translates to decreased Glut4 translocation and activity, or even decreased Glut4 expression (i.e. decreased levels of Glut4 protein) (Reaven 1999; Funaki et al. 2004).

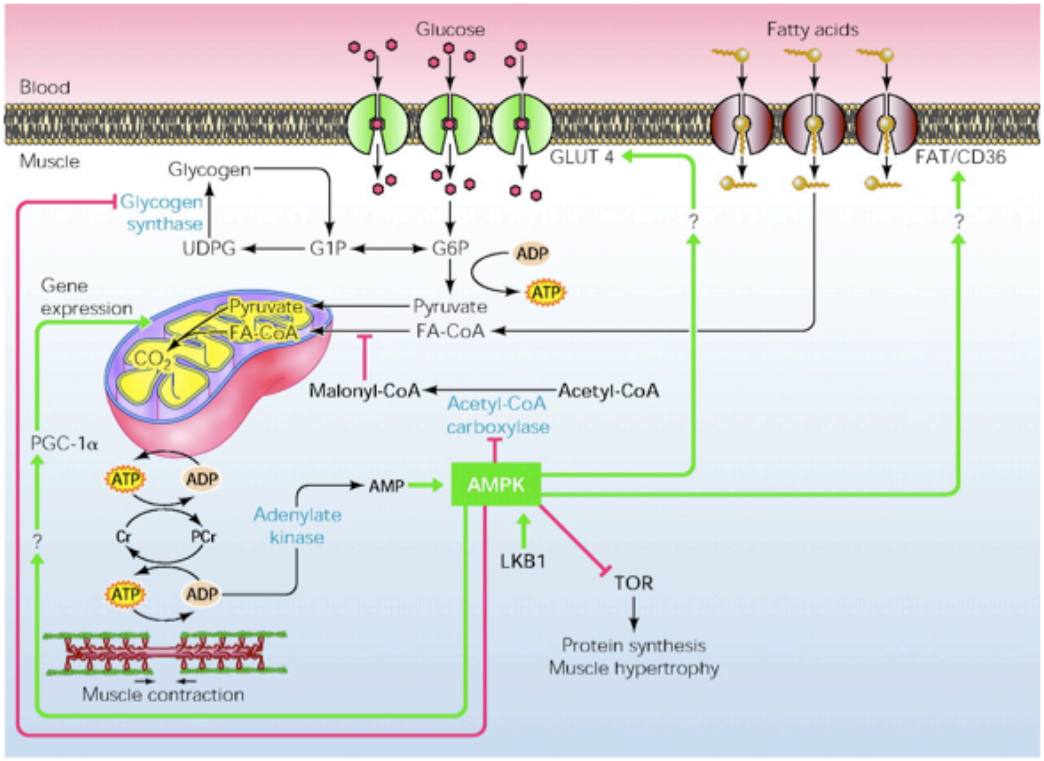


Figure 13: AMPK pathway modulates glucose and lipid metabolism (taken from:(Hardie and Sakamoto 2006)).

1.3.3 SGLT transporters

There are 3 types of SGLTs, which transport glucose against their concentration gradient using energy derived from co-transport of sodium down its electrochemical gradient (Shepherd

and Kahn 1999). SGLT-1 present in the small intestine and kidney and SGLT-2 found mainly in kidney are responsible for glucose absorption and reabsorption. Concerning SGLT-3, it is found in small intestine and skeletal muscle. Although it was thought to be a glucose transporter in the beginning, it is now considered to represent a glucose sensor involved in the depolarization of the cells in which it is present. The exact intracellular cascade initiated with SGLT3 is still being elucidated (Diez-Sampedro et al. 2003; Fairchild 2008).

1.4 Insulin

Insulin is a peptide hormone that plays an important role in carbohydrate and fat metabolism. Although glucose is the most potent stimulus of insulin release, other substances also modulate its pancreatic production, including dietary protein-derived amino acids, neurotransmitters, and gastrointestinal hormones such as glucose-dependent insulinotropic peptide (GIP) (Porte 2003; Kahn 2005). Insulin is composed of 51 amino acids (AA) consisting of an A chain (21 AA) and B chain (30 AA) joined together by two disulfide bonds (Katzung 2004; Kahn 2005).

1.4.1 Insulin synthesis

It is synthesized, stored and secreted from β-cells of the pancreatic islets. Insulin is synthesized from preproinsulin (110 AA), a protein precursor for proinsulin that possesses a signal peptide (24 AA), rich in hydrophobic residues, allowing for translocation of the nascent protein into the rough endoplasmic reticulum (RER). The signal peptide is then removed by signal peptidases within the RER (Kumar 2003; Voet 2004; Kahn 2005), forming proinsulin (84 AA), which undergoes proper folding (aligning disulfide bonds between A and B chains) in the

RER (Figure 14) .

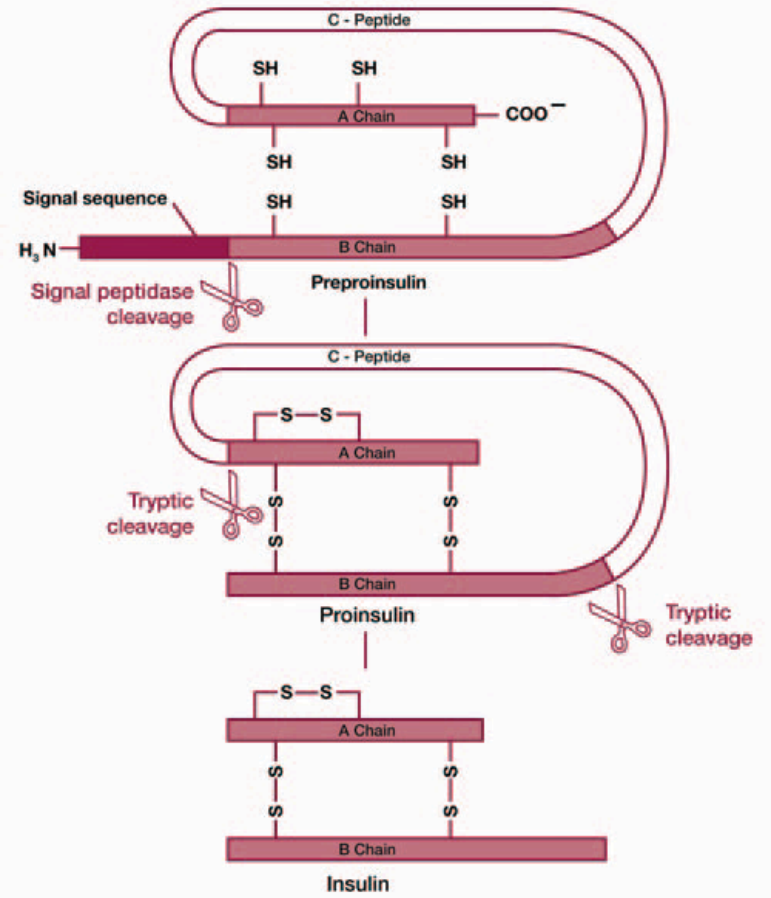


Figure 14: Insulin synthesis from preproinsulin to insulin (taken from:(Kramer and Sauer 2010)).

Properly folded proinsulin is then delivered to cis-Golgi in an ATP-dependent process and undergoes trafficking from the cis to the trans stacks of the Golgi apparatus. Proinsulin is then almost exclusively destined to the regulatory vesicular pathway, triggered in response to a secretagogue. However, very little if any also goes through the constitutive vesicular pathway, present in all cells and used for secretion (Kahn 2005). The regulatory pathway consists in packaging proinsulin into secretory granules followed by exocytosis in response to a stimulus, undergoing granule maturation in the process. It starts with a clathrin-coated vesicle containing proinsulin and involving a process of acidification. The cleavage of proinsulin is exerted by

proteolytic enzymes, such as prohormone convertase (PC1 and PC2) and the exoprotease carboxypeptidase E. Insulin and C-peptide are formed, resulting in insulin-rich mature uncoated granules (Steiner 1967; Kahn 2005; Sriram 2010)(Figure 15).

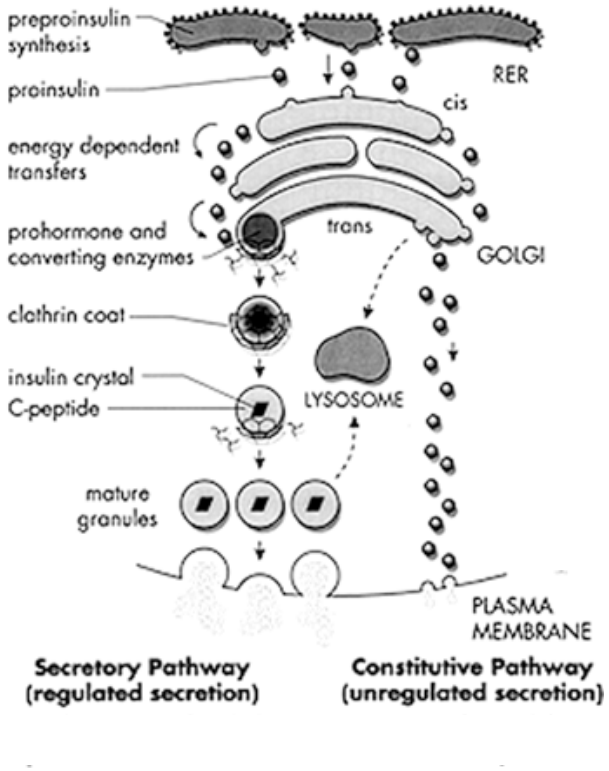


Figure 15 : The secretory pathway in which proinsulin through a process of acidification, prohormone and converting enzymes, and granule maturation, results in the formation of insulin-rich mature granules and C-peptide (taken from:(Steiner 2011)).

C-peptide is used clinically as an indicator of the insulin-secreting potential of β-cells of the pancreatic islets of Langerhans. C-peptide has also been shown to have effects on microvascular blood flow and tissue health (Forst et al. 2008; Nordquist and Johansson 2008). Concerning proinsulin, it is said to be predictive of non-insulin dependent diabetes mellitus (NIDDM), since it has been found that the ratio of proinsulin to insulin is increased in NIDDM and even in

insulin-dependent diabetes mellitus (IDDM). This observed increase may be the result of impairment in the enzymatic conversion of proinsulin to insulin, or even due to rapid granule turnover where proinsulin does not have enough time to be converted before it is released (Kahn 2005).

1.4.2 Insulin secretion

The blood glucose threshold required to stimulate insulin secretion is 4-6 mM. In contrast, the one for insulin biosynthesis is even lower ranging from 2-4 mM, biosynthesis reaching its maximum peak at levels of 10-12 mM (Kahn 2005). When β-cells are exposed to glucose, there is a 20 minute lag period before an observed significant increase in proinsulin biosynthesis. After 60 minutes, there is 10-20 fold increase in the rate of synthesis, and once the stimulus is removed it takes around 1 hour to return to normal levels (Kahn 2005).

The following steps lead to insulin release: glucose enters the β-cells in the pancreas through Glut2 transporters. It is then metabolized (glycolysis) and goes through oxidative phosphorylation where high amounts of ATP are formed. This leads to the closing of ATP-dependent K+ channels, triggering cell membrane depolarization and allowing for an influx of Ca2+ through voltage-controlled calcium channels (Figure 16).

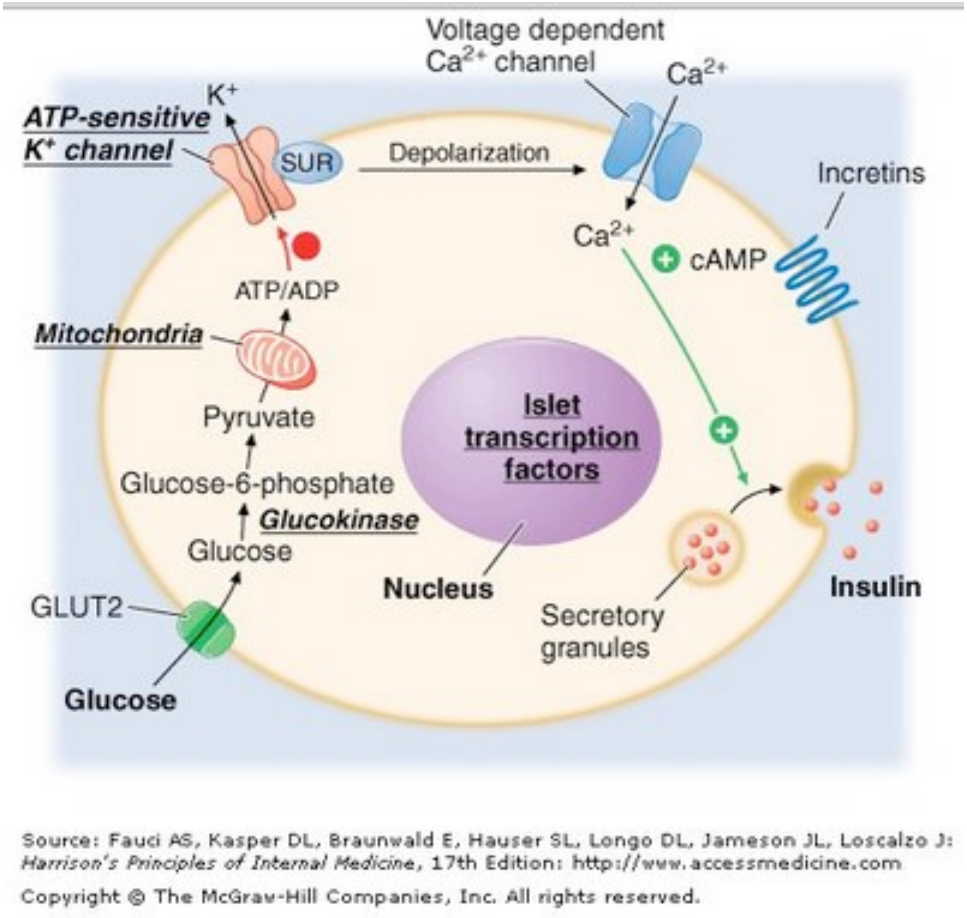


Figure 16: Insulin secretion (taken from:(Fauci 2008)).

The increase in Ca2+ levels is also believed to activate phospholipase C, which cleaves phosphatidyl inositol 4,5-biphosphate (PIP2) into inositol 1, 4, 5-triphosphate (IP3) and diacylglycerol (DAG) (Thore 2005). IP3 binds to receptor proteins in the ER allowing Ca2+ to be released from ER via IP3-gated channels, increasing Ca2+ levels even more and causing previously formed and stored insulin to be released (Belfiore 2000; Thore 2005)(Figure 17).

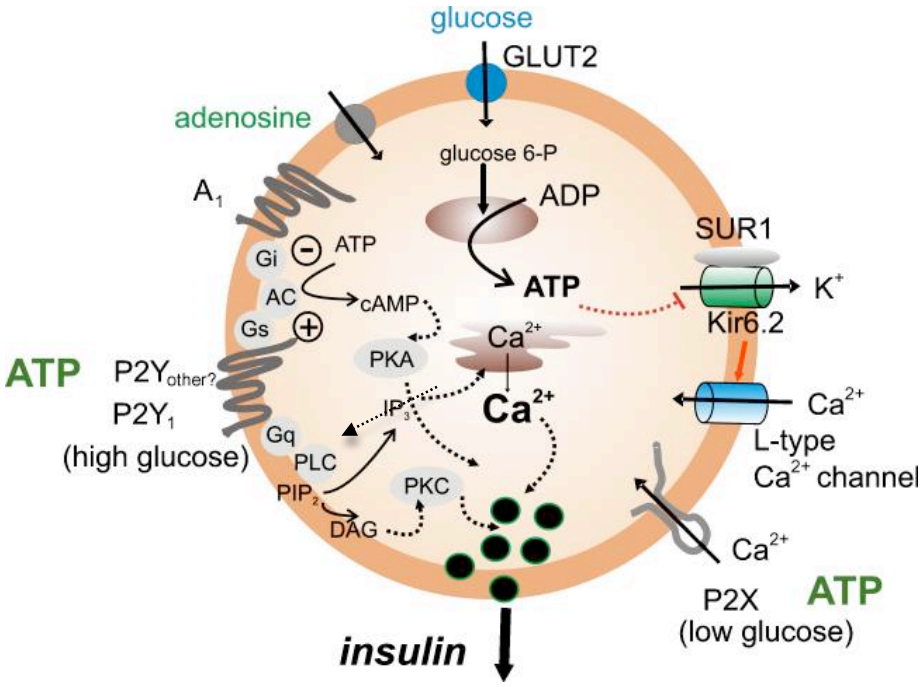


Figure 17 : Other factors involved in stimulating insulin secretion such as IP3, DAG, PKC (adapted from:(Novak 2008)).

Concerning DAG, it activates PKC, which is also known to stimulate insulin secretion. It does so probably by regulating proteins involved in the core machinery for vesicular docking on target membrane, thereby favoring membrane fusion (ex. SNARE, VAMP, SNAP25, syntaxin-1A) (Uchida et al. 2007). Other effector proteins involved in PKC action include granuphilin, Noc2, collectrin (Uchida et al. 2007; Gauthier and Wollheim 2008), or even myristoylated alanine rich PKC substrate (MARCKS). The latter binds actin and calcium-calmodulin, thus inducing insulin exocytosis (Calle et al. 1992; Donohoue 2008).

Insulin release is pulsatile in nature and is considered either as basal or stimulated. Basal release occurs after absorption is complete in order to maintain glycemia at normal levels between meals. Stimulated release occurs postprandially in order to respond to increased glucose loads. In either case, insulin favors glucose disposal into peripheral tissues such as the muscle or

adipose tissue and inhibits hepatic glucose production. Moreover, stimulated insulin release is biphasic, with a first phase lasting 10 minutes where β-cells promptly release insulin, followed by a second, more steady and sustained, phase that lasts until normoglycemia is restored (Del Prato et al. 2002). Even though both phases are important in regulating glucose levels, it is the first phase that is weakened in patients with impaired glucose tolerance or in those with diabetes (Saltiel and Kahn 2001; Del Prato et al. 2002; Cavaghan 2004).

1.5 Insulin signaling

The insulin receptor is a transmembrane receptor belonging to the family of tyrosine kinase receptors. It is composed of 2α extracellular binding domains, and 2β transmembrane subunits kept together by three disulfide bonds. The β-subunits contain extracellular and transmembrane portions, as well as an intracellular domain consisting of an ATP binding domain and a tyrosine kinase catalytic domain (Dorrestijn et al. 1998; Hunter and Garvey 1998; Perz and Torlinska 2001). Binding of insulin to the α-subunits initiates a cascade of intracellular mechanisms. First, it creates a conformational change in the receptor, allowing ATP to bind to the intracellular domain of the β-subunit. This activates the tyrosine kinase domains residing on the intracellular portion of the β-subunits. Autophosphorylation ensues at the level of tyrosine residues found on the C-terminus of the β-subunit. This event enables the insulin receptor tyrosine kinase to phosphorylate the tyrosine residues found on the insulin receptor substrate (IRS-1/2) (Mantzoros 2011).

Once the insulin receptor is activated, the signal gets propagated to many downstream intracellular protein effectors and pathways, the two main ones being MAPk and PI3K (see Figure 12).

1.5.1 The MAPk pathway

The mitogen-activated protein kinase pathway (MAPk) regulates the transcription of genes implicated in the cell cycle, mediating insulin's effects on growth and proliferation (Kido et al. 2001; Mantzoros 2011). It is activated by a signaling cascade involving either the binding of growth factor binding protein 2 (Grb2) through the SH2 domain to phosphorylated IRS-1/2, or to phosphorylated Shc (src homologous and collagen protein). This allows SOS (son of sevenless; guanine nucleotide exchange factor) to bind to Grb2 through its Sh3 domain and to catalyze the exchange of GDP with GTP on Ras (small G protein) (Olefsky 2004). This leads to activation of Raf, Map kinase kinase (MEK or MAPKK), and MAPk Erk1/2, which goes on to phosphorylate a variety of transcription factors involved in cellular proliferation and differentiation (Saltiel and Kahn 2001; Capeau 2003)(see Figure 12). MAPk activation in the adipose tissue has been shown to inhibit adipocyte differentiation both *in vitro* and *in vivo*, mediating its effect by inhibiting PPARγ and activating SOX9, which decreases the expression of C/EBPβ and δ (Finucane et al. 2009; Sul 2009; Donzelli et al. 2010). It has also been associated with more intense pre-replicative phases of hepatocyte proliferation, hyperplasia, leading to the development of non-alcoholic fatty liver disease (NAFLD) (Chavez-Tapia et al. 2009).

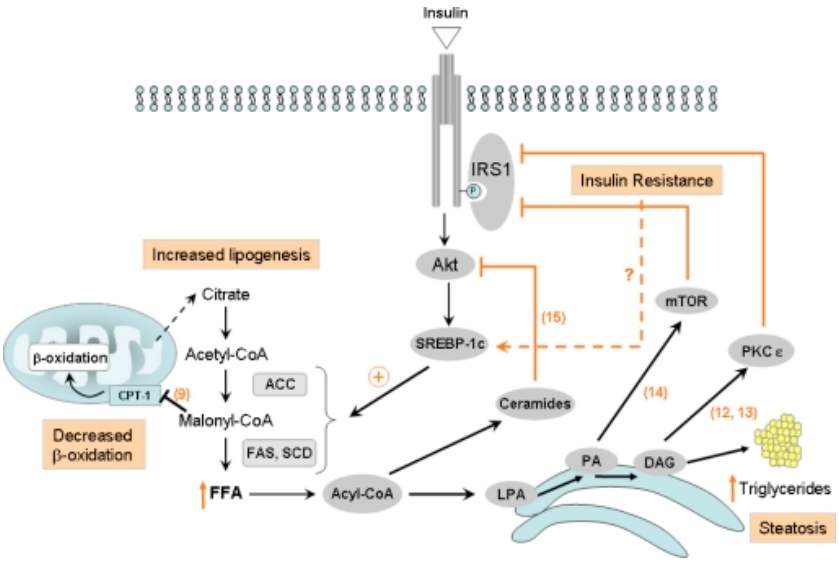
1.5.2 The PI3K pathway

Phosphorylated IRS-1/2 can also activate the phosphoinositol-3-kinase (PI3K) pathway, by recruiting PI3K through its regulatory subunit p85 SH2 domain. The p110 catalytic subunit of PI3K then phosphorylates phosphatidylinositol (4,5) biphosphate (PI(4,5)P2) generating phosphatidylinositol (PI(3,4,5)P3). Protein kinase 3-phosphoinositide dependent protein kinase-1 (PDK-1) goes on to activate protein kinase (PKB)/Akt (containing a pleckstrin homology domain

(PH) that allows for binding to PIP3) or atypical PKC (both of which are serine/threonine kinases). Activation of both the Akt and atypical PKC (aPKC) kinases lead to the translocation of Glut4 to the membrane in order to increase glucose transport in tissues such as the muscle or adipose tissue (Czech and Corvera 1999; Tirosh et al. 2000). In the liver, activating aPKC stimulates storage pathways such as lipogenesis, which involves upregulating the expression of transcription factors such as SREBP1-c (Chattopadhyay et al.). Concerning hepatic Akt activation, it leads to mechanisms that promote glucose metabolism (glycolysis) and storage (glycogenesis), as well as lipogenesis, while inhibiting glucose production through gluconeogenesis or glycogenolysis. Therefore, Akt downregulates enzymes such as glucose-6-phosphatase or PEPCK. It also phosphorylates GSK-3, thus allowing the activation of GS and promoting glycogen formation. Akt activation also phosphorylates FOXO-1. This prevents the latter from binding to PGC-1α, resulting in the inhibition of gluconeogenesis pathways (see Figure 11). Without going into tissue specific regulation, Akt can also go on to modulate the mTOR (mammalian target of rapamycin) pathway. Akt phosphorylates and inactivates TSC2 of the tuberus sclerosis complex (TSC1/2) decreasing RHEB GTPase activity and increasing RHEB-GTP, resulting in mTOR activation (Pelengaris 2006). The mTOR pathway is involved in protein synthesis, cell size, survival (suppressing apoptotic pathways) and proliferation. It promotes ribosomal biogenesis, as well as transcription and translation, activating 40S ribosomal protein S6 kinase (p70s6k) and the eukaryotic initiation factor 4E-binding protein 1 (EIF4E)(phosphorylation of EIF4EBP, a translation repressor protein, allows for EIF4E to dissociate and for translation to proceed) (Pelengaris 2006) (see Figure 12).

1.5.3 Downregulation of the PI3K/Akt/PKB pathway

The Akt pathway can be inactivated in many ways, such as receptor internalization, ubiquitination and subsequent degradation by proteasomes, or by dephosphorylation of phosphotyrosine residues of the insulin receptor by protein tyrosine phosphatases (PTP) such as PTP1B (Saltiel and Kahn 2001; Zinker et al. 2002). Downregulation of the insulin signaling pathway can also occur through dephosphorylation of PIP3 by 3-phosphoinositide phosphatase (PTEN) (Saltiel and Kahn 2001; Zinker et al. 2002). Other ways of halting activation of the insulin pathway include serine-threonine phosphorylation of the insulin receptor (Coba et al. 2004), or of the IRS by PKC-β (inhibits catalytic activity of insulin receptor) (Aguirre et al. 2002; Ishizuka et al. 2004; Liberman et al. 2008). FFA metabolism is also involved in insulin signal impairment by increasing DAG levels and concomitantly activating conventional and novel PKC (Dey et al. 2006). In summary, different PKC isoforms, through different mechanisms, lead to insulin signaling impairment by acting either on the insulin receptor, IRS, or Akt, or even by activating pathways as the mTOR or MAPk, which can negatively regulate the pathway (feedback mechanism in situations of prolonged insulin stimulation) (Engelman et al. 2000; Tremblay et al. 2007; Fox et al. 2010).



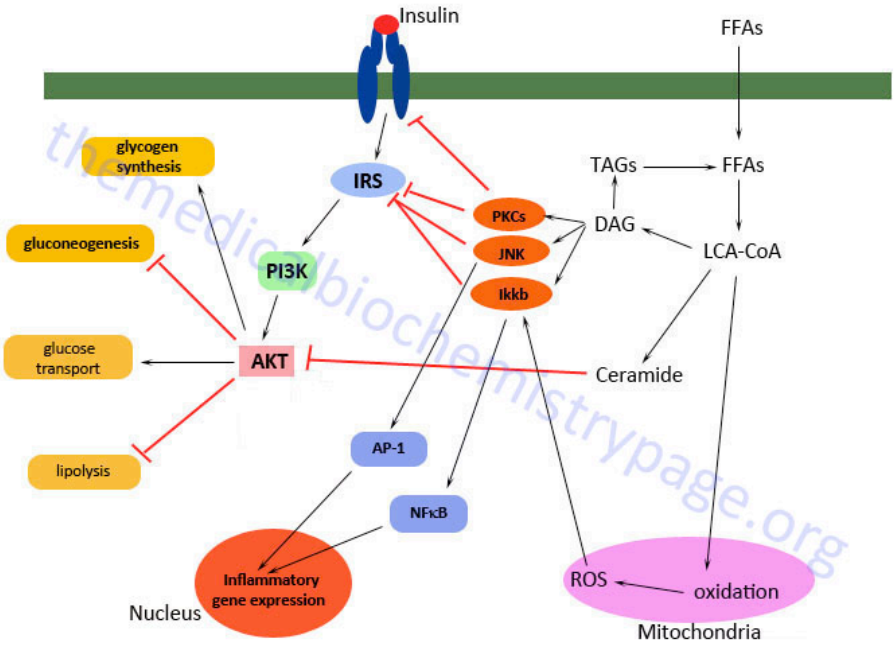


Figure 18 : Examples of factors involved in negatively regulating the insulin pathway (taken from:(Clement et al. 2009; King 2012)).

1.5.4 The TC10 pathway

The TC10 pathway functions in parallel but independently of the PI3K to stimulate Glut4 translocation in response to insulin in tissues such as skeletal and cardiac muscle (Bernard et al. 2006; Gupte and Mora 2006) as well as adipose tissue (Chiang et al. 2001) (see Figure 12). Tyrosine phosphorylation of the insulin receptor allows for Cbl (proto-oncogene)/CAP (Cbl-associated adaptor protein) to be recruited to the receptor in the presence of APS (adaptor protein). Firstly, the phosphorylated tyrosine residues of the insulin receptor allow for APS binding via its SH2 domain. APS gets phosphorylated in the process, and recruits Cbl via its SH2 domain. Cbl then interacts with CAP that belongs to the sorbin homology family (SoHo) of adaptor proteins. The APS-Cbl-CAP complex, once phosphorylated dissociates and translocates to lipid rafts. Once there, CAP binds to flotillin through its SoHo domain, while Cbl via its SH2 domain recruits the SH2/SH3 adaptor protein CrkII. CrkII via its SH3 domain forms a complex

with C3G, a guanine nucleotide exchange protein that catalyzes the exchange from GTP to GDP on TC10 (Rho family of GTPase). TC10 is then activated and can interact with effector proteins such as Cdc42-interacting protein 4/2 (CIP4/2), located on intracellular compartments. Once activated they favor translocation of Glut4 containing vesicles to the plasma membrane (Baumann et al. 2000; Saltiel and Kahn 2001; Kimura et al. 2002; Chang et al. 2004).

1.6 Other mechanistic pathways involved in glucose and lipid metabolism

1.6.1 AMPK pathway

In general, AMPK activation stimulates energy producing pathways such as glycolysis and fatty acid oxidation. It inhibits energy-consuming pathways, for example lipogenesis, and steroid or protein synthesis (Hardie et al. 2003; Viollet et al. 2003). Even cell growth is inhibited, with AMPK activating and phosphorylating TSC2, and inhibiting mTOR activity (Hahn-Windgassen et al. 2005). The effects of AMPK on glucose and lipid metabolism can be beneficial in the treatment of diabetes, and obesity. It has been observed that the two commonly prescribed classes of antidiabetic medications, biguanides and thiazolidinediones, exert their beneficial effects through activation of this pathway (Viollet et al. 2003).

1.6.1.1 AMPK pathway activation

The AMPK pathway is activated when the AMP/ATP ratio is increased. When the muscle is contracting ATP is broken down to ADP. In order to regenerate ATP, the formed ADP combines with another ADP creating ATP and increasing concentrations of AMP in the process.

This can occur in conditions of hypoxia, low glucose, exercise, and can even be activated by adipocytokines such as leptin and adiponectin, or substances such as AICAR, metformin, and mitochondrial uncouplers (Chen et al. 1999; Winder and Hardie 1999; Ruderman and Prentki 2004; Fujii et al. 2006). There has even been recent evidence suggesting that long-chain fatty acids (LCFA) regulate AMPK/ACC activity (Kawaguchi et al. 2002; Clark et al. 2004; Taylor et al. 2005; Fediuc et al. 2006). There is no clear consensus on how LCFAs regulate AMPK/ACC activity (Fediuc et al. 2006). Some claim that LCFAs enhance the AMPK pathway, by increasing phosphorylation of AMPK and/or ACC (Kawaguchi et al. 2002; Clark et al. 2004; Fediuc et al. 2006), while others report that they inhibit phosphorylation and activation of AMPK by its upstream kinase LKB1/STRAD/MO25 (Taylor et al. 2005). Other suggestions pertaining to the mode of activation by LCFAs include inhibition of dephosphorylation of Thr-172 on AMPK by protein phosphatases, or inhibition of dephosphorylation and activation of ACC once again by phosphatases (Winder 2001; Carling 2005; Fediuc et al. 2006). These findings clearly demonstrate that AMPK is more than just an energy sensor. It can also be considered as a mediator of extracellular signals and a metabolic sensor (Allard et al. 2007). Therefore, it is evident that many factors are involved in the regulation of AMPK activity and that of its downstream effectors (such as ACC).

AMPK, a stress-sensing protein kinase, is composed of 3 subunits. The α subunit containing a serine/threonine protein kinase catalytic domain, where upstream kinases phosphorylate Thr/172. The β regulatory subunit has glycogen-binding domains that stabilize the interaction between α and γ subunits. The γ regulatory subunit has the ability to detect changes in the AMP/ATP ratio through four cystathionine beta synthase domains (CBS). The γ subunit binds both AMP and ATP in a competitive manner. If ATP displaces AMP then

activation of AMPK is reversed (Wong and Lodish 2006; Xiao et al. 2007; Garrett 2010). The four CBS domains create two AMP binding sites also referred to as Bateman domains. Once the first AMP binds to the Bateman domain, this increases binding affinity for the second AMP. This creates a conformation change in the γ subunit, which exposes the catalytic domain of the α subunit. It is in the α subunit that AMPK becomes activated through phosphorylation on threonine-172 by an upstream kinase termed AMPK kinase (AMPKK), such as liver kinase B1 (LKB1/MO25/STRAD) (Hardie 2005). Calcium calmodulin dependent protein kinase-β (CaMKKβ) has also been shown to activate AMPK (Shen et al. 2007). There have also been reports of other kinases that may exist upstream of AMPK and play a role in its activation and phosphorylation. One example is Tak1, a member of the MAPKKK family. However their physiological role and relevance are still being investigated (Shen et al. 2007; Carling et al. 2008) (Figure 19).

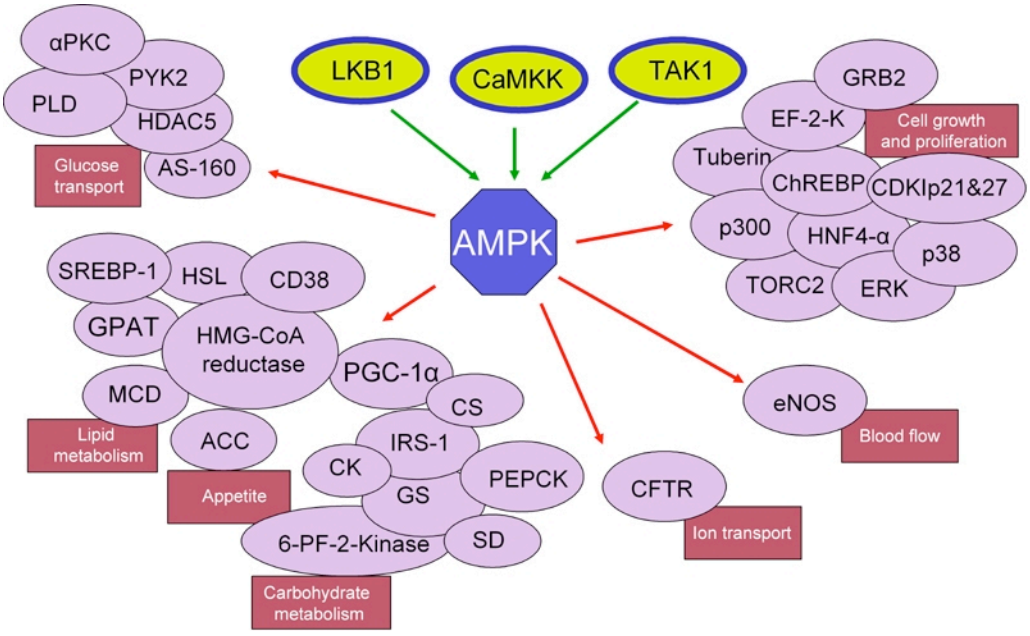


Figure 19 : Kinases involved in activating and phosphorylating AMPK as well as the downstream effectors it regulates (taken from:(Gruzman et al. 2009)).

There has been evidence that allosteric modulators such as 3-phosphoglycerate, a glycolysis intermediate, can also influence AMPK activation. It can enhance AMPK activation by acting on AMPKK (LKB1-STRAD-MO25), or on AMPK itself, making it a better substrate for LKB1-STRAD-MO25 (less accessible to phosphatases)(Ellingson et al. 2007).

1.6.1.2 AMPK pathway on glucose metabolism

AMPK regulates glucose metabolism by increasing glucose transport and enhancing Glut4 translocation to the plasma membrane through an insulin-independent pathway. AMPK has been shown to act on downstream effectors such as the Akt substrate 160 (AS-160), a Rab-GTPase activator protein and a key regulator of glucose trafficking (found predominantly in muscle and to some extent in adipose tissue) (Marette 2008). Once AS-160 is phosphorylated it inhibits Rab-GTPase activator protein activity. Activated GTP-bound Rab can promote Glut4 translocation in the muscle and adipose tissue (Nascimento et al. 2006; Marette 2008; Nascimento 2010) (Figure 20). AS-160 is a point of convergence of both the AMPK and Akt pathway, naturally implying its importance in regulating glucose transporter translocation (Treebak et al. 2006) (Figure 20).

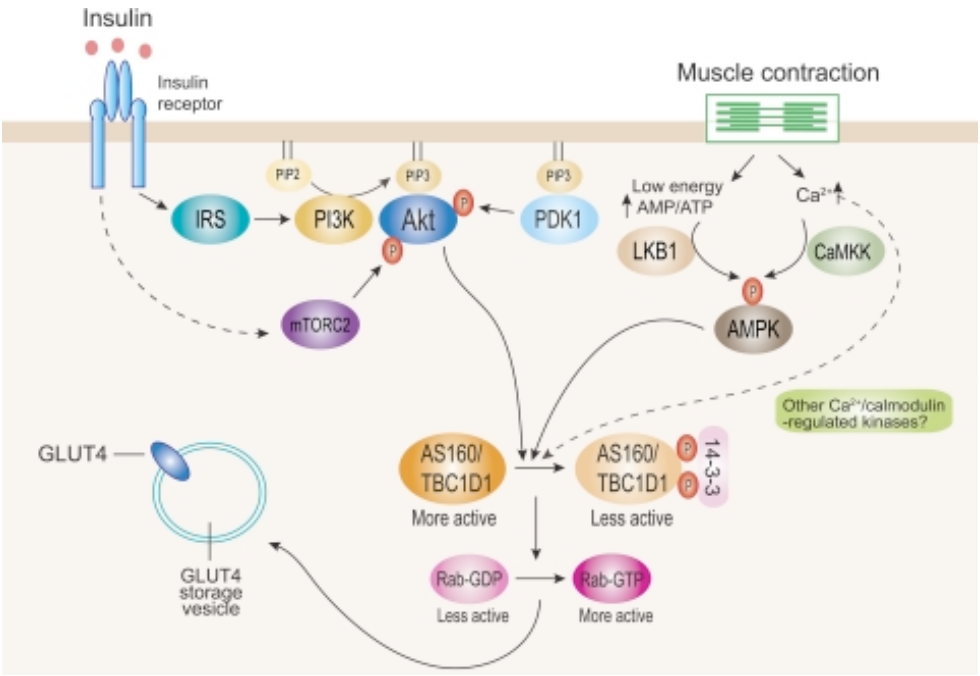


Figure 20: This scheme is an example of how AMPK activates Glut4 translocation in muscle, through AS-160. AS-160 is a point of convergence between the Akt and AMPK pathway resulting in increased Glut4 translocation (taken from:(Sakamoto and Holman 2008)).

AMPK may also upregulate Glut4 expression and transcription by acting on myocyte enhancer factor-2 (MEF2) and Glut4-enhancer factor (GEF; associates to MEF2), or even on PGC-1α (maybe binds and coactivates MEF2) (Karnieli and Armoni 2008) (Figure 21). AMPK can either directly activate Glut4 translocation, or it can interact with downstream activators. One such example is AMPK activation of MKK3/6, an upstream kinase that phosphorylates p38 MAPk. PGC-1α and MEF-2 are then activated, inducing mitochondrial biogenesis, as well as Glut4 translocation (Karnieli and Armoni 2008). P38 MAPk is activated in response to many of the same conditions that stimulate AMPK, including AICAR, and mitochondrial uncouplers. It is involved in the inflammatory process, cell growth, cell differentiation, cell cycle, cell death, and glucose transport (Xi et al. 2001; Lemieux et al. 2003; Pelletier et al. 2005; Ribe et al. 2005). In

the muscle, AMPK activation leads to inhibition of glycogen synthesis, and increased fatty acid oxidation (Winder and Hardie 1999; Hardie et al. 2003; Jorgensen et al. 2004; Andreelli 2005; Korbonits 2008; Grimaldi 2009).

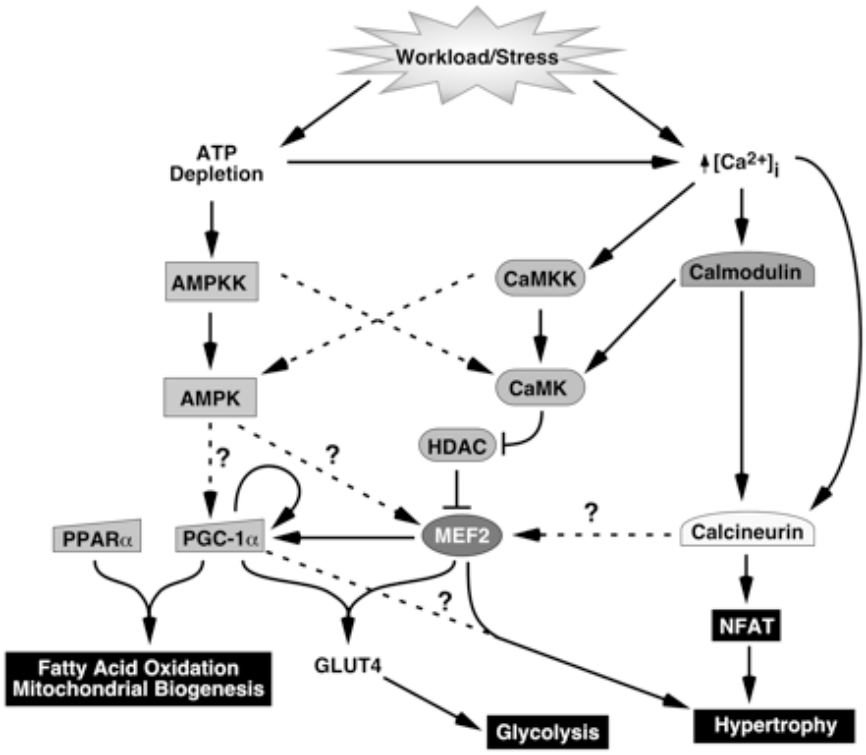


Figure 21: AMPK, in the muscle, acts on MEF2 and PGC-1α to increase Glut4, and activates fatty acid oxidation and mitochondrial biogenesis (taken from:(Czubryt and Olson 2004)).

In the liver, AMPK inhibits hepatic glucose production (gluconeogenesis), lipogenic enzymes (FAS, ACC-1), and stimulates FA oxidation (Viollet et al. 2003). AMPK upregulates transcriptional factors such as PGC1-α, modulating mitochondrial biogenesis and fatty acid oxidation (Steinberg and Kemp 2009; Lustig et al.).

Hepatic glucose production is actually inhibited through the action of AMPK on PEPCK and G-6-Pase. AMPK induces an orphan nuclear receptor called small heterodimer partner (SHP), which is a transcriptional repressor of many nuclear receptors and transcription factors. Some

examples include cAMP-response element binding protein (CREB), hepatocyte nuclear factor 4 alpha (HNF-4α), and Foxo-1, all implicated in the expression of gluconeogenic enzymes PEPCK and G-6-Pase (Kim et al. 2008; Lee et al. 2010)(see Figure 19).

1.6.1.3 AMPK pathway on lipid metabolism

In adipose tissue, the AMPK pathway increases fatty acid oxidation, while inhibiting energy-consuming pathways such us lipogenesis and lipolysis (Korbonits 2008; Gaidhu and Ceddia 2011). Lipolysis is increased during exercise. This activates the AMPK pathway in order to limiting further TG breakdown, while promoting fatty acid oxidation of the already present fatty acids. Fatty acids that are released need to be oxidized by adipose or other tissues, otherwise they will be reesterified into triglycerides creating an energy-futile consuming cycle (Gaidhu and Ceddia 2011).

AMPK plays a role in lipid metabolism by phosphorylating acetyl-CoA carboxylase (ACC) inactivating it in the process. There are two isoforms of ACC. ACC-1 is expressed in all tissues, mostly lipogenic ones, and is the prevalent isoform in the adipose tissue and liver. It is implicated in fatty acid synthesis. ACC-2, prevalent in cardiac and skeletal muscle, and to a lesser extent liver, is involved in mitochondrial fatty acid oxidation (Wakil and Abu-Elheiga 2009). AMPK phosphorylates ACC-1 on Serine (ser)-79, Ser-1200, and Ser-1215, and ACC2 on Ser-218. Once ACC is phosphorylated it does not allow the formation of malonyl-CoA, a building block of fatty acids. Malonyl-CoA inhibits the transfer of the fatty acyl group from acyl CoA to carnitine by inhibiting the activity of carnitine palmitoyl transferase (CPT-1). CPT-1 facilitates the transport of fatty acids to the mitochondria for beta-oxidation. In summary, by phosphorylating ACC and inactivating it, malonyl-CoA production is decreased allowing CPT-1 to function properly and

conduct fatty acids to beta-oxidation in the mitochondria. This process allows for decreased lipid accumulation in insulin sensitive tissues such as the muscle, liver, and adipose tissue contributing to the maintenance of insulin sensitivity (Winder and Hardie 1999; Zhou et al. 2009; Fogarty and Hardie 2010). AMPK also downregulates transcription factors involved in the synthesis of enzymes promoting fatty acid or cholesterol synthesis. Some examples include carbohydrate responsive element-binding protein (ChREBP), sterol regulatory element binding protein (SREBP-1c), and 3-hydroxy-methyl-glutaryl-CoA-reductase (HMGR; cholesterol synthesis). In the presence of insulin there is activation of phosphatases that remove the inhibitory effect on ACC and allow for fatty acid synthesis (Witters et al. 1988; Hansmannel et al. 2006)(see Figure 9).

AMPK is also said to act upstream of SIRT-1 (sirtuin-1), which is a NAD-dependent deacetylase. SIRT-1 has different roles depending on the tissue (liver, muscle, pancreas, adipose tissue) in which it is activated, and on different stimuli (i.e. whether fasted or fed state). SIRT-1 is said to be involved in a variety of metabolic processes (see Figure 9). It improves FA mobilization and oxidation, mitochondrial biogenesis, glucose uptake, insulin sensitivity (represses PTP1B), and plays a role in insulin secretion (Fulco and Sartorelli 2008; Nunn et al. 2009; Valentino et al. 2011).

1.6.2 PPAR pathways

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptor proteins that function as transcription factors (Michalik et al. 2006). There are a variety of endogenous ligands that bind and activate PPARs. They include saturated or unsaturated FA, eicosanoids, leukotrienes (PPARα) or prostaglandins (PPARγ) (Tjokroprawiro 2006). When a ligand is bound

PPARs heterodimerize with retinoid X receptor (RXR). This complex then binds to DNA sequences termed peroxisome proliferator response elements (PPRE) of target genes involved in a variety of cellular processes. Examples include cell differentiation, adipocyte differentiation, metabolism of lipids, carbohydrates and proteins, tumorigenesis, and inflammation (Shi et al. 2002; Tjokroprawiro 2006; Belfiore et al. 2009). There are three types of PPAR that have been identified each one having different functions and expressed in different tissues: PPARα, PPARβ/δ, PPARγ (Berger and Moller 2002)(Figure 22).

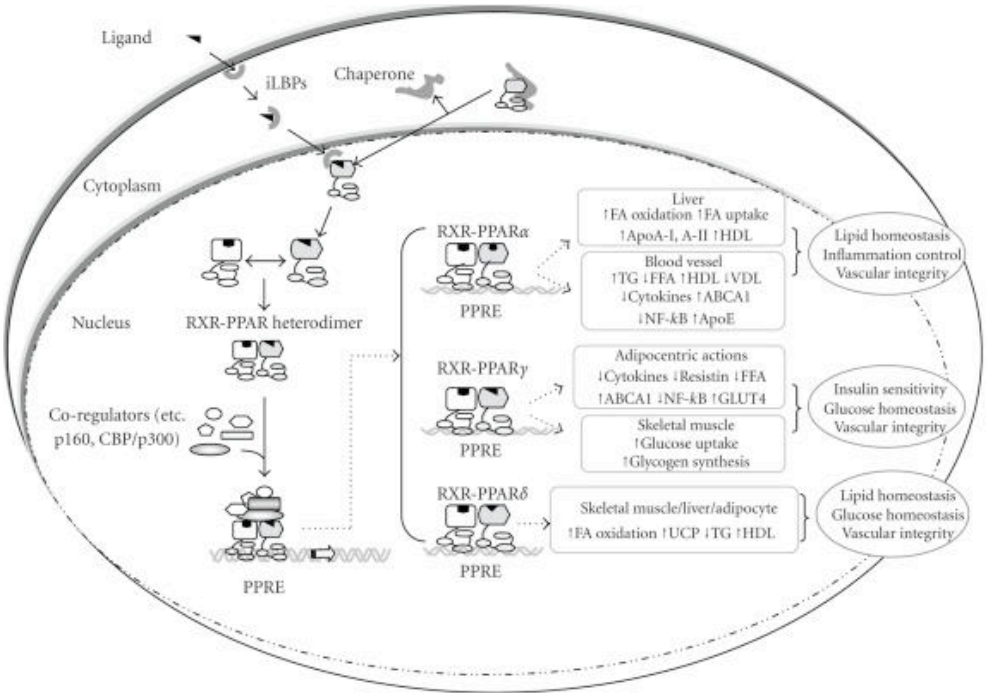


Figure 22: Summary of PPAR α, β, and γ activation and their functions (taken from:(Cho et al. 2008)).

PPARα is expressed in liver, kidney, gut, heart, muscle, and adipose tissue (Tjokroprawiro 2006). PPARα promotes lipid metabolism in tissues such as the liver and muscle. It activates genes implicated in fatty acid transport, and mitochondrial or peroxisome mediated fatty acid oxidation (Tjokroprawiro 2006; Wanders et al. 2011). It is also the target of fibrate

drugs, which aim to lower cholesterol and TG levels (Tjokroprawiro 2006), while improving insulin sensitivity (Fatehi-Hassanabad and Chan 2005).

PPARβ/δ is found in many tissues including the brain, colon, liver, adipose tissue, and skin (Berger and Moller 2002; Shi et al. 2002; Tjokroprawiro 2006). Its function remains elusive. However there has been evidence that it may act as a potent inhibitor of ligand-induced transcription activity of PPARα and γ by binding to PPREs. It interacts in the process with corepressor silencing mediator for retinoid or thyroid hormone receptors (SMRT) and SHARP (SMRT and histone deacetylase associated repressor protein) (Shi et al. 2002). Another function associated to PPARβ/δ was discovered with the use of an agonist GW501516. It consists in changing the body's fuel preference from glucose to lipids (Stienstra et al. 2007; Adeghate et al. 2011). This can be beneficial in reducing tissue lipid levels and the risks associated with hyperlipidemia (Adeghate et al. 2011).

PPARγ has three subtypes PPARγ1, PPARγ2, and PPARγ3, which are obtained through alternative splicing from a single gene (Tjokroprawiro 2006). In general, PPARγ activates genes that are implicated in lipid uptake (FATP and aP2/FABP4), fatty acid storage, and adipogenesis in fat cells (lipoprotein lipase and acyl-CoA). It even plays a role in glucose metabolism, modulating expression of genes for insulin receptor substrates, the p85 subunit of PI3K, and Glut4 (Picard and Auwerx 2002; Tjokroprawiro 2006)(Figure 23).

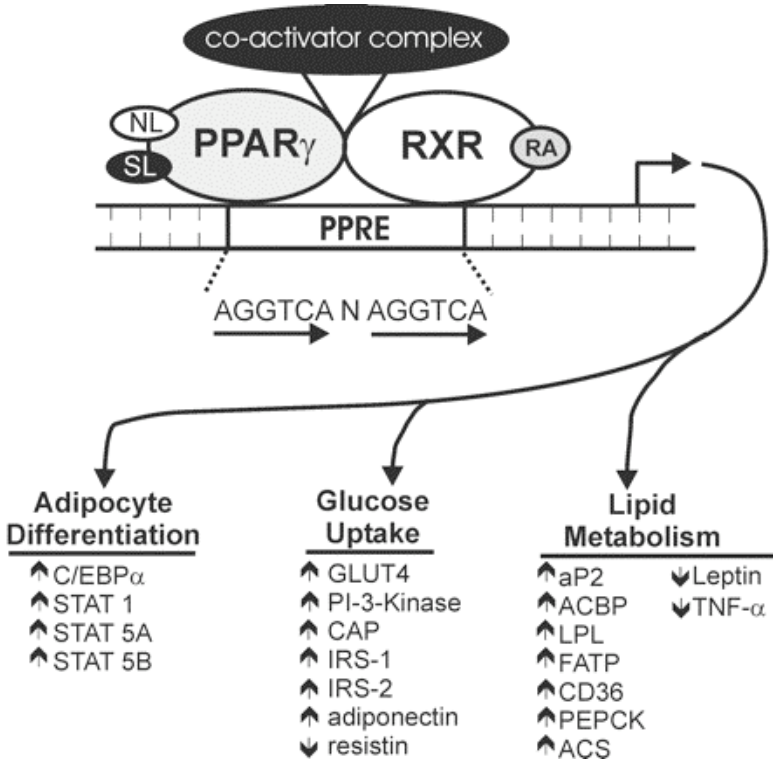


Figure 23 : PPARγ activates genes involved in adipocyte differentiation, glucose uptake, lipid metabolism (taken from:(Brown and McIntosh 2003)).

PPARγ1 is expressed in heart, muscle, colon, kidney, pancreas and spleen (Berger and Moller 2002; Tjokroprawiro 2006). PPARγ2 is 30 amino acids longer than PPARγ1 and it is expressed in the adipose tissue (Berger and Moller 2002; Tjokroprawiro 2006). PPARγ3 is expressed in macrophages, large intestine, and white adipose tissue (WAT) (Berger and Moller 2002; Tjokroprawiro 2006). PPARγ modulators include insulin, which increases PPARγ mRNA expression, and SREBP1-c, which increases the production of lipophilic molecules with potent PPARγ ligand activity (Farmer 2005a). PPARγ is also the target of thiazolidinediones (Tzds), a class of oral hypoglycemic medications. PPARγ modulators are used to treat hyperlipidemia, by increasing ATP-binding cassette transporter (ABCA1). This increases the transport of cholesterol to HDL therefore increasing uptake and excretion from the liver (Adeghate et al. 2011). There

also exists a class of dual/pan-PPAR agonists. They are defined by non-selective action on PPARs by binding two or more of the PPAR isoforms, PPARα, δ/β, γ. PPARα and PPARγ dual agonists are called glitazars and include aleglitazar, muraglitzar, tesaglitazar (Calkin and Thomas 2008; Adeghate et al. 2011). Glitazars are being investigated for their use in treating a larger subset of symptoms of the metabolic syndrome since they have the lipid benefits of PPARα agonists (fibrates), and the glycemic benefits of PPARγ agonists (Tzds) (Calkin and Thomas 2008; Adeghate et al. 2011).

1.6.3 Uncoupling proteins (UCPs)

Uncoupling proteins (UCPs) are transmembrane proteins that are part of the larger family of mitochondrial anion carrier proteins (MACP) that uncouple oxidative phosphorylation from ATP synthesis. UCPs increase the permeability of the mitochondrial membrane, allowing protons from the intermembrane space to leak back in to the mitochondrial matrix without going through the ATP synthase complex (Figure 24). Therefore, this allows for fast substrate oxidation with low ATP production. There have been 5 subtypes identified thus far (UCP-1 to 5), with UCP-1 to 3 more extensively studied. UCP-1, termed thermogenin, has been identified in brown adipose tissue (BAT), and has been associated with non-shivering thermogenesis (Krauss et al. 2005).

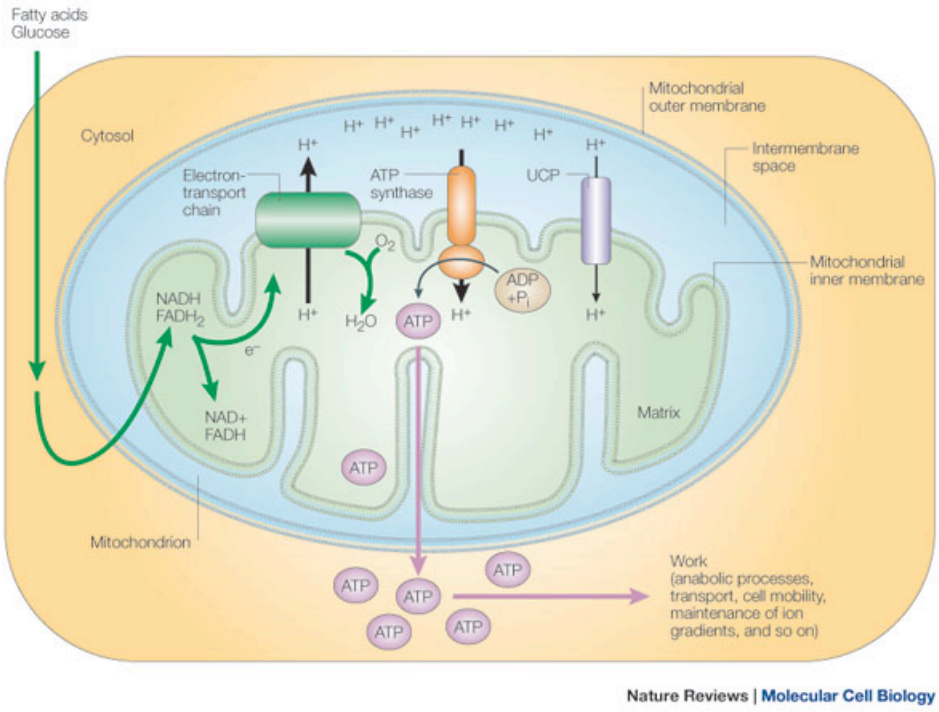


Figure 24 : UCPs increase permeability of mitochondrial membrane, allowing protons to be pumped back in to the mitochondrial matrix without going through the ATP synthase complex (taken from:(Krauss et al. 2005)).

The sympathetic nervous system increases the transport, mobilization, and oxidation of fuel, increasing the production of NADH, and FADH2, essential for UCP-1 mediated proton leak. It stimulates β3-adrenergic receptors activating a cAMP/PKA mediated signaling cascade. This leads to phosphorylation and activation of lipases transforming TG into FFAs. FFAs can be metabolized for example through β-oxidation and subsequent entering into the CAC, serving as substrate for mitochondrial oxidative respiration (Krauss et al. 2005). FFAs can also directly activate UCP-1. However, the exact mechanism is unknown. Two mechanisms have been proposed (Krauss et al. 2005). In the first hypothesis, it is thought that FFAs act as a prosthetic group within the UCP-1 translocation channel donating protons to proton-buffering amino acids

that aid in the translocation of protons through the channel (Krauss et al. 2005)(Figure 25 a). The second hypothesis is termed the fatty-acid cycling model (Krauss et al. 2005). Without a carrier protein, the fatty-acid anion remains in the mitochondrial matrix. It binds to UCP-1, which brings it to inner mitochondrial membrane. This exposes it to the intermembrane space where it accepts a proton (Krauss et al. 2005). It then flips back across the inner mitochondrial membrane toward the matrix where it releases the proton. The cycle then starts over again with UCP-1 transporting the fatty-acid anion back across the inner mitochondrial membrane and exposing to intermembrane space and protons (Krauss et al. 2005) (Figure 25 b).

a) b)

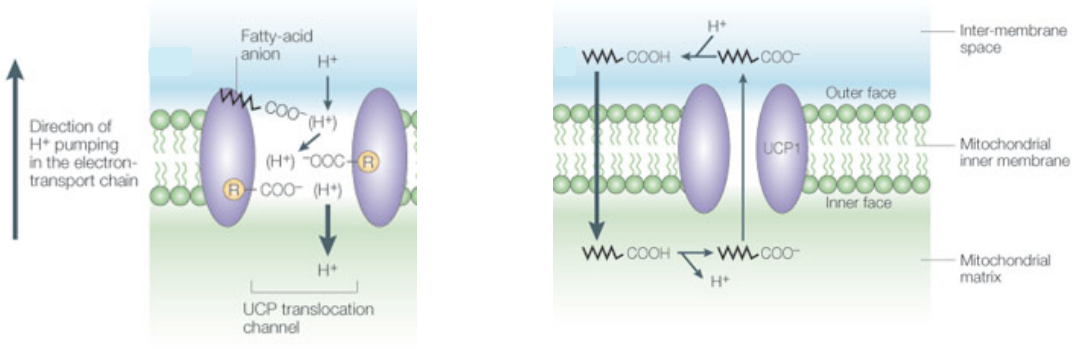


Figure 25 : FFA can activate UCP-1 through two distinct mechanisms detailed above (taken from:(Krauss et al. 2005)).

UCP-2 is found in skeletal muscle and liver and controls the generation of mitochondria-derived reactive oxygen species (ROS) (Krauss et al. 2005). ROS formation is highly dependent on proton-motive force, therefore promoting proton leak through UCP-2 expression can limit it (Krauss et al. 2005). UCP-3, predominant in BAT and skeletal muscle, is thought to play a role in the handling of lipids as fuel, having important implications in obesity (Krauss et al. 2005). Fatty acids can accumulate in the mitochondria due to increased lipid metabolism, and this can produce

toxicity (Krauss et al. 2005). It has been reported that mutations in UCP3 have led to increased respiratory quotients indicative of decreased fatty acid oxidation. This suggests that UCP3 expression and function is important in lipid metabolism. An increase in UCP3 expression is associated with increased fat metabolism seen in fasting or high-fat diet animals (Krauss et al. 2005).

1.7 Lipid metabolism

1.7.1 Lipogenesis

There are three major sources of fatty acids (FA): from dietary components (glycerides, phospholipids, sterols), from peripheral lipolysis, or from de novo lipogenesis. Fatty acids can then either be oxidized through beta-oxidation, lipolytic pathways, or ketosis, or they can form TG through esterifcation of FA with glycerol. Lipid accumulation therefore occurs when there is an imbalance between the amount of FA being formed and stored as TG, and the amount that is being used as fuel. This imbalance can lead to the development of obesity, insulin resistance, type 2 diabetes, and cardiovascular disease (Saltiel and Kahn 2001). The main sites of lipogenesis comprise the liver, adipose tissue and intestinal mucosa (Vernon et al. 1999). The liver has a central clearing role, taking up and esterifying FA. It can also synthesize FA de novo from acetyl-CoA derived from the metabolism of carbohydrates, and to a lesser extent of amino acids (Vernon et al. 1999; Ramesh and Sanyal 2004; Patel 2011; King 2012). An abundance in dietary carbohydrates leads to TG formation. Indeed the action of acetyl CoA carboxylase (ACC) on the glycolytic end-product acetyl-CoA forms malonyl-CoA and activates FAS. Alternatively, the glycolysis intermediate glyceraldehyde-3-phosphate is used to form glycerol-3-phosphate (esterification leads to TG) (Neville and Picciano 1997)(Figure 26).

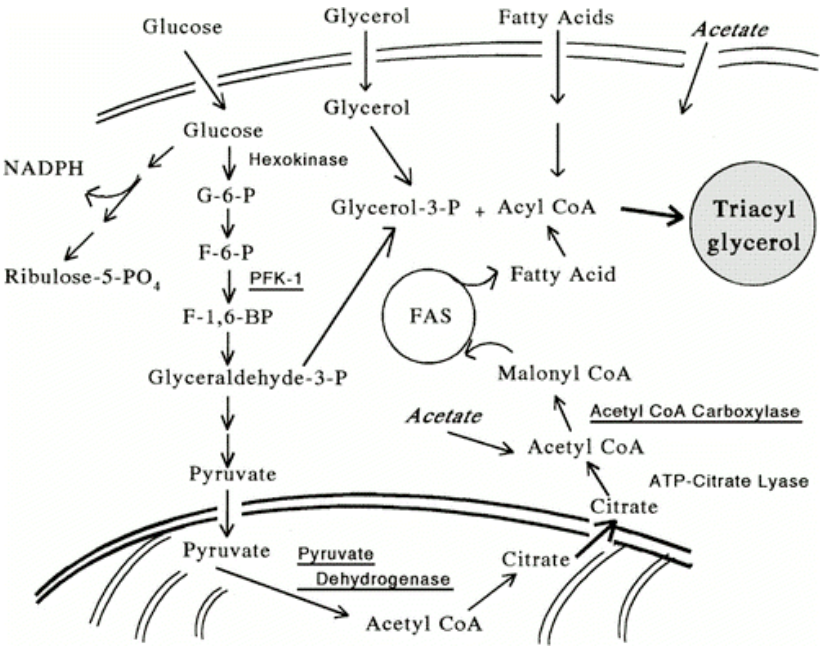


Figure 26: Summary of different pathways that lead up to TG formation (taken from:(Neville and Picciano 1997)).

Very little de novo lipogenesis occurs in individuals on a high-fat diet. Excess FA is converted to TG in the liver. Alternatively it can also be stored in the adipose tissue through the action of lipoprotein lipase on the chylomicrons or VLDL particles carrying them. The adipose tissue obtains FA through the action of lipoprotein lipase. This enzyme hydrolyses TG coming from the liver or intestine and is also implicated in de novo FA synthesis (Vernon et al. 1999). On the other hand, the intestinal mucosa is involved in handling FAs that are absorbed from diet. It is to note that the synthesized TG are exported into circulation either as chylomicrons, coming from the intestine, or as VLDL, coming from liver (Vernon et al. 1999)(Figure 27). Fatty acid synthesis occurs in the cytoplasm as compared to fatty acid oxidation occurring in mitochondria.

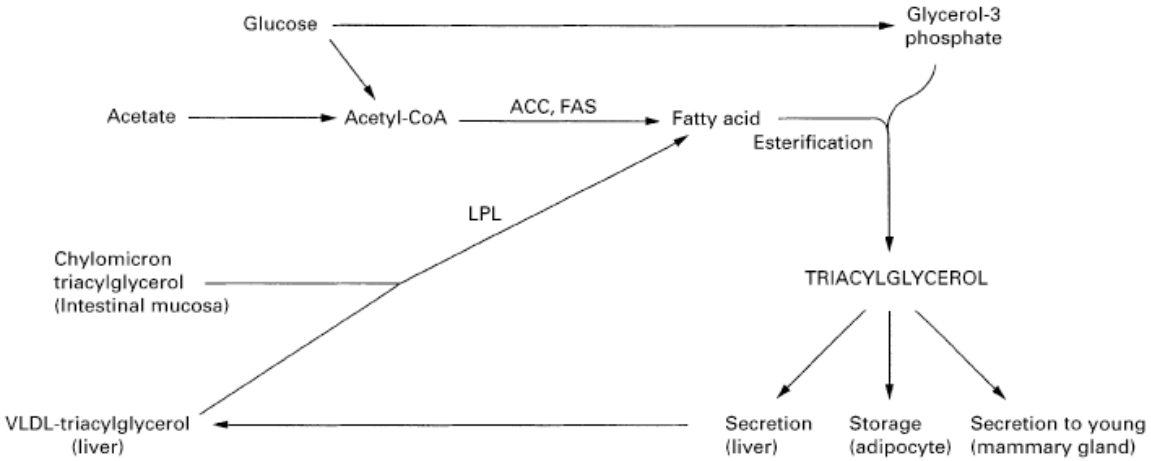


Figure 27 : Action of lipoprotein lipase on chylomicron and VLDL leads to FA release followed by TG esterification destined to secretion by the liver or storage into adipose tissue (taken from:(Vernon et al. 1999)).

The presence of insulin is indicative of a fed state, promoting pathways of storage, such as lipogenesis and FA synthesis. Insulin induces lipogenesis in the liver, while inhibiting hormone-sensitive lipase and therefore lipolysis in the adipose tissue. Consequently, this limits the release of fatty acids and glycerol, while promoting TG storage (Sakakura et al. 2001; Kraemer and Shen 2002; Postic et al. 2004). Insulin induces sterol-regulatory-element binding protein (SREBP1-c), which regulates the transcription of genes involved in cholesterol and FA biosynthesis. Activation of SREBP1-c by insulin or chREBP/Mlx by high blood glucose levels can also modulate the transcriptional effects of insulin on lipogenic or glycolytic enzymes. Examples include fatty acid synthase (FAS), ACC, pyruvate kinase and glycerol-3-phosphate acyltransferase (first step in process of TG formation from glycerol-3-phosphate) (Shimano 2001; Bildirici et al. 2003; Clement et al. 2009). PPARγ is also known to regulate fatty acid storage by stimulating lipid uptake and adipogenesis in adipose tissue (Bildirici et al. 2003).

1.7.2 Lipolysis

Hormones such as glucagon, norepinephrine, epinephrine, growth hormone, testosterone, cortisol, and leptin, as well as the AMPK pathway stimulate lipolysis. The latter occurs when energy stores need to be mobilized. It is the process by which TG are converted to FA, by lipases such as hormone-sensitive lipase (in adipose tissue) or other lipoprotein lipases. FA then go through β-oxidation producing acetyl-CoA, which can then enter the CAC (ATP production) or go into the gluconeogenic pathway (Lass et al. 2011). Fatty acids arrive in the cell, and in the cytoplasm they get linked to coenzyme A, forming fatty acyl coenzyme A. The latter is then transported by CPT-1 into the mitochondria, where it undergoes cycles of β-oxidation. Each cycle releases a molecule of acetyl-CoA and a fatty acyl-CoA shortened by 2 carbon atoms. This process continues until all is converted to acetyl-CoA, which proceeds into the CAC (Lodish 2000). Beta-oxidation occurs in the mitochondria of the cells in question, or it can also occur in peroxisome when the fatty acid chains are too long to be processed by mitochondria. The difference between β-oxidation in the peroxisome as compared to the mitochondria is that it is not coupled to ATP synthesis. More specifically, the electrons transferred on O_2 yield H_2O_2. Catalase, present in peroxisomes, converts H_2O_2 to water and oxygen (Lodish 2000). It is also believed that these long chain fatty acids may undergo initial oxidation in the peroxisomes followed by mitochondrial oxidation.

In addition, medium chain fatty acids can undergo CYP4A mediated ω-oxidation that takes place in the endoplasmic reticulum of the liver or kidney (Reddy and Hashimoto 2001). It involves oxidation on the ω-carbon, the most distant carboxyl group of the fatty acid, instead of the β-carbon, involved in β-oxidation. First step in omega-oxidation, FA go through a process of

hydroxylation by mixed function oxidases (members of CYP450 family). This is followed by oxidation of the hydroxylated FA, with alcohol dehydrogenase and aldehyde dehydrogenase (Kundu et al. 1991). At the end of this cycle, the formed dicarboxylic acid can enter mitochondria or peroxisome as a CoA-ester. It undergoes β-oxidation, producing succinic acid as an end product, which can then enter CAC or the gluconeogenic pathway (Hunt and Alexson 2008; Wanders et al. 2011). Omega-oxidation represents a minor pathway, responsible for 5-10% of FA oxidation. However, during starvation or diabetes it gains importance. Alpha-oxidation occurs in peroxisome and only its end products go on in mitochondria for full oxidation. Alpha and omega-oxidation occur when carbon-3 of the fatty acid has a methyl or other functional group attached to it (Kundu et al. 1991; Wanders et al. 2011).

TGs are transported in the blood through the means of chylomicrons or lipoproteins to tissues such as the muscle and adipose tissue. Once they reach these tissues the cellular lipoprotein lipases (LPL), found in the endothelial cells of the capillary beds of the adipose, muscle and cardiac tissues, lyse the TG from the chylomicrons or lipoproteins into glycerol and FFA. The FFA are then either taken up by the cell or transported by binding to albumin to tissues requiring energy (Brody 1999; Lodish 2000). Concerning glycerol it is absorbed by the liver and kidney. Through the action of glycerol kinase it is converted to glycerol-3-phosphate. In the liver, glycerol-3-phosphate is converted to dihydroxyacetonephosphate (DHAP) and then isomerized to glyceraldehyde-3-phosphate (GA3P). The latter can be used for glycolysis or gluconeogenesis (Berg 2002).

1.7.3 Lipoproteins

Chylomicrons belong to the family of lipoproteins, which also include very low density lipoprotein (VLDL), low density lipoprotein (LDL), high-density lipoprotein (HDL), and intermediate density lipoprotein (IDL). Chylomicrons are composed of nascent TG synthesized from absorbed FFA and monoacylglycerol along with other lipids and apolipoprotein B48 (Wasan et al. 2008). Chylomicrons are produced in absorptive cells of small intestine, more specifically in epithelial cells within the villi of the duodenum. Their purpose is to transport exogenous (i.e. dietary source) fats to appropriate tissues in order to be used for fuel or storage if in excess (Widmaier 2004; Wasan et al. 2008). VLDL is assembled in the liver from TG, cholesterol and apolipoproteins (B100, C1, E), transporting endogenous fats and cholesterol to tissues (Wasan et al. 2008). VLDL is converted to LDL in the blood. VLDL comes into contact with lipoprotein lipase, which removes TG from VLDL for storage or energy production. As TG get removed from VLDL its composition changes, and it becomes an IDL. 50% of IDL is endocytosed by liver through ApoB-100 and ApoE, and the remaining 50% lose ApoE and become LDL (cholesterol content greater than TG). LDL can then be endocytosed. Its contents are either stored, used for membrane structure, or converted to steroid hormones or bile acids. HDL has the highest protein to cholesterol ratio of all lipoproteins. The main apolipoprotein in HDL is ApoA. It is cleared from the blood by scavenger receptor B1 (SRB1), which is present on hepatocytes (Wasan et al. 2008). The role of HDL is to transport cholesterol back to the liver. It is then excreted into bile and intestine directly, or indirectly after conversion to bile acids. HDL can also be delivered to steroidogenic organs such as ovary and testes (Kingsbury and Bondy 2003) (Figure 28).

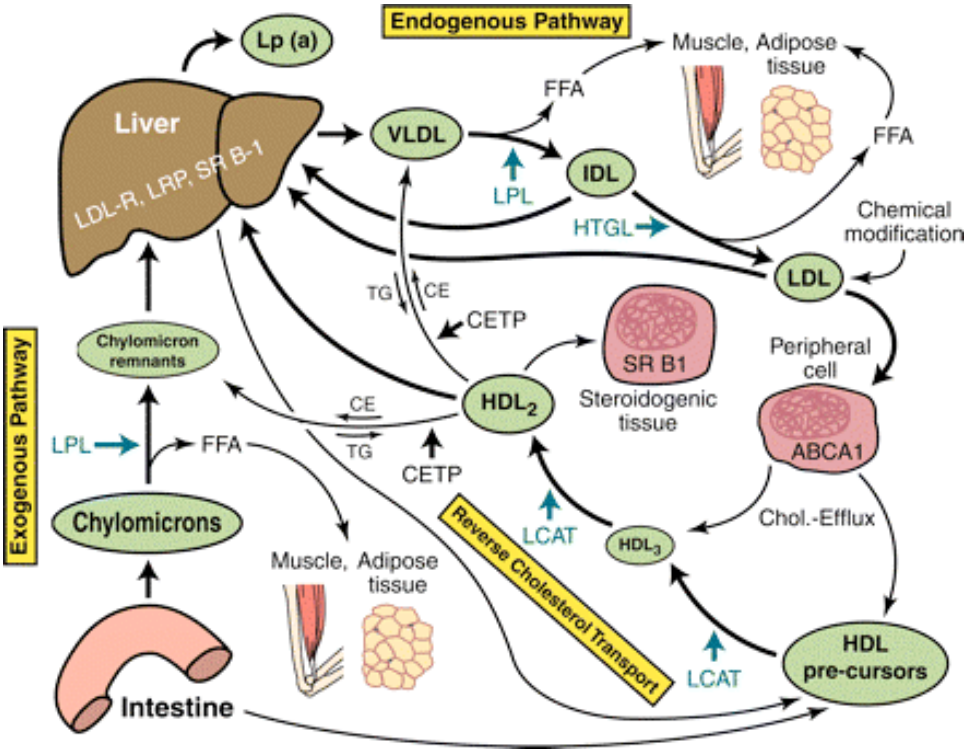


Figure 28: Summary of the formation and fate of chylomicrons and lipoproteins (Kwan et al. 2007).

1.7.4 Alternative fuel through ketogenesis

Ketogenesis occurs mainly in the mitochondria of hepatocytes under conditions of starvation, overnight fast (increased glucagon and decreased insulin secretion), low blood glucose levels and after exhaustion of glycogen stores (Voet 2004; Soeters et al. 2009). During starvation, gluconeogenesis can take place for several days. It promotes TG breakdown forming glycerol and dihydroxyacetone phosphate in the process, or increases proteolytic degradation of proteins, the major source being muscle mass (Voet 2004). There is also mobilization of FA stores from adipose tissue, where FA are broken down into acetyl-CoA through β-oxidation. All these mechanisms have the objective of restoring energy production, and eventually leading to alternate

pathways, such as ketone body formation (Voet 2004). The CAC can get overwhelmed due to large amounts of acetyl-CoA produced by β-oxidation. In addition, CAC activity can be decreased due to lack of intermediates, such as oxaloacetate used in pathways of gluconeogenesis. Therefore, this leads to acetyl-CoA being used in the biosynthesis of ketone bodies. Two molecules of acetyl-CoA through the action of thiolase form acetoacyl-CoA, which interacts with a third molecule of acetyl-CoA forming β-hydroxy-β-methylglutaryl-CoA (HMG-CoA; precursor of cholesterol biosynthesis) by HMG-CoA synthase. This leads up to the formation of ketone bodies such as acetoacetate (action of HMG-CoA lyase on HMG-CoA), and β-hydroxybutyrate (action of β-hydroxybutyrate dehydrogenase on acetoacetate)(Voet 2004)(Figure 29).

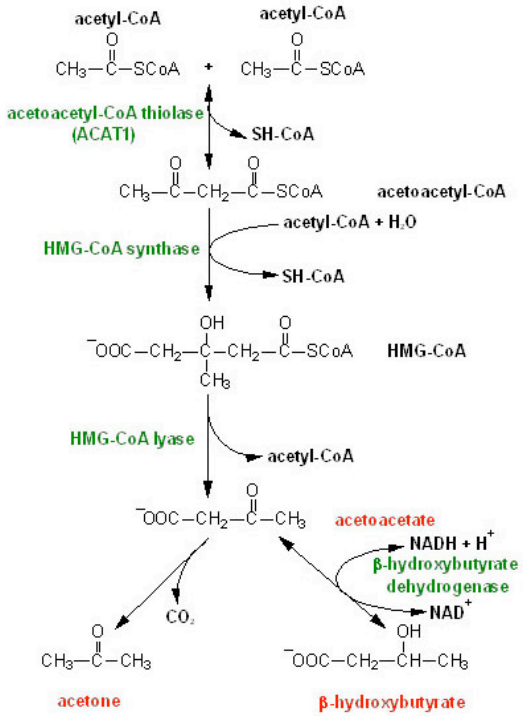


Figure 29: The pathway leading up to the formation of ketone bodies (taken from:(King 2012)).

The liver releases these ketone bodies into circulation, in order to be used as fuel in peripheral tissues, such as heart and skeletal muscle. The brain's main source of energy is glucose. However during prolonged periods of starvation, since fatty acids cannot penetrate the blood-brain barrier, the main source of fuel become the ketone bodies (Voet 2004). Once the ketone bodies have reached the peripheral tissues, they are converted by a series of enzymes to acetyl-CoA. Acetoacetate, a β-keto acid, can undergo non-enzymatic decarboxylation, forming acetone and CO_2. When it is produced faster than it is metabolized, it can lead to ketosis/ketoacidosis, giving individuals acetone-smelling breath (Voet 2004; Soeters et al. 2009). In healthy individuals, the ketogenesis pathways is kept under control. However, in patients with type 1 diabetes (lacking the presence of insulin which inhibits ketogenesis), this pathway can lead to increased amounts of acetoacetate ketone body formation, causing ketoacidosis, and a pH drop in the blood. Symptoms of ketoacidosis include vomiting, dehydration, confusion and can lead to coma. Ketoacidosis can also be present among type 2 diabetic patients, where it is termed ketosis-proned type 2 diabetes. This occurs in T2D patients, since the amount of insulin produced is insufficient, or its action on target tissues is impaired due to established state of insulin resistance, leading to decreased glucose uptake, and enhancement of the ketogenesis pathway (Voet 2004)(Figure 30).

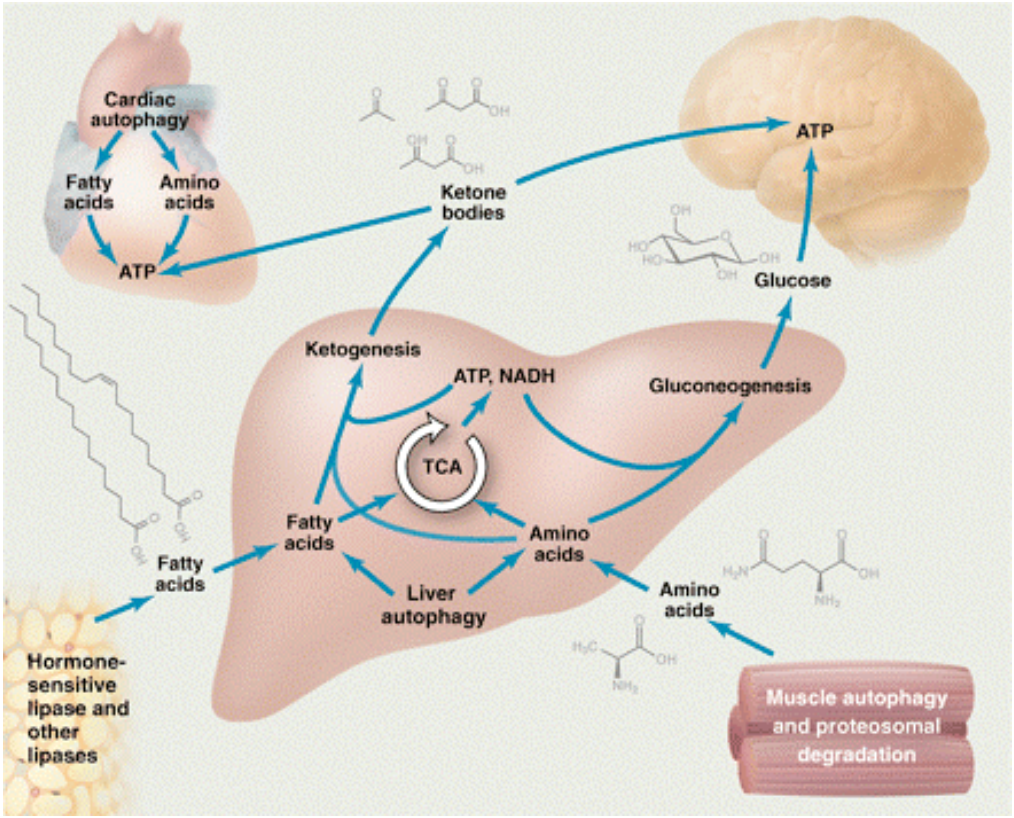


Figure 30: Ketone bodies used as a source of fuel and energy production in tissues such as the brain and the heart (taken from:(Rabinowitz and White 2010)).

1.8 The metabolic syndrome and obesity

1.8.1 The metabolic syndrome

The metabolic syndrome, also known as syndrome X, is characterized by the presence of 3 out of these 5 symptoms: central obesity defined by waist circumference or waist-to-hip ratio of respectively over 102 cm and 0.90 for males, and of over 88 cm and 0.85 for females; elevated circulating triglycerides levels (over 150 mg/dL or 1.7 mmol/L); decreased HDL (good cholesterol) levels (under 40 mg/dL or 1.03 mmol/L in males, and 50 mg/dL or 1.29 mmol/L in females); increased blood pressure (over 130/85 mmHg); and increased fasting blood glucose

levels (varies according to guidelines followed, but in general over 5.6 mmol/L) or use of medications for hyperglycemia (Alberti 2006). There are a variety of factors that are involved in the development of the metabolic syndrome. These include a sedentary lifestyle with decreased physical activity and increased caloric intake, body weight, genetics, age, and stress, which is said to increase cortisol levels, which in turn increase glucose and as a result insulin levels (Bouchard 1995; Groop 2000; Poulsen et al. 2001; Tsigos and Chrousos 2002; Grundy et al. 2004; Pollex and Hegele 2006). Hyperinsulinemia has been associated with raising blood pressure by increasing the sympathetic nervous system and release of catecholamine levels (Abdulla et al. 2011).

1.8.2 Obesity

The body mass index (BMI), which takes into account the weight and height of an individual, is used as a tool to determine the degree of obesity: between 25-30 kg/m2 an individual is considered overweight and pre-obese, over 30 kg/m2 they are considered obese, and with a BMI over 35 kg/m2 they are morbidly obese. Obesity is defined by an excess in fat accumulation not only in the adipose tissue, but also ectopically in the liver and muscle. An imbalance in the liver either of fatty acid uptake, endogenous FA synthesis, TG synthesis, FA oxidation, or TG export, increases the chances of developing non-alcoholic fatty liver disease (NAFLD) (Stienstra et al. 2007). Obesity, more specifically central, i.e. visceral adipose tissue, increases the risk of developing the many metabolic and systemic disorders that characterize or originate from the metabolic syndrome. These include T2D, dyslipidemia, and cardiovascular health problems, the latter being a major cause of mortality in these patients. Obesity is linked to

a state of chronic low-grade inflammation, initiated by morphological changes in adipose tissue. This contributes to the development of insulin resistance (Figure 31).

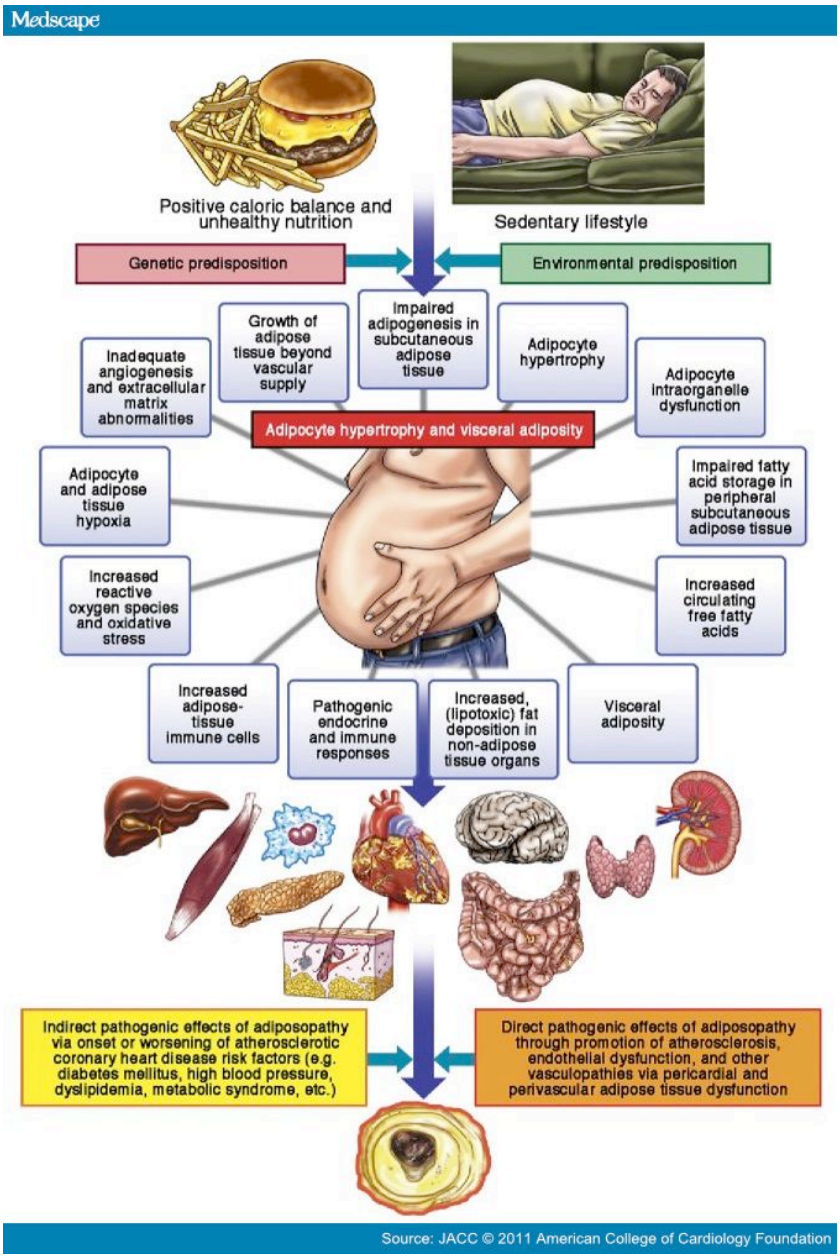


Figure 31: Summary of factors leading to obesity and its associated metabolic disturbances (taken from:(Bays 2011)).

1.8.3 Obesity and inflammation

Obesity leads to adipose tissue/adipocyte hypertrophy, stimulating necrotic and apoptotic pathways (Cinti et al. 2005; Sun et al. 2011). This upregulates the phagocytic stimuli involved in macrophage infiltration, inducing chemokines (MCP-1, CXCL14, MIP1α, MCP-2 and MCP-3), T-lymphocytes, neutrophils. Secretion of proinflammatory cytokines (TNF-α, IL-6, MCP-1, etc.) is also increased (Figure 32).

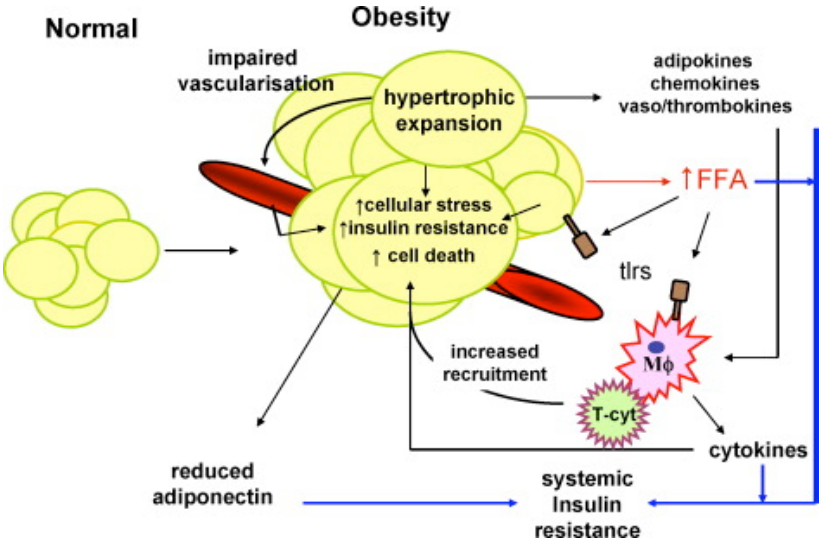


Figure 32 : Obesity lead to hypertrophy and hypoxia leading to increased secretion of pro-inflammatory cytokines and macrophage infiltration (taken from:(Morton 2009)).

Whereas, anti-inflammatory cytokines, such as adiponectin and IL-10 are down-regulated (Suganami and Ogawa 2010; Mandal et al. 2011; Sun et al. 2011)(Table 2).

Adipokine	Primary source(s)	Binding partner or receptor	Function
Leptin	Adipocytes	Leptin receptor	Appetite control through the central nervous system
Resistin	Peripheral blood mononuclear cells (human), adipocytes (rodent)	Unknown	Promotes insulin resistance and inflammation through IL-6 and TNF secretion from macrophages
RBP4	Liver, adipocytes, macrophages	Retinol (vitamin A), transthyretin	Implicated in systemic insulin resistance
Lipocalin 2	Adipocytes, macrophages	Unknown	Promotes insulin resistance and inflammation through TNF secretion from adipocytes
ANGPTL2	Adipocytes, other cells	Unknown	Local and vascular inflammation
TNF	Stromal vascular fraction cells, adipocytes	TNF receptor	Inflammation, antagonism of insulin signalling
IL-6	Adipocytes, stromal vascular fraction cells, liver, muscle	IL-6 receptor	Changes with source and target tissue
IL-18	Stromal vascular fraction cells	IL-18 receptor, IL-18 binding protein	Broad-spectrum inflammation
CCL2	Adipocytes, stromal vascular fraction cells	CCR2	Monocyte recruitment
CXCL5	Stromal vascular fraction cells (macrophages)	CXCR2	Antagonism of insulin signalling through the JAK–STAT pathway
NAMPT	Adipocytes, macrophages, other cells	Unknown	Monocyte chemotactic activity
Adiponectin	Adipocytes	Adiponectin receptors 1 and 2, T-cadherin, calreticulin–CD91	Insulin sensitizer, anti-inflammatory
SFRP5	Adipocytes	WNT5a	Suppression of pro-inflammatory WNT signalling

ANGPTL2, angiopoietin-like protein 2; CCL2, CC-chemokine ligand 2; CXCL5, CXC-chemokine ligand 5; IL, interleukin; JAK, Janus kinase; NAMPT, nicotinamide phosphoribosyltransferase; RBP4, retinol-binding protein 4; SFRP5, secreted frizzled-related protein 5; STAT, signal transducer and activator of transcription; TNF, tumour necrosis factor.

Table 2: Summary of factors that are upregulated and released during obesity (taken from:(Ouchi et al. 2011)).

The observed hypertrophy also leads to poorly oxygenated adipose tissue and therefore hypoxia. This upregulates factors such as macrophage migration inhibitory factor (MIF), matrix metalloproteinases MMP2 and MMP9, IL-6, ANGPTL4, PAI-1, VEGF and leptin. The two latter are directly regulated by the hypoxia inducible factor (HIF) (Sun et al. 2011). VEGF and ANGPTL4 are involved in angiogenesis. They are said to be the target of peroxisome proliferator activators, such as the PPARγ agonists thiazolidinediones, and to promote insulin sensitivity (regulating glucose and lipid metabolism) (Gealekman et al. 2008). PAI-1 is an inhibitor of fibrinolysis/degradation of blood clots. Its high levels in obese patients have been linked with a

higher incidence of fibrosis, arterial remodeling, hypertension, and atherothrombotic events (Vaughan 2011). PAI-1 is also reportedly high in type 2 diabetics, and can be used as a predictor of the development of this disease. However the exact causality relationship is under investigation (Vaughan 2011). Treatment with metformin or thiazolidinediones in diabetic patients effectively reduces circulating PAI-1 levels (Vaughan 2011).

1.8.4 Adipose tissue as an endocrine organ

The adipose tissue acts as an endocrine organ, able to release cytokines, also known as adipokines, into the bloodstream. This allows it to communicate with the other insulin-sensitive organs, i.e. the muscle and the liver. These adipokines, which include leptin, adiponectin, resistin and TNF-α, are secreted from the adipose tissue and function in an endocrine (regulating energy homeostasis), autocrine or paracrine manner (regulating adipocyte metabolism). Palmitoleate or palmitoleic acid (C16:1 n-7) is a monounsaturated fatty acid derived from plant and marine sources. Current studies have classified palmitoleate as an adipose tissue-derived lipid hormone, termed as lipokine (Cao et al. 2008; Lodhi et al. 2010; Mozaffarian et al. 2010; Zhang and Zhang 2010; Fabbrini et al. 2011; Fernandez et al. 2011; Yang et al. 2011; Zechner et al. 2012). This lipokine is produced during de novo lipogenesis in the adipose tissue (through the action of SCD-1 on palmitic acid/palmitate). It is a circulating factor that in animal models is involved in improving insulin sensitivity, as well as regulating glucose and lipid metabolism in insulin-sensitive tissues such as the liver and muscle. It has been associated with preventing β-cell apoptosis, promoting β-cell proliferation, and improving the circulating lipid profile (Cao et al. 2008; Lodhi et al. 2010; Mozaffarian et al. 2010; Zhang and Zhang 2010; Fabbrini et al. 2011; Fernandez et al. 2011; Yang et al. 2011; Zechner et al. 2012). It has been reported to alter the

adipocytokine profile, for example decreasing circulating levels of TNF-α and resistin, which are associated with insulin resistance. Other beneficial effects include reducing lipid accumulation in the liver. This is achieved by decreasing expression of enzymes or transcription factors involved in de novo lipogenesis and triglyceride synthesis, such as fatty acid synthase (FAS), SCD-1 (stearoyl-CoA desaturase-1), and SREBP-1. It has also been suggested that palmitoleate modifies food intake, however its exact mechanism remains unresolved (Cao et al. 2008; Lodhi et al. 2010; Mozaffarian et al. 2010; Zhang and Zhang 2010; Fabbrini et al. 2011; Fernandez et al. 2011; Yang et al. 2011; Zechner et al. 2012). The next important step is validating the aforementioned metabolic benefits of palmitoleate in humans.

1.8.4.1 Leptin

Leptin is a hormone secreted primarily from white adipose tissue (WAT). Other sources include brown adipose tissue (BAT), placenta, ovaries, skeletal muscle, stomach, gastric mucosa, bone marrow, pituitary and liver. It is secreted in levels proportional to body fat, making it a biomarker for adiposity. Its action on the hypothalamus allows for regulation of energy intake and expenditure, body temperature, body weight, and reproductive function (acting on gonadotropin-releasing hormone, luteinizing and follicle stimulating hormone) (Matarese et al. 2002; Meli et al. 2004; Hafeezullah 2006). Leptin binds to neuropeptide Y (NPY) and agouti-related protein (AgRP) neurons in the arcuate nucleus of the hypothalamus. This decreases the activity of these neurons, signaling to the body that satiety has been reached. In return, it increases expression of anorexigenic peptides POMC (proopiomelanocortin) and CART (cocaine and amphetamine regulated transcript). Orexigenic peptides, both NPY and AgRP, are involved in the regulation and stimulation of appetite. Leptin also acts on and increases the activity of α-

melanocyte-stimulating hormone (α-MSH) neurons signaling satiety. In obese individuals, leptin circulates in high concentrations in the plasma leading to leptin-receptor desensitization, consequently not allowing the body to properly perceive signals of satiety (Zimmet et al. 1996). Leptin has also been shown to activate the AMPK pathway, promoting glucose uptake, fatty-acid oxidation, inhibiting lipogenesis and ectopic fat storage in non-adipose tissues, thereby enhancing insulin sensitivity (Margetic et al. 2002; Castracane 2006; Hardie 2008)(Figure 33).

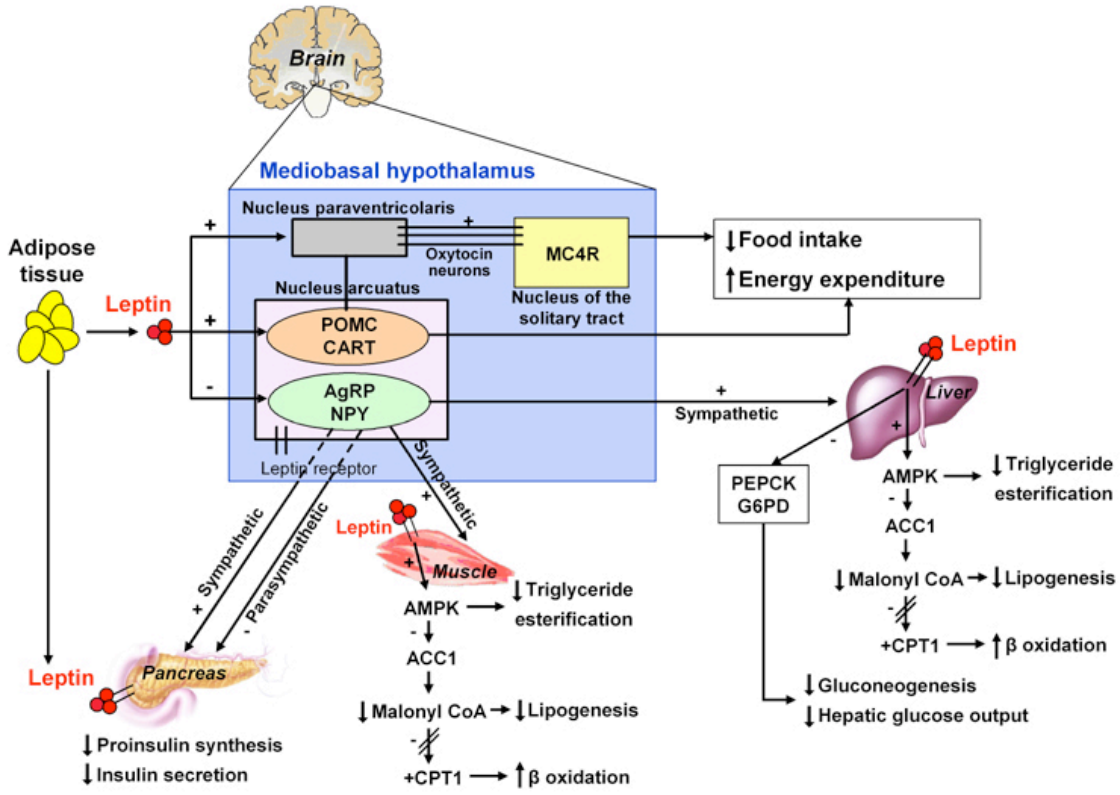


Figure 33 : Summary of the actions of leptin on peripheral tissues (taken from:(Maiorana et al. 2007)).

1.8.4.2 Adiponectin

Adiponectin is secreted into the bloodstream where it accounts for 0.01% of total serum protein. Its concentrations range from 5-10 μg/ml, being higher in females than in males (Trujillo and Scherer 2005). It was thought to be exclusively secreted from adipose tissue. However recent evidence supports that it is also expressed and secreted from cardiomyocytes and vascular smooth muscle cells, which also express adiponectin receptors (Pineiro et al. 2005; Ding et al. 2007). Adiponectin has antidiabetic, anti-atherogenic, anti-hypertensive, insulin-sensitizing properties, as well as anti-inflammatory ones. It is also involved in modulating a number of metabolic processes implicated in glucose regulation and fatty acid metabolism. As opposed to leptin, it is inversely correlated to the percentage of body fat (Ding et al. 2012). Diabetes and obesity lead to decreased circulating adiponectin levels, whereas weight loss promotes its secretion. Its expression is up-regulated during adipogenesis (Trujillo and Scherer 2005), and also by antidiabetic medications, such as the thiazolidinediones (Lindsay et al. 2002). It stimulates the AMPK pathway. This promotes glucose and fatty acid uptake (Liu et al. 2010), increases fatty acid oxidation in tissues such as the muscle and adipose tissue, and inhibits hepatic glucose production (Figure 34). Therefore, in obese individuals the low circulating adiponectin levels further contribute to the development of insulin resistance by a reduction of the beneficial effects of AMPK pathway activation (Kadowaki et al. 2008).

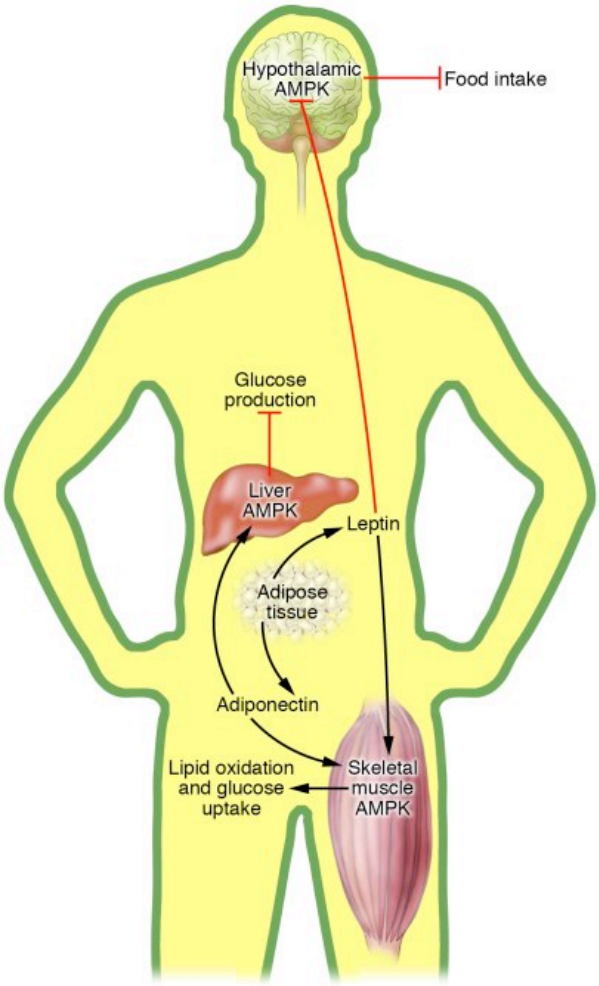


Figure 34: Adiponectin stimulates the AMPK pathway in both the liver and skeletal muscle, decreasing glucose production, promoting lipid oxidation and glucose uptake (taken from:(Long and Zierath 2006)).

1.8.4.3 Pro-inflammatory cytokines, particularly TNF-α

Increased visceral fat and adipocyte hypertrophy increase the degree of inflammation, through macrophage infiltration into WAT. This leads to production of pro-inflammatory cytokines (Stienstra et al. 2007). Examples include interleukins 1b, 6, and 8 (IL-1β, IL-6, IL-8), and monocyte-chemoattractant protein-1 (MCP-1), MIF (proinflammatory process), MIP

(regulates leukocyte migration and infiltration in inflammatory process), as well as C-reactive protein (biomarker of system inflammation)(Fruhbeck 2008). MCP-1 is similar to resistin in that it is secreted by the adipose tissue, increases with obesity, and has immune modulating functions (Stienstra et al. 2007).

The most important cytokine, however, is called tumor necrosis factor-alpha (TNF-α). It is secreted in levels proportional to adipose tissue size, while also regulating cell size. This means that as adipocytes get bigger, TNF-α levels increase. This in turn initiate changes to limit adipocyte size or induce apoptosis (Coppack 2001). Hence it is implicated in regulating its own expression, while increasing the synthesis of other cytokines such as IL-6. TNF-α and IL-6 decrease lipid accumulation in adipose tissue. This is achieved through the inhibition of lipoprotein lipase and stimulation of hormone-sensitive lipase, which induces uncoupling protein expression (Coppack 2001). TNF-α contributes to the development of insulin resistance by downregulating insulin-stimulated glucose uptake. This occurs through a reduction of Glut4 expression, or through effects on insulin receptor or IRS-1 (Coppack 2001). Its actions on the insulin signaling pathway include lowering tyrosine phosphorylation of the insulin receptor, and increasing serine phosphorylation at 307 through phosphorylated IKKβ (well-known downstream kinase of TNF-α) or even JNK, or by phosphorylating other serine or threonine residues of IRS-1 (Coppack 2001; Jiang et al. 2003; Gual et al. 2005; Huang et al. 2009). IKKβ can also be activated by PKC isoforms, which are involved in IRS-1 ser/thr phosphorylation in response to FFA concentrations. FFA activate PKC, which goes on to stimulate IKK, or they can directly act on JNK, with the end result being serine phosphorylation (Ser307) on IRS-1 (Gual et al. 2005). It is to note, that salicylates (found in *Populus balsamifera*, one of the plant extracts studied in this thesis) inhibit IKKβ. This can lead to an improvement in glucose tolerance by preventing the

decrease in tyrosine phosphorylation (Gual et al. 2005). TNF-α has also been reported to inhibit CCAAT-enhancer binding protein and PPARγ, which in turn decrease the expression of genes such as FABP, FAS, ACC, glucose-6-phosphate dehydrogenase, as well as lipoprotein lipase and Glut4 (Coppack 2001). The inflammatory cytokine increases the mobilization of lipids by activating hormone sensitive lipase, thereby increasing lipolysis of TG in the adipose tissue and releasing FFAs into circulation. This promotes ectopic fat storage. IL-1 and IL-6 are also involved in increasing lipolysis and inhibiting lipoprotein lipase (Coppack 2001)(Figure 35).

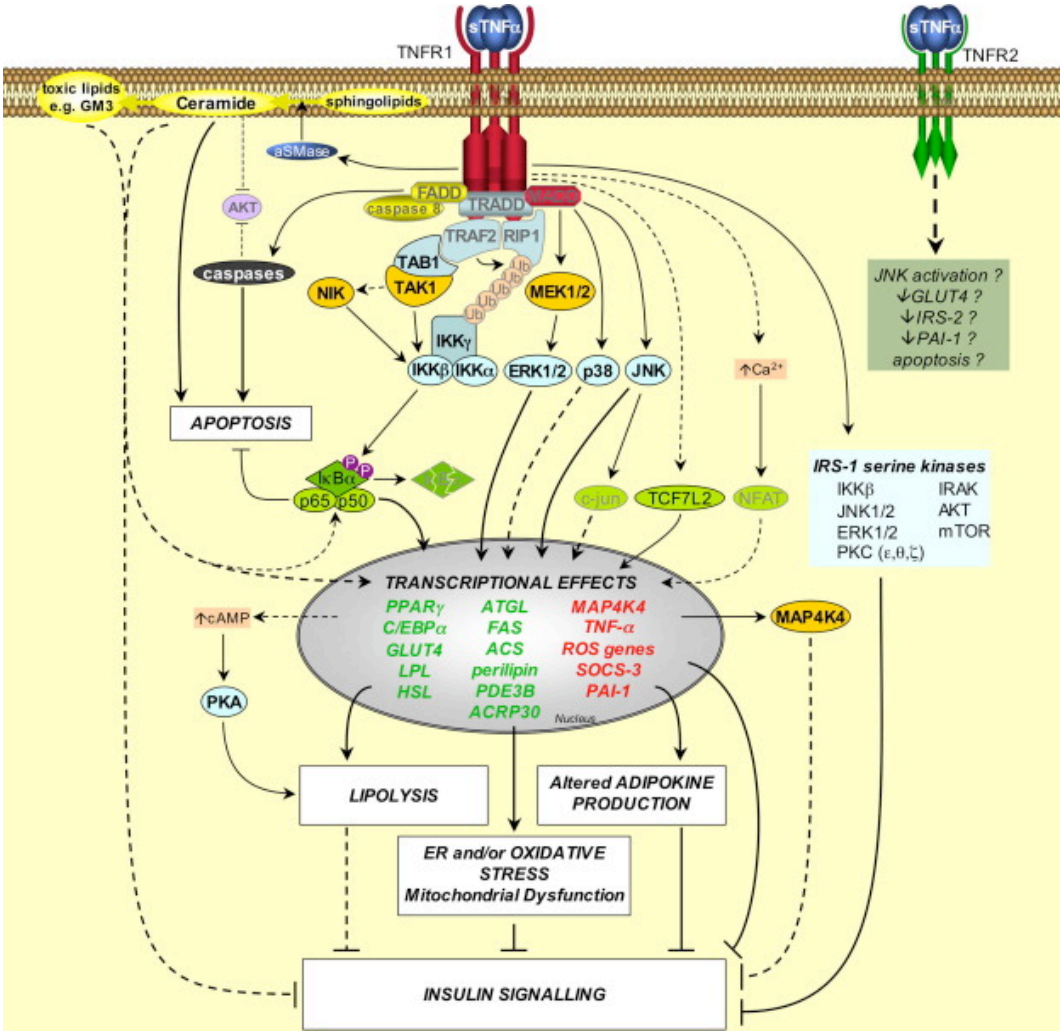


Figure 35 : TNF-α leads to insulin resistance by acting on various downstream effectors (taken from:(Cawthorn and Sethi 2008))).

1.8.4.4 Resistin

Resistin is produced and released by adipose tissue, as well as mammary gland, heart, brain, lungs, and gastrointestinal tract (Pantsulaia et al. 2007). It is said to play a role in inflammation, energy homeostasis (not completely well-defined), and insulin resistance (Vasudevan 2011). There have also been studies that have revealed that resistin induces β-cell apoptosis in rat insulinoma RINmF5 cells, whereas in many cell lines it can lead to a reduction of Akt phosphorylation and attenuation of insulin signal (Gao et al. 2009). There are many contradictory studies on the role of resistin on obesity and T2D. However, in many cases, there have been reported increased levels present in obese patients (particularly with central obesity), associated with increased insulin resistance. It is also said to be implicated in the transcriptional events that lead up to increased expression of pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6 (Calabro et al. 2009).

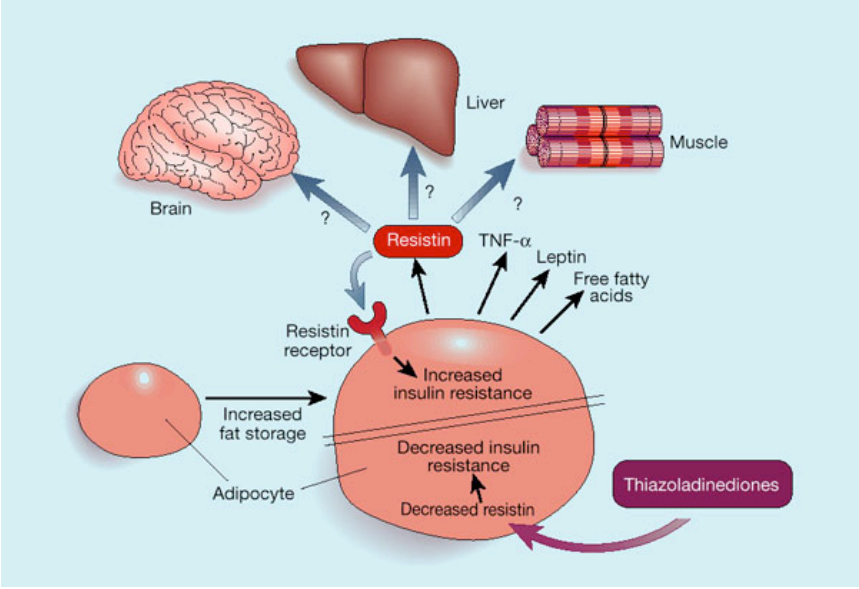


Figure 36: Resistin leads to insulin resistance and increases expression of pro-inflammatory cytokines (taken from:(Flier 2001)).

In addition, transcription of the resistin gene is decreased in subjects treated with agonists of PPARγ, such as the antidiabetic Tzd medications, thereby contributing to increase the action of insulin (Kumar 2003; Calabro et al. 2009)(Figure 36).

1.8.4.5 Newly identified inflammatory cytokines

There are many other newly identified inflammatory cytokines that are being investigated for their role in obesity and type 2 diabetes. Retinol binding protein 4 (RBP4), a hepatocyte-secreted factor that transports retinol (vitamin A) throughout the body, has also been reported to be released from adipocytes and macrophages (Ouchi et al. 2011). Its expression is inversely correlated with that of Glut4. Its presence inhibits insulin-induced IRS-1 phosphorylation. Therefore increased RBP4 serum concentrations are associated with features of the metabolic syndrome: increased blood pressure, low HDL levels, increased cholesterol and triglyceride levels, and increased body mass index/BMI (Ouchi et al. 2011). Mostly produced by visceral adipose tissue in states of obesity and insulin resistance, RBP4 can be used as a marker of intra-abdominal adipose tissue expansion and subclinical inflammation (Ouchi et al. 2011). Lipocalin 2 is also known as neutrophil gelatinase associated lipocalin or 24p3. It belongs to the lipocalin protein family, which also includes RBP4. It is expressed abundantly in adipose tissue, is induced by inflammatory stimuli, and positively correlates to the degree of obesity and adiposity, hyperglycemia, insulin resistance and C-reactive protein (Ouchi et al. 2011).

ANGPTL2 is also a marker that is positively associated with adiposity, markers of insulin resistance and C-reactive protein levels, and it activates inflammatory and phagocytic (monocytes and macrophages) responses (Ouchi et al. 2011).

CXCL5 is associated with obesity, insulin resistance, and inflammation. It is expressed at high levels in adipose tissue. It decreases insulin-induced glucose transport in the muscle by activating the JAK/STAT pathway and increasing expression of SOCS2, which inhibits insulin signaling cascade (Chavey et al. 2009).

NAMPT, also known as pre-B cell colony enhancing factor or visfatin, is expressed by adipose tissue. It circulates in high levels in patients with obesity and type 2 diabetes. Its serum levels positively correlate to IL-6 and C-reactive protein levels(Ouchi et al. 2011). However, there have been conflicting results regarding its physiological role, since some studies report that it has glucose-lowering properties and promotes insulin secretion from pancreatic β-cells (Revollo et al. 2007).

1.8.4.6 Obesity treatments

The best way to prevent or even treat obesity is through diet and exercise. In extreme cases of obesity, bariatric surgery may be recommended or even necessary. However, many antiobesity treatments are available on the market or currently under investigation in clinical trials. They are geared to control or decrease body weight, by altering either appetite, metabolism, or interfering with the body's ability to absorb certain nutrients (Melnikova and Wages 2006). Throughout history, mankind has focused on finding the ultimate solution to weight loss, these included the use of laxatives, purgatives, thyroid hormone in euthyroid individuals, dinitrophenol (DNP), amphetamines, Fen-phen, and ephedra. Many of these treatments were removed from the market due to their serious adverse effects (Valentino et al. 2011). For example, DNP, which uncouples oxidative phosphorylation from ATP production producing heat instead, lead to warmth and frequent sweating, and in some cases to fatal

hyperthermia (Valentino et al. 2011). It can even cause agranulocytosis (leucopenia where individuals lack major class of infection-fighting white blood cells), dermatitis, and cataracts (Valentino et al. 2011). Those taking thyroid hormone had hyperthyroid-like symptoms such as palpitations, and difficulty sleeping. Amphetamines (enhance release of neurotransmitters) used for their appetite-suppressing effects and increased alertness were very popular and eventually became a part of the 'rainbow pill regime', which included amphetamines, thyroid hormone, diuretics, digitalis, laxatives and barbiturates (to suppress the stimulant effects of the amphetamines) (Valentino et al. 2011). Fen-phen, a combination of fenfluramine and phentermine, was very popular in the 90's, but was removed from the market in 1997 due to reports of valvular heart disease (Valentino et al. 2011). Ephedra was removed because of concerns that it increased blood pressure and the risk of stroke and death (Valentino et al. 2011).

More recent antiobesity treatments include Orlistat, sibutramine, rimonabant, and exenatide (Valentino et al. 2011). Orlistat (Xenical®), a lipase inhibitor, acts by inhibiting intestinal fat absorption through the inhibition of pancreatic lipase. This treatment comes with undesirable side-effects such as oily bowl movements (can be decreased if dietary fat content is reduced), stomach pain, and flatulence (Bays 2004; Valentino et al. 2011). Sibutramine (Meridia®) is a neurotransmitter reuptake inhibitor, increasing concentrations of serotonin, norepinephrine and dopamine in the synapse, resulting in decreased appetite (satiety), and increased thermogenesis (Li and Cheung 2009). It has been pulled off the market because its risks outweigh the benefits. Indeed, it increases blood pressure, and can cause myocardial infarction, stroke, dry mouth, constipation, headache and insomnia (Li and Cheung 2009). Rimonabant is a CB1 receptor antagonist, acting centrally in the brain by decreasing appetite, and peripherally by increasing thermogenesis and energy expenditure (Li and Cheung 2009; Verty et al. 2009). It has not been approved for use in Canada or USA due to serious psychiatric adverse effects (ex.

depression and suicide) (Valentino et al. 2011). TM38837 is in clinical trials, and is an inverse agonist of the CB1 receptor (acts in periphery) (Sink et al. 2009). Exenatide (Byetta®) is a long-acting GLP-1 analogue. GLP-1 is secreted by the intestine L-cells in the presence of food, delaying gastric emptying and promoting the feeling of satiety (Bays 2004; Hainer 2011; Valentino et al. 2011). The disadvantage of this treatment is that it needs to be injected twice a day and causes severe nausea, and gastrointestinal symptoms.

There are numerous treatments that are being researched and are undergoing clinical trial for their antiobesity potential. These include pramlintide (symlin), ZGN-433, lorcaserin, new combinations of selective serotonin reuptake inhibitors (SSRIs) with phentermine, and other lipase inhibitors such as GT 389-255 (without the oily stools side-effect as seen with Orlistat). Pramlintide is administered subcutaneously and is a synthetic analogue of the hormone amylin, secreted by the pancreas in response to food intake, delaying gastric emptying while promoting satiety (Bays 2004; Li and Cheung 2009; Valentino et al. 2011). Although it is approved for use in combination with insulin for the treatment of T1D and T2D, it is now being evaluated for its antiobesity potential (Bays 2004; Pullman et al. 2006; Li and Cheung 2009; Valentino et al. 2011). ZGN-433, also known as beloranib, an analog of the natural compound fumagillin (known for its anti-angiogenic properties), is an inhibitor of methionine aminopeptidase (METAP2; binding to its active site). Through a mechanism that remains unclear at this point, it re-establishes the balance in the metabolism of fat, decreasing body weight and retroperitoneal fat pad weight, while restoring glycemia in the process (Powell et al.). Lorcaserin is a selective agonist of the 5-HT2c receptor, found exclusively in the brain in regions such as the hypothalamus. Activation of these receptors leads to increased production of POMC increasing satiety and promoting weight loss (Bays 2009). FDA did not approve the use of lorcaserin due to

cancer promoting properties that could not be ruled out, and for its marginal effect on weight loss (Alavi 2010; Colman 2010; Valentino et al. 2011).

There are many potential treatment avenues currently being investigated for their use against obesity. There have been studies that demonstrate the importance of studying the interactions between nuclear hormone receptors and regulatory cofactors. For example, when the interaction between SMRT (nuclear hormone corepressor) and the nuclear hormone receptor proteins was inhibited, this lead to increased adiposity and decreased metabolic rate (Nofsinger et al. 2008). Another interesting venue is the use of ribonucleic interference (RNAi), in silencing genes implicated in fat metabolism. One gene that has been explored in mouse models is the RIP140 gene, a nuclear hormone corepressor, which regulates fat accumulation. Genetically manipulated mice devoid of RIP140 exhibited a lean profile, were resistant to diet-induced obesity, and increased metabolic rate (Leonardsson et al. 2004; Christian et al. 2006; Kiskinis et al. 2007).

1.9 Diabetes classifications and antidiabetic treatments

1.9.1 Diabetes prevalence, symptoms, diagnosis

Diabetes mellitus, generally referred to as diabetes, currently affects 346 million people worldwide (WHO 2011a). It is defined by increased blood glucose levels due either to inadequate insulin secretion or to the lack of response by the cells to the insulin produced. Individuals experience symptoms such as polyuria (increase urination), polydypsia (increase thirst), polyphagia (increased appetite), and weight loss, as well as vision changes, and fatigue (WHO 2011a).

A person is diagnosed as diabetic when they have a fasting blood glucose concentration of 7 mmol/L (126 mg/dL) or more, or when their glycemia during random testing is 11 mmol/L (200 mg/dL) or more (WHO 2006). Glycated hemoglobin (HbA1C) is also used in the diagnosis of diabetes, giving an index of average blood glucose levels over a period of 120 days (lifespan of erythrocytes) (Fourlanos et al. 2006). Individuals with an HbA1C of over 6.5% are considered diabetic (ADA 2010b). An oral glucose tolerance test (OGTT) consists in the administration of an oral glucose load of 75g after an overnight fast followed by measuring blood glucose levels after 2h. If glycemia levels are over 11 mmol/L (200 mg/dL) two hours after an OGTT, the individual is considered diabetic (WHO 1999; WHO 2006; Goldstein 2008; Vijan 2010). If, however, they are between 7.8 mmol/L (140 mg/dL) and 11 mmol/L (200 mg/dL) the individuals are considered as having impaired glucose tolerance (IGT) (WHO 1999; WHO 2006; Goldstein 2008; Vijan 2010). On the other hand, if fasting levels range from 5.6 to 6.9 mmol/L (100 to 125 mg/dL), they are considered as having impaired fasting glucose (IFG) (WHO 1999; WHO 2006; Goldstein 2008; Vijan 2010). Both IGT and IFG are considered prediabetic conditions, yet only individuals with impaired glucose tolerance have a higher risk of developing full-blown diabetes and the complications that arise from it (WHO 1999; WHO 2006; Goldstein 2008; Vijan 2010).

1.9.2 Type 1 diabetes

Type 1 diabetes (T1D), also known as insulin-dependent diabetes mellitus (IDDM) or juvenile diabetes, results in increased blood and urine glucose levels, and in some cases ketoacidosis. This is due to a lack of insulin, caused either by autoimmune or idiopathic destruction of β-cells of the islets of Langerhans in the pancreas. T1D corresponds to 5-10% of all cases of diabetes, and is divided into two subtypes: type 1A, corresponding to autoimmune destruction of β-cells, and type 1B idiopathic, of unknown origin (Imagawa et al. 2000; ADA

2004; ADA 2009). Genetics and environmental factors are implicated in the development of T1D. There are many genes (polygenic) involved in the onset, for example HLA-DQ or DR gene mutations, HLA being cell surface receptor on antigen presenting cells and involved in antigen presentation. In terms of environmental factors, they include viral infections (Fairweather and Rose 2002), where the immune system attacks the virus and the β-cells in the pancreas. Dietary components influencing the gut flora, intestinal permeability and immune function of the gut, may lead to β-cell autoimmunity and to the progression of T1D (Knip 2009). Chemicals (toxins) such as streptozotocin (zanosar), used as an antibiotic and antineoplastic agent (chemotherapy for pancreatic cancer), kills β-cells resulting in decreased insulin production(Bolzan 2002). Other factors that may lead to diabetes are diseases of the exocrine pancreas, where damage to the pancreas is extensive. Examples of such problems include trauma to the pancreas, pancreatitis, and tumors (malignant or benign) (ADA 2004; ADA 2010a). It is to note that autoantibodies can be detected prior to the onset of hyperglycemia, and can be present for 5-10 years before the development of T1D (Knip et al. 2005). The antibodies can target islet cells, insulin, the 65 kDa glutamic acid decarboxylase (GAD; responsible for pancreatic GABA synthesis with a role on the endocrine function of pancreas)(Franklin and Wollheim 2004), tyrosine phosphatase IA-1 and IA-2b (suggested role in pancreatic development and insulin secretion), and other antigens. (Roberts et al. 2001; ADA 2010a). Within this time frame, some individuals may develop T1D as adults, often misdiagnosed as T2D because of their age, creating another subtype of diabetes termed latent autoimmune diabetes of adults (LADA). LADA appears after age 35 and is associated with other autoimmune diseases, such as autoimmune thyroid, celiac disease, rheumatoid arthritis, pernicious anemia, and autoimmune hepatitis (Fourlanos et al. 2006; Dib and Gomes 2009). Even though T1D is not preventable, there exist many treatments to control

hyperglycemia and to minimize the long-term complications associated with it. These include the utilization of immunosuppressive drugs, insulin therapy, pancreas transplantation, and islet cell transplantation (Shapiro et al. 2000). There have even been reports that breastfeeding (affecting microbial flora), as well as administration of vitamin D (acts as immunosuppressive) in early childhood decreases the risk of developing T1D (Akerblom et al. 2005; Wahlberg et al. 2006; Holmberg et al. 2007; Rosenbauer et al. 2008; Siljander 2010).

1.9.3 Type 2 diabetes

Type 2 diabetes (T2D), also referred to as non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes, accounts for 90-95% of all cases of diabetes. It is characterized by hyperglycemia, insulin resistance, and relative insulin deficiency (Patel and Macerollo 2010). An individual is diagnosed with T2D if they have a single raised glycemia level along with presence of symptoms (similar to those observed with T1D), or if they have increased blood glucose levels on two separate occasions (fasting over 7 mmol/L and OGTT over 11 mmol/L)(Patel and Macerollo 2010).

There are many culprits that lead to the development of T2D. These include lifestyle (high-caloric diet and physical inactivity) changes, and environmental factors (epidemiological, industrialized countries or those with recent increased wealth as seen in Asia) (Caterson et al. 2004; Scholl 2012). Genetic mutations (polygenic) are also involved. Examples include mutations in the TCF7L2 gene, involved in regulating expression of proglucagon and glucagon like peptide-1 (GLP-1), or in the KCNJ11 gene, which encodes islet ATP-sensing K+ channel Kir6.2 (Kirkpatrick et al. 2010). Other culprits include medications, and certain medical

conditions, such as the metabolic syndrome, chronic pancreatitis, cancer (NDIC 2008; NIDDK 2011).

Many factors lead to β-cell dysfunction, and therefore to insulin deficiency in T2D. These include: increased demands of insulin secretion induced by insulin resistance, or age-related impairment, or deposition of the islet amyloid polypeptide (IAPP or amylin). IAPP leads to β-cells becoming refractory to the glucose signals or even to β-cell apoptosis (Hoppener and Lips 2006; Ritzel et al. 2007). IAPP is a neuroendocrine hormone and member of the calcitonin family of polypeptide hormones. Under normal conditions, amylin (produced by β-cells) is secreted along with insulin in response to meal ingestion. However hyperinsulinemia leads to increased IAPP production which deposits as amyloid fibrils in β-cells and is toxic to them (Marzban et al. 2003; Marzban 2004; Valentino et al. 2011). Glucotoxicity and lipotoxicity of the β-cell also lead to decreased glucose-induced insulin secretion, impaired insulin gene expression, and increased β-cell death (Robertson et al. 2004). Since obesity leads to 55% of observed cases of T2D, increasing physical activity, reducing consumption of saturated or trans fats and replacing them with unsaturated fats, helps prevent or treat T2D (Eberhardt 2004). In extreme cases, even gastric bypass surgery proves to be extremely beneficial. There are also many antidiabetic therapies available that can be administered in combination with insulin, to help manage hyperglycemia.

T2D was also known as adult-onset diabetes, since it was more common among those of middle age or older (due to decreased lean mass, increased accumulation of abdominal fat, and decreased tissue sensitivity to insulin). However, it is now being seen more frequently in children and young adults due to the increased prevalence of obesity in this demographic group (CDC 2001; Copeland et al. 2005; Snijder et al. 2006). Lean individuals may also develop diabetes and this may be due to genetics, fatty liver disease, inflammation, or autoimmunity (more likely

qualified as LADA and often misdiagnosed as T2D). Even stress, during which time excessive amounts of cortisol are released, increases blood sugar. Cortisol release is beneficial in times of fight or flight, but detrimental when secreted chronically, eventually leading to T2D.

1.9.3.1 Insulin resistance and diabetes

Insulin resistance is characterized by an inability of normal levels of insulin to maintain blood glucose levels in check, leading to impaired glucose tolerance expressed as postprandial hyperglycemia. This stimulates β-cells to secrete more insulin, causing hyperinsulinemia, which compensates for the decrease in insulin sensitivity (Leslie 1993; Kumar 2003). As diabetes progresses, the β-cells are no longer able to produce enough insulin, due to β-cell exhaustion, intrinsic β-cell defect (lose glucose-recognition ability), and loss of 20-50% in β-cell mass (Bonner-Weir 2000; Porte and Kahn 2001; Kumar 2003). Insulin resistance consists in a diminished response of insulin-sensitive tissues, such as liver, muscle and adipose tissue, to physiological concentrations of insulin. This may be due to a variety of qualitative or quantitative defects. Examples include changes in insulin receptor content or function, or at the level of certain intracellular mechanisms (ex. phosphorylation on serine instead of tyrosine residues on targets downstream of insulin receptor activation) (Kumar 2003), or as mentioned can occur at the level of the β-cell (affecting insulin production or release). Environmental factors such as age, obesity and level of exercise are involved in the development of insulin resistance and T2D.

Obesity, especially central obesity, is linked to a state of low-grade inflammation. This implies that there is dysregulation of a variety of adipokines and inflammatory cytokines that are implicated in the development of insulin resistance and T2D (Zimmet 1982; Manson 1991; Shimokata 1991; Leslie 1993). Obesity also gives rise to an atherogenic lipoprotein profile,

increased TG in circulation, and increased FFA (result of increased lipolysis from the adipose tissue), as well as increased LDL and decreased HDL (van de Woestijne et al. 2011). All these metabolic abnormalities favor increased ectopic fat storage in organs such as the liver, muscle, and β-cells. The process downregulates crucial intracellular mechanisms, involved in glucose production (liver), glucose uptake (muscle), or glucose-sensing (pancreas)(Yaney 2003). It is to note that the inflammatory cytokines such as TNF-α, along with FFA activate either JNK/SAPK (Prada et al. 2005), IKKαβ, or PKC pathways (Kim et al. 2004), that are all implicated in phosphorylating serine residues on the IRS, in turn downregulating the insulin pathway (Cusi 2010).

It is to note that branched-chain amino acids (BCAA) and their related metabolites are also strongly associated with the incidence, progression, and remission of insulin resistance, diabetes, and cardiovascular disease (Newgard 2012). BCAA, which include leucine, isoleucine, and valine, are essential amino acids acquired by the body through the ingestion of specific foods or as supplements. Amino acids, the building blocks of proteins, are required for normal growth, tissue repair and maintenance, hormone production, immune function, as an energy source (during starvation). BCAA metabolism is strongly linked to circulating levels of insulin, as well as to the action of insulin on tissues (liver, muscle, adipose tissue) (Adeva et al. 2011). In healthy individuals, insulin facilitates disposal of amino acids into peripheral tissues. Whereas in diabetics and obese individuals, where there is presence of insulin deficiency or resistance to insulin action, BCAA metabolism is disturbed and their plasma concentrations are increased (Pereira et al. 2008; Adeva et al. 2011). BCAA act similarly to FA in that they both interfere with insulin signaling and secretion, promoting the development of insulin resistance. There have been reports that BCAA chronically activate mTOR, p70-S6K-1, c-Jun N-terminal kinase (JNK), and

phosphorylate Ser307 on IRS-1, all leading to the downregulation of the insulin receptor pathway (Ha and Zemel 2003; Adeva et al. 2011; Newgard 2012). Excess of BCAA and FA can also clog β-oxidative pathways, filling the TCA cycle in a process called anaplerosis. This can lead to decreased glucose utilization and eventually glucose intolerance. It has also been stated that chronically elevated levels of BCAA and its related metabolites may synergize with increased levels of FA to promote chronic hyperinsulinemia. This leads to increased secretory pressure on β-cells and may contribute to β-cell dysfunction, observed in T2D (Newgard 2012). BCAA concentrations can also be strongly influenced either from ones diet, genetic variations in catabolic enzymes, as well as variations in gut microbiome (bacterial species are capable of de novo BCAA synthesis) (Newgard 2012). There has been evidence suggesting that increased plasma BCAA levels occur years before diabetes is diagnosed, reinforcing their predictive value (Adeva et al. 2011; Valerio et al. 2011). In addition, plasma concentrations of BCAA and their metabolites are highly responsive to intervention outcomes, which are reported to enhance the BCAA catabolic pathway. Such examples include antidiabetic medications (for example thiazolidinediones) or even gastric bypass surgery (Adeva et al. 2011; Newgard 2012).

1.9.3.2 Complications arising from T2D

If left unmanaged or if managed improperly, T2D gives rise to long-term complications, such as retinopathy, neuropathy, nephropathy, loss in cognitive ability, and to cardiovascular complications (ADA 2010b; ADA 2010a). These microvascular complications can be the result of the activation of the polyol pathway, advanced glycation end product formation (AGEP), PKC activation, VEGF, reactive oxygen species (ROS) production, and the hexosamine pathway. Hyperglycemia leads to activation of the polyol pathway, activating aldose reductase and

consuming NADPH, which leads to decreased glutathione reductase activity, and therefore increased oxidative stress and likelihood of developing cellular damage (Nathan 1993; Cumbie and Hermayer 2007). This pathway's derived intermediates, along with non-enzymatic reactions (Maillard) between amino groups and glucose or highly reactive glucose derivatives (dicarbonyls), can also lead to the production of AGEP. AGEPs are heterogeneous group of modified proteins, lipids and nucleic acids, which alter intracellular and extracellular proteins and their function. They are implicated in the aging process and in diabetes. AGEPs bind to receptors of AGEP, and lead to NF-kβ mediated production of pro-coagulatory and pro-inflammatory cytokines, and to increased vascular permeability (Nathan 1993; Cumbie and Hermayer 2007). Hyperglycemia can also lead to increased de novo DAG formation (from glyceraldehyde-3-phosphate), which is an upstream activator of the majority of PKC isoforms, such as PKCβ and δ. PKC can also be stimulated by AGEP and hyperglycemia-induced superoxides. PKCβ and δ activation are responsible for the deleterious effects observed in the retinal, neural and renal tissues. They do so by impairing blood flow, increasing vascular permeability, and increasing inflammatory cytokine production (Nathan 1993; Cumbie and Hermayer 2007). The PKC pathway can also lead to increased production of vascular endothelial growth factor (VEGF), which is implicated in retinopathy (increased VEGF levels in vitreous humor), through its action on VEGF-1 and VEGF-2 tyrosine kinase receptors, stimulating angiogenesis, capillary permeability and leakage. Increased VEGF levels may also be implicated in the development of nephropathy (Nathan 1993; Cumbie and Hermayer 2007). Hyperglycemia leads to mitochondrial electron transport chain overload, which induces mitochondrial superoxide overproduction, possibly activating all four pathways involved in hyperglycemia damage, i.e. the polyol pathway, hexosamine pathway, PKC isoform activation, and AGEP formation (Nathan 1993; Cumbie and

Hermayer 2007). In the presence of high concentrations of glucose, some of it gets diverted from the glycolytic pathway to that of hexosamine. In the hexosamine pathway, fructose-6-phosphate (from glycolysis) is converted to glucosamine-6-phosphate and UDP-N-acetyl-glucosamine by fructose-6-phosphate amidotransferase (Brownlee 2005). The end products of the hexosamine pathway can lead to pathologic changes in gene expression, for example increasing expression of PAI-1, leading to vascular complications in diabetics (Brownlee 2005). Macrovascular complications associated with T2D include peripheral arterial disease, stroke (increase risk by 150-400%), and coronary artery disease. There is also an increased incidence of myocardial infarction due to the context in which T2D occurs, i.e. abdominal obesity, hypertension, hyperlipidemia, and increased coagulability, all defining parameters of the metabolic syndrome (Fowler 2008)(Figure 37).

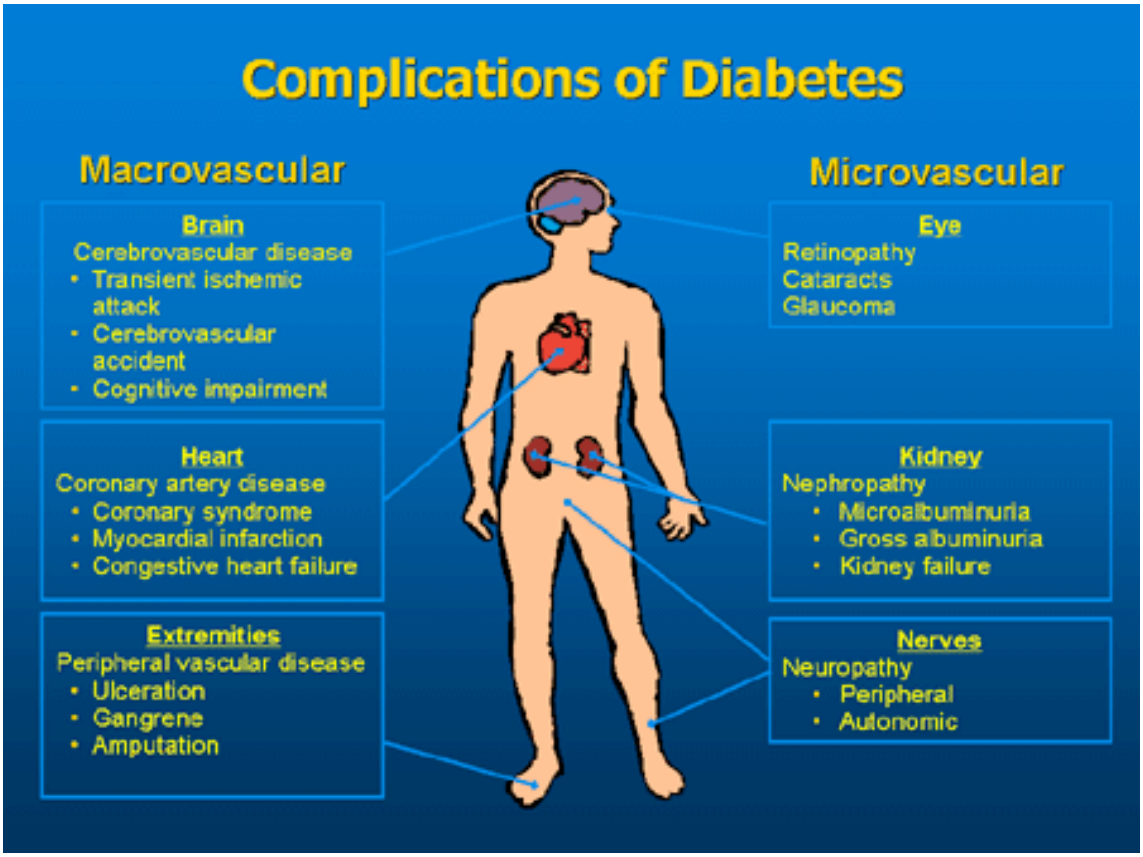


Figure 37: Summary of macrovascular and microvascular complications associated with diabetes/hyperglycemia (taken from:(Cefalu 2006b)).

1.9.4 Gestational diabetes

Gestational diabetes refers to increased blood glucose levels detected during pregnancy, without necessarily having any previous history of diabetes. It affects around 4% of pregnancies worldwide, but this varies greatly depending on the population (Bose 2005). For instance, the prevalence of gestational diabetes among the Cree is 12.8%, which is twice as high as the rest of the North American population, and the second highest among aboriginal populations worldwide (Rodrigues 1999). Gestational diabetes can be categorized into two groups. The first group consists in mothers developing diabetes during pregnancy. The second group refers to a state

where diabetes was present prior to pregnancy. Group 1 is further divided into two subtypes. In type A1, pregnant mothers have an abnormal OGTT with normal fasting blood glucose levels. This can be controlled with diet. Type A2, consists in abnormal OGTT and fasting glucose levels. This type needs antidiabetic medication or insulin. Group 2 is subdivided into many more types (B, C, D, E, F, R, RF, H, T) depending on the age of onset and duration of diabetes, and the complications that may have arisen from it (the earlier the onset the greater the health risks) (White 1949; Gabbe 2007; Rahman 2009). There are many identifiable risk factors involved in the development of gestational diabetes. These include previous diagnosis of gestational diabetes, or prediabetes, impaired glucose tolerance, impaired fasting glycemia, women of 35 years of age and over, ethnic background, overweight, obese or severely obese, just to name a few (Ross 2006). The symptoms associated to it resemble those of T1D or T2D: increased thirst, urination, fatigue, nausea, bladder infections, yeast infection, and blurred vision (WHO 2011a). Gestational diabetes incurs many potential health concerns for both mother and child, during pregnancy (pre-eclampsia and caesarian section) or after birth. It increases the likelihood for both to develop T2D. It even leads to babies with high birth weights (chance of developing childhood obesity later on in life), and low blood glucose levels (hypoglycemia shortly after birth). It can be treated with diet and exercise, and if necessary with antidiabetic medication or insulin (Donovan 2010; CDC 2011c)(Figure 38).

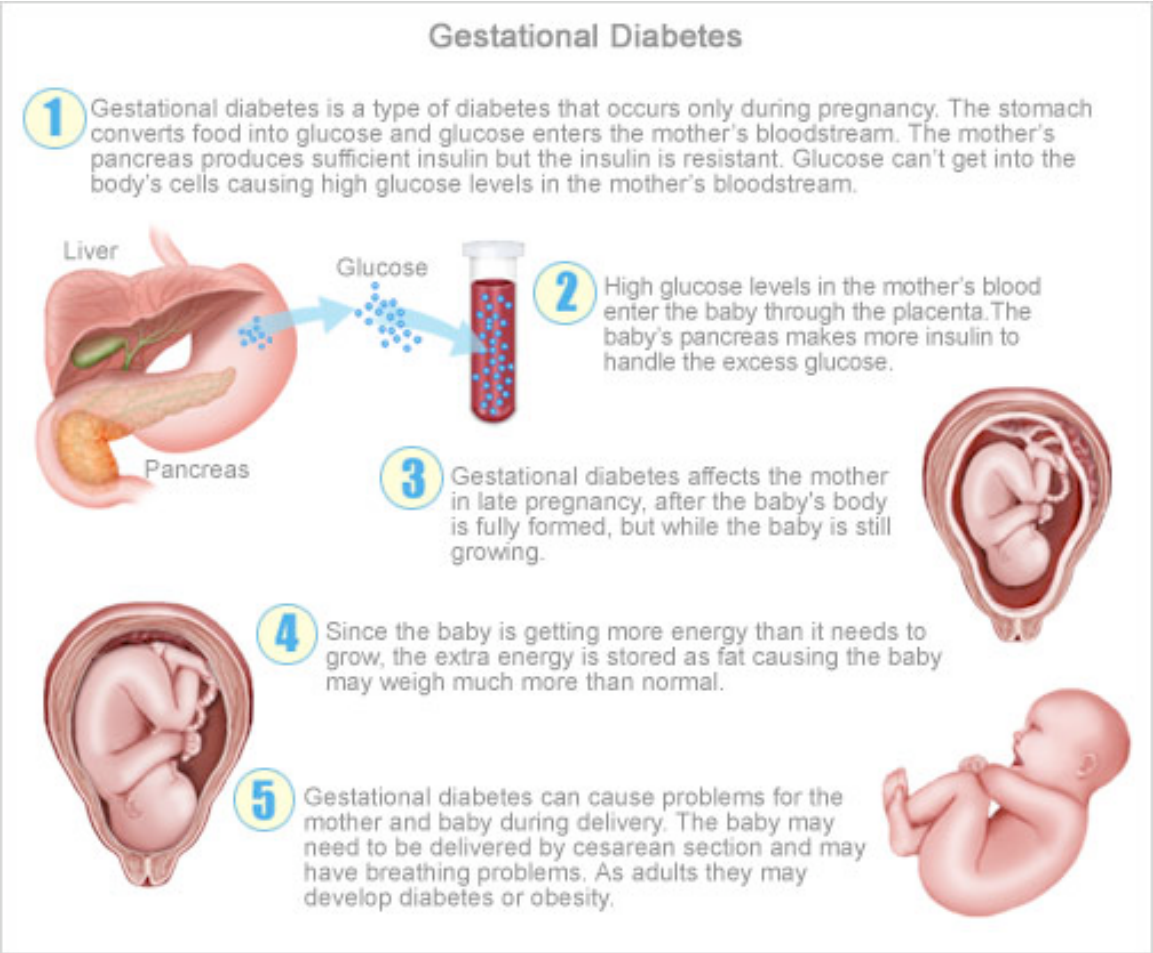


Figure 38: Gestational diabetes and the problems it incurs on mother and child (taken from: http://www.tree.com/health/diabetes-types-gestational.aspx)

1.9.5 Maturity onset diabetes of the young

Maturity onset diabetes of the young (MODY) corresponds to 5% of cases presumed to be T1D or T2D. It produces mild to moderate hyperglycemia at an early age (before 25), with ineffective insulin production and release from pancreatic beta-cells, with minimal or inexistent effects on insulin action(ADA 2004). It is a hereditary form of diabetes, with mutations occurring in an autosomal dominant gene (sex-independent), and it is also qualified as monogenic, affecting one gene (ADA 2004). An example of one of the most common forms of MODY is mutation in

the glucokinase gene, glucokinase being implicated in the conversion of glucose to glucose-6-phosphate, the metabolism of which triggers insulin secretion from the β-cell(ADA 2004). Therefore, a mutation in glucokinase, which is considered a glucose sensor for the beta-cell, requires increased plasma glucose levels in order to elicit insulin secretion (ADA 2004). Other types of MODY include gene mutations in hepatocyte nuclear factors (HNF-1a, HNF-1b, HNF-4a; regulate transcription of genes involved in glucose, fatty acid, and cholesterol transport and metabolism), or in the insulin promoter factor (IPF-1; necessary for pancreatic development and β-cell maturation)(ADA 2004). Treatments for MODY are similar to those for T1D and T2D, and include diet, exercise, antidiabetic medications and insulin injections.

1.9.6 Treatments for diabetes

Lifestyle changes alone or in combination with pharmacological agents can help manage T2D and minimize its complications. Weight loss through diet, decreasing saturated or trans fats, increasing fiber and polyunsaturated fat intake, and exercise reduce the risk of developing T2D or at least help in the treatment of it (Widmaier 2004; Bantle et al. 2008). Difficulty of sustainable implementation of lifestyle changes requires the use of antidiabetic medications. Those available include insulin secretagogues (sulfonylureas, and rapid-acting secretagogues such as meglitinides), insulin sensitizers (biguanides, and thiazolidinediones), inhibitors of intestinal carbohydrate absorption and digestion (α-glucosidase inhibitors), and the new class of incretin-based therapies (incretin mimetics, and DPPIV inhibitors).

1.9.6.1 Sulfonylureas

Sulfonylureas bind and close ATP-sensitive potassium (KATP) channels, binding with high affinity to sulfonylurea receptors (SUR-1) and increasing insulin secretion. SUR belong to

the family of ATP-binding cassette transporters and are subunits of the KATP channel. The KATP channel is a hetero-octameric complex composed of two different types of protein subunits: four inward-rectifier K+ ion channels Kir6.x (6.1 and 6.2) (ATP binding to this tetramer closes the channel), associated with four SUR subunits. KATP channels are also found in heart, skeletal and smooth muscle, with different SUR subunits, giving different drug sensitivities. For example tolbutamide and gliclazide bind and block SUR-1 containing channels in β-cells but not SUR-2 found in cardiac and smooth muscle. In contrast, glibenclamide, glimepiride, repaglinide, meglitinide bind and block both SUR-1 and SUR-2 containing channels (Proks et al. 2002). The binding of sulfonylureas to SUR-1 blocks ATP-dependent K+ channels, depolarizes the membrane and opens voltage-gated calcium channels, allowing for a calcium influx. The rise in intracellular calcium levels promotes exocytosis of insulin from storage granules. There are first and second-generation sulfonylureas available on the market. Although both increase insulin secretion from pancreatic β-cells, they vary in terms of their potency and adverse effects (Katzung 2004)(Figure 39).

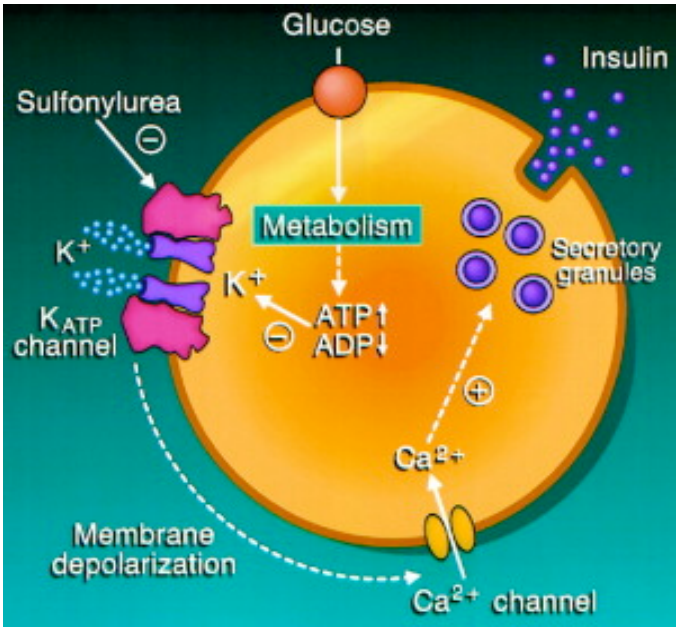


Figure 39 : Mechanism of action of sulfonylureas (taken from:(Brady and Terzic 1998)).

Examples of first-generation sulfonylureas include tolbutamide, chlopropamide, and tolazamide are. Whereas glyburide, glipizide, and glimepiride belong to the second-generation and have fewer side-effects and drug interactions. The main adverse effects of sulfonylureas include hypoglycemia, weight gain, and potential cardiovascular implications. It is noteworthy that sulfonylureas are associated with primary and secondary failure. Primary failure is characterized by the patient not responding to the highest dose of medication. Whereas during secondary failure, the patient initially responds but fails to as time goes on. Therefore, secondary failure may be the consequence of β-cell exhaustion caused by the treatment. Other factors that promote secondary failure include decreased β-cell mass, reduction in physical activity, decline in lean body mass and increase in ectopic fat deposition; the latter caused in part by progression of chronic type 2 diabetes (Wilson Rodger 1999; Katzung 2004; Moran 2010).

1.9.6.2 Meglitinides (rapid-acting insulin secretagogues)

Meglitinides or glinides, such as the carbamoyl methyl benzoic acid derivative repaglinide, or the d-phenylalanine derivative nateglinide, are rapid in onset and improve postprandial glycemic control. They can be used by diabetics who are allergic to sulfurs or sulfonylureas. Repaglinide reaches its peak effect 1hour after ingestion and its duration of action is 5-8 hours. In contrast, nateglinide is absorbed within 20 minutes, reaches its peak after 1 hour, and its effect lasts less than 4 hours (Katzung 2004). They bind to SUR1 in the plasma membrane of the β-pancreatic cells, but on a different binding site than that of sulfonylureas. Nateglinide has also been reported to partially restore initial insulin release (partially lost in T2D which results in increased postprandial glucose) (Katzung 2004). Both repaglinide and nateglinide can be used

effectively either alone or in combination therapy with non-secretagogue antidiabetic agents, such as biguanides or thiazolidinediones (Katzung 2004; Shigeto et al. 2007)(Figure 40).

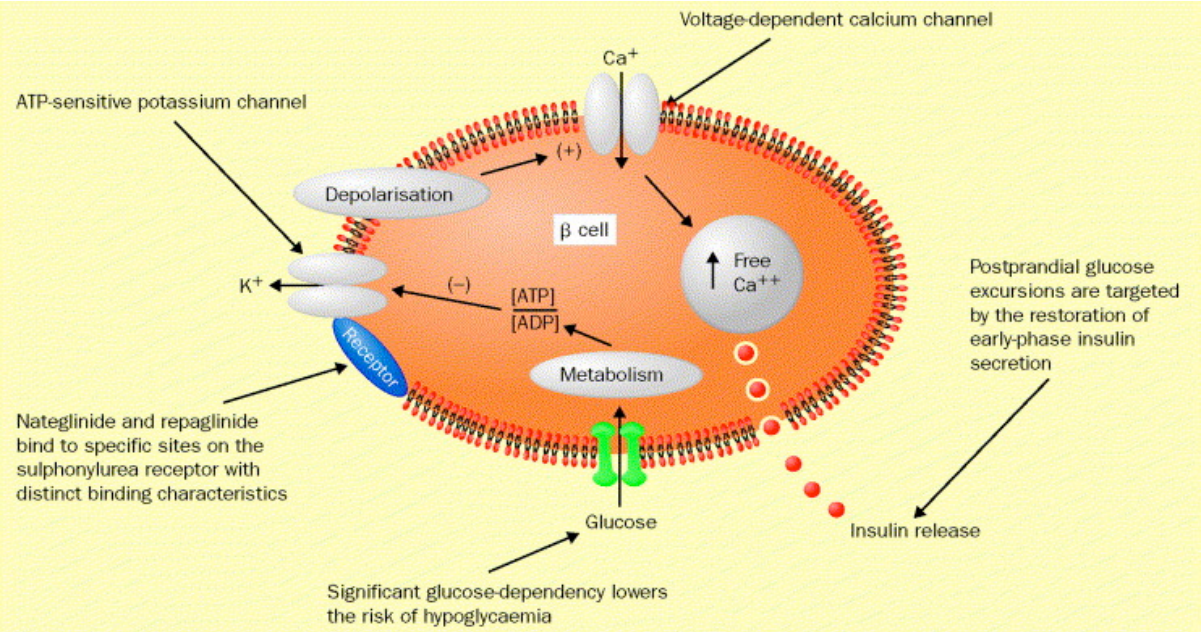


Figure 40 : Mechanism of action of meglitinides (taken from:(Dornhorst 2001)).

1.9.6.3 Biguanides

Biguanides do not depend on functioning pancreatic β-cells in order to mediate their effects (Katzung 2004). They act by potentiating the effects of insulin on its target tissues. They inhibit gluconeogenesis in the liver, and increase glucose uptake, through increased Glut4 translocation, in the muscle and adipose tissue. They also stimulate glycolysis (Katzung 2004). Since hepatic and renal gluconeogenesis are inhibited by biguanides, there is a greater risk of developing lactic acidosis, especially in patients with renal insufficiency (Katzung 2004). This is one of the main reasons that phenformin and buformin were removed from the market since they were more prone to cause acidosis, with the former being associated with sometimes fatal lactic acidosis (Fisman and Tenenbaum 2009). Metformin has a better safety profile, and as a result is

still used today as pharmacotherapy toward diabetes. Some of its adverse side effects include nausea, diarrhea, dyspepsia, anorexia, and although uncommon, hypoglycemia, especially if taken with other oral antidiabetic medication (Katzung 2004). It is reported that, through the insulin-independent AMPK pathway, metformin induces fatty acid oxidation, suppresses expression of lipogenic enzymes, and decreases circulating TG levels (Zhou et al. 2001; Krentz and Bailey 2005)(Figure 41).

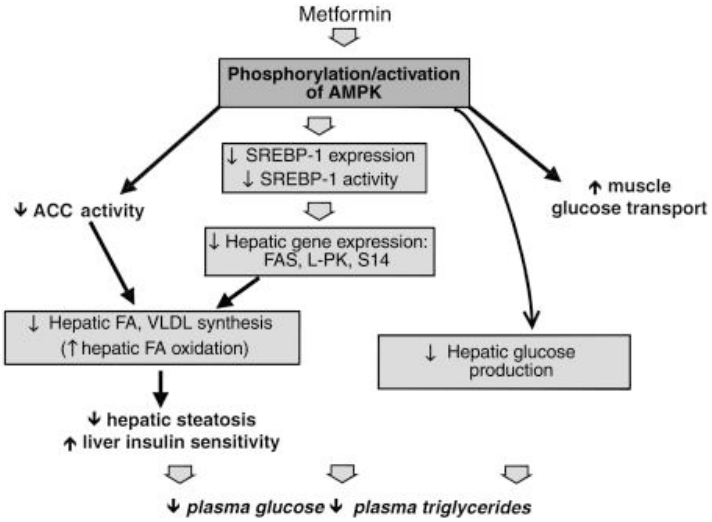


Figure 41: Mechanism of action of biguanides, such as Metformin (taken from:(Zhou et al. 2001)).

1.9.6.4 Thiazolidinediones

Thiazolidinediones (Tzds), also known as glitazones, are ligands of the peroxisome proliferator activated receptor gamma (PPARγ). PPARγ is a nuclear receptor, involved in the regulation of genes implicated in glucose and lipid metabolism, insulin signaling, adipocyte differentiation (pre-adipocyte to adipocyte accompanied with fat accumulation). PPARγ is also involved in modulating the synthesis of adipokines (increasing adiponectin levels) and inhibiting pro-inflammatory cytokines (ex. TNF-α)(Katzung 2004; Yki-Jarvinen 2004)(Figure 42).

Glitazones have been shown to activate the AMPK pathway in the liver, muscle, and adipose tissue (Fryer et al. 2002; Hardie et al. 2006). There are three drugs that belong to this family of medications, namely troglitazone, pioglitazone, and rosiglitazone. Although they have all been associated with serious adverse effects, only troglitazone has been completely removed from the market for its increased risk of causing drug-induced hepatitis (Cluxton et al. 2005). Both pioglitazone and rosiglitazone have been suspended in Europe due to potential risks of causing bladder cancer and cardiovascular complications respectively (McCulloch 2011).

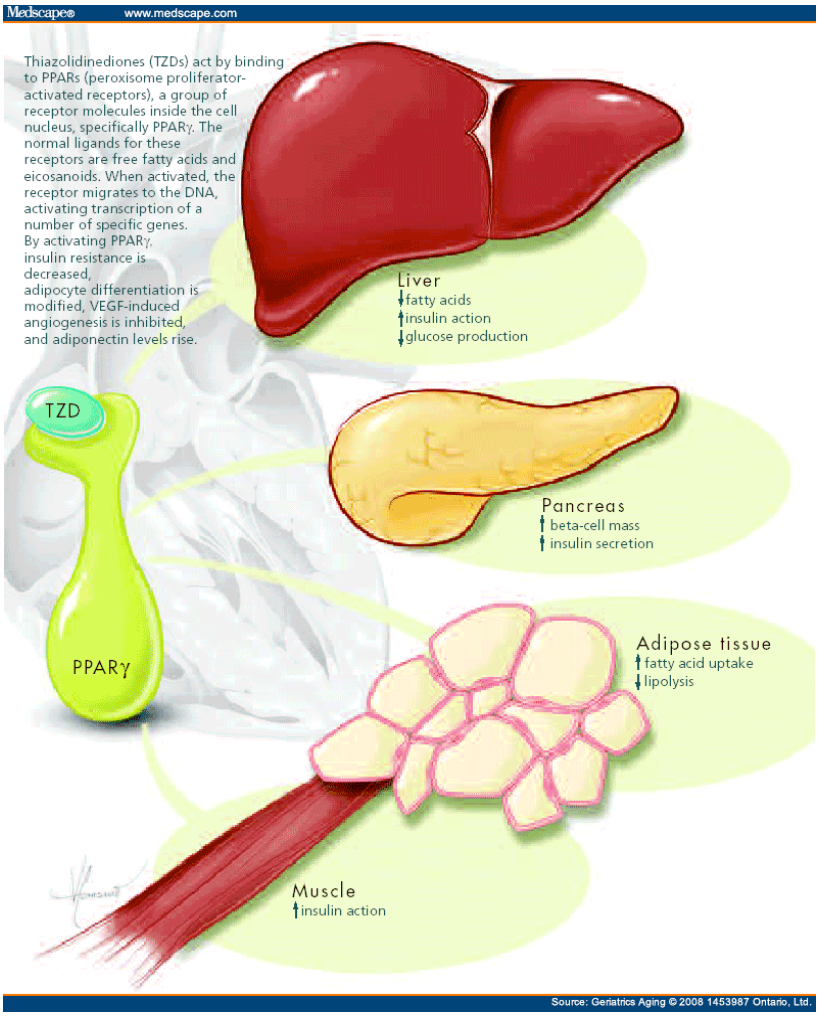


Figure 42: Mechanism of action of thiazolidinediones on peripheral tissues such as liver, muscle, and adipose tissue (taken from:(Singh 2008)).

1.9.6.5 Alpha-glucosidase inhibitors

Alpha-glucosidase inhibitors, which include acarbose, miglitol, and voglibose (newest in its class), help in lowering postprandial hyperglycemia while having an insulin-sparing effect (Katzung 2004). They act as inhibitors of α-glucosidases such as sucrase, maltase, glycoamylase, and dextranase. These are enteric enzymes found on the brush border membrane of the intestinal cells. These enzymes aid in the digestion of complex starches such as oligosaccharides and convert disaccharides into monosaccharides, allowing them to be absorbed in the duodenum and upper jejunum (Katzung 2004)(Figure 43).

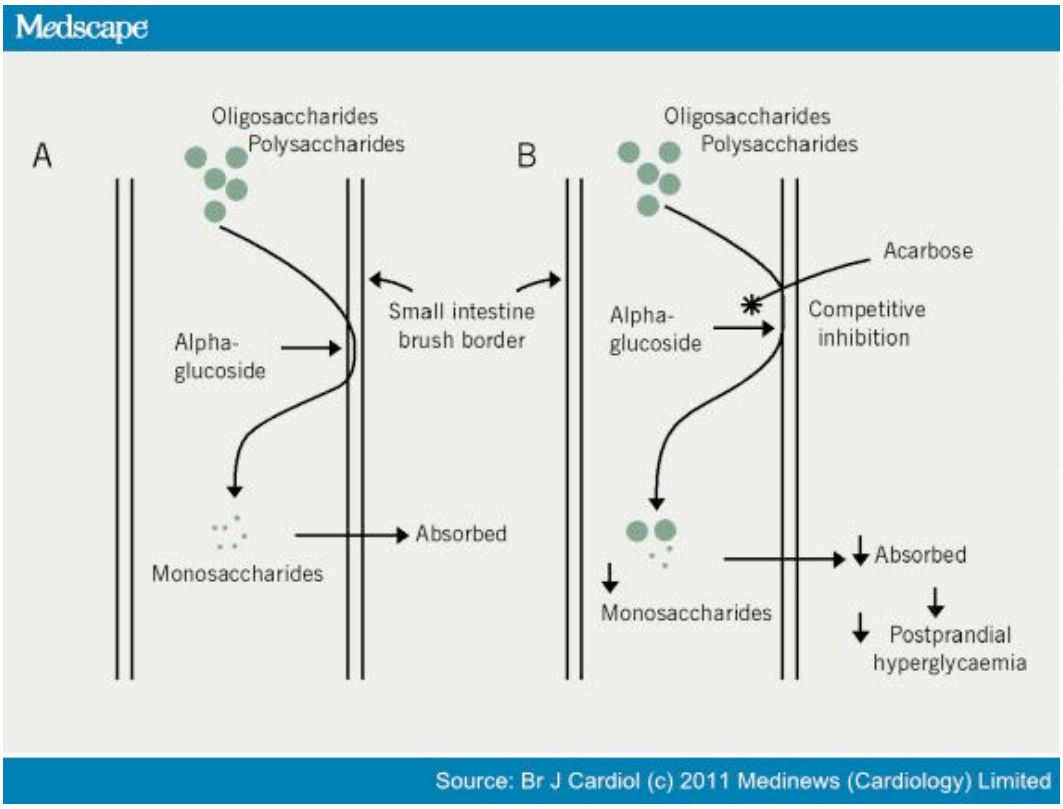


Figure 43: Mechanism of action of α-glucosidase inhibitors (taken from:(Arungarinathan 2011)).

By inhibiting these enzymes, carbohydrates will not be digested in the upper small intestine and instead will undergo bacterial digestion/fermentation in the colon, causing undesirable side effects such as bloating, flatulence and diarrhea. Other adverse effects of α-glucosidase inhibitors include nausea, vomiting, and hepatic injury (Hanefeld 1998; Harrigan 2001; Rodes 2007). In situations where a patient taking α-glucosidase inhibitors goes into hypoglycemia, it is advised for them to eat something containing monosaccharides such as glucose tablets. Indeed food containing complex starches will not be digested and will not relieve low blood glucose levels. Treatment with these medications can also lead to decreased glucose-insulinotropic polypeptide (GIP). The latter responds to rate of nutrient absorption rather than presence of nutrients as is the case with glucagon-like peptide-1 (GLP-1), which is increased during treatment (Moritoh et al. 2009).

1.9.6.6 Incretins

Glucagon like peptide-1 (GLP-1) and gastric inhibitor protein, also known as glucose-dependent insulinotropic polypeptide (GIP), belong to the group of gastrointestinal hormones termed incretins. They are both secreted within minutes of food intake and are degraded almost as quickly by the enzyme dipeptidyl peptidase-4 (DPP-4) (Baggio and Drucker 2007). GLP-1 is secreted from L-cells of the distal gut (ileum and colon) and GIP from K-cells of the proximal intestine (duodenum and jejunum)(Baggio and Drucker 2007). They both exert their effects on pancreatic β cells by promoting glucose-dependent insulin secretion, inducing β-cell proliferation and enhancing resistance to apoptosis (Bouwens and Rooman 2005; Baggio and Drucker 2007; Moritoh et al. 2009). GLP-1 slows down gastric emptying and increases satiety. Sustained GLP-1 receptor activation potentially results in weight loss (Baggio and Drucker 2007). It is also

implicated in glucose-dependent inhibition of glucagon secretion, consequently decreasing hepatic glucose output (Baggio and Drucker 2007). GIP is reported to modulate energy storage pathways, in part by increasing glucose uptake and storage in muscle and adipose tissue. It also stimulates components involved in fatty acid metabolism, such as lipoprotein lipase and fatty acid synthesis (Gallwitz 2006; McClean et al. 2007; Kim and Egan 2008). Regardless of their individual functions, they work together to keep blood glucose levels under control. The potential of these hormones as novel antidiabetic medications has led to the development of two new classes of oral hypoglycemic drugs. Exenatide and liraglutide are GLP-1 mimetics/analogs that are resistant to the action of DPP-4. The second class comprises DPP-4 inhibitors such as sitagliptin, vildagliptin, saxagliptin, linagliptin, with many more under clinical development (dutogliptin, gemigliptin) (Baggio and Drucker 2007; Seewoodhary and Bain 2011)(Figure 44).

a) b)

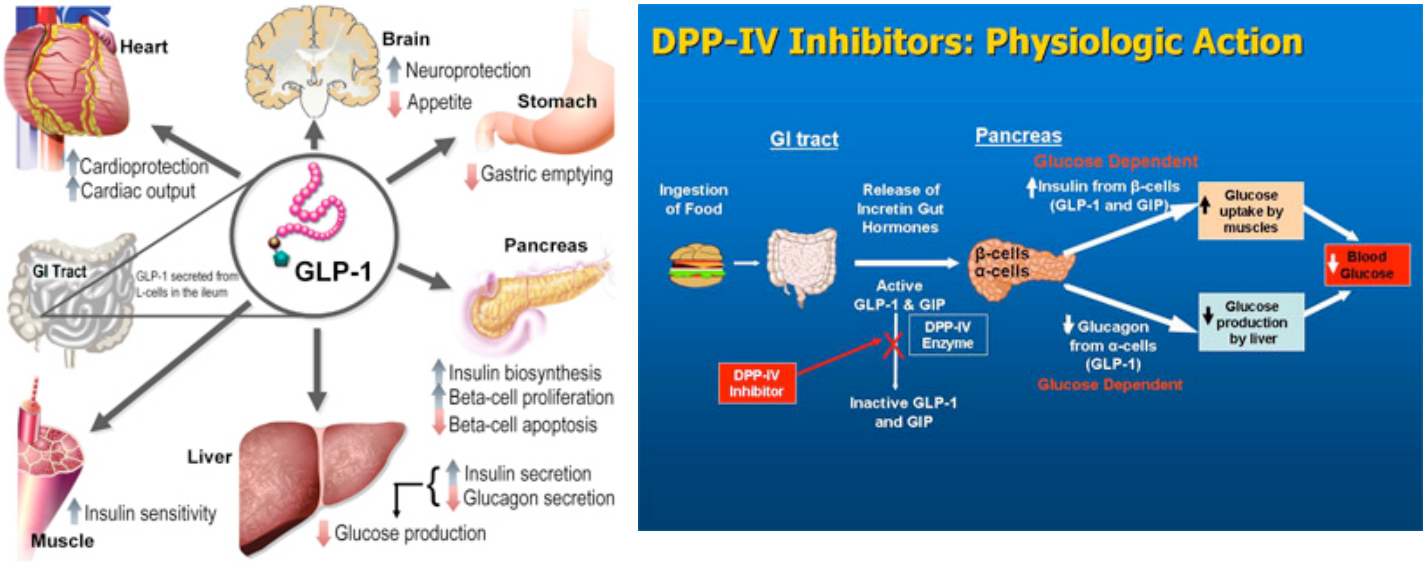


Figure 44: a) Mechanism of action of incretin mimetics, b) DPP-IV inhibitors (taken from:(Abrahamson 2006; Pratley and Gilbert 2008)).

1.10 Complementary and alternative medicine

Despite the availability of modern medicine and of educational programs on lifestyle intervention, proper treatment and management of T2D, obesity, and even other ailments is not always achieved. This has encouraged many to consider the use of complementary and alternative medicine. Until the middle of the 20th century and the advent of the so called modern medicines, traditional medicine were the only means widely used and available (Robinson 2011). Whether it be in developing countries, such as Asia, Africa, Latin America and the Middle East, or in industrialized nations such as Canada, France, Germany, and Italy, around 70-95% of the citizens use traditional medicines (complementary, alternative, non-conventional) to respond to their primary health care needs and concerns (Robinson 2011). This growing popularity has created a worldwide annual market of 60 billion US dollars. It has therefore prompted a growing interest among government bodies, and pharmaceutical industries, which are now investing more money in order to identify promising medicinal herbs and novel chemical compounds (Tilburt and Kaptchuk 2008).

There are many plant species that have been identified throughout the world as having antidiabetic potential. Some of these plants have even lead to the development of pharmaceutical drugs, such as Metformin, which is derived from the naturally occurring compound guanidine, found in *Galega officinalis* (French Lilac, or goat's rue) (Oubré 1997; Yeh et al. 2003). Other examples of some widely used herbal medicines for treating diabetes are *Trigonella foenum graecum* (Fenugreek) (Haddad et al. 2001; Broca 2004), used in North Africa, India and Middle East, *Gymnema sylvestre* (gur-mar; sugar destroyer) (Haddad et al. 2001; Ananthan et al. 2004) originating from an ancient Indian healing system, and *Momordica charantia* (bitter melon)

(Basch et al. 2003; Yeh et al. 2003; Grover and Yadav 2004) used in Asia, Africa, India, and South America.

1.10.1 Ethnobotanical survey and identification of plant species with antidiabetic potential within the CEI traditional pharmacopoeia

As mentioned, an extensive ethnobotanical survey was conducted in 6 communities of northern Quebec, that identified over 30 plants from the CEI traditional pharmacopoeia, as having potential to treat diabetes and its associated symptoms (Leduc et al. 2006). The CEI have witnessed a soaring prevalence of T2D, which can in part be explained from lifestyle changes (decreased physical activity and adoption of western diet), accompanied by a cultural disconnect to modern medicine. This reinforces the need in identifying effective treatment options that the CEI can identify to in terms of culture and lifestyle. Therefore, validating the culturally acceptable use of plant species stemming from the CEI traditional pharmacopoeia as antidiabetic treatments is of primary importance. There were a number of *in vitro* screening studies that were carried out, in order to bring forth the plant species portraying the strongest antidiabetic activities (Spoor et al. 2006; Harbilas et al. 2009). The assays used assessed the plants abilities to increase glucose uptake in muscle cells and adipocytes in the presence or absence of insulin while comparing them to the commonly used antidiabetic drug Metformin. Their effects on adipogenesis (compared to the Tzd Rosiglitazone), and insulin secretion, as well as their neuroprotective and anti-oxidant capacities were also evaluated. Based on the results of these studies, further experiments were conducted in order to identify the signalling pathways implicated (Eid et al. 2010a; Eid et al. 2010b; Martineau 2010a; Martineau 2010b; Martineau et al. 2010; Nistor Baldea et al. 2010). Although roughly half of the plants exhibited a strong antidiabetic potential, there were 2 in particular out of 17 plant species, namely *P. balsamifera*

and *L. laricina* that became of interest based on their potential of being both antiobesity and antidiabetic agents.

1.10.2 *Populus balsamifera* L. (Salicaceae): traditional uses

Populus balsamifera L. (*P. balsamifera*), also known as balsam poplar, belongs to the family of Salicaceae (Figure 45). It is a deciduous tree that needs full sun and a moist rich soil. It can grow on average up to 30m in height in many regions of North America, up to the arctic slope of Alaska (Rook.org 2006; PFAF 2008b). Different parts of the species have been used as various concoctions for many years by North American Indian tribes, such as the CEI, for their medicinal properties. Leaf buds have antiscorbutic, antiseptic, diuretic, expectorant, stimulant and tonic properties (Uphof 1968; Grieve 1971; Usher 1974; Mills 1985; Schofield 2003). The resin from the leaf buds can be used as salve and wash for sores, rheumatism, and wounds (Moerman 1998; Foster 1999), or made into a tea that can be used as a wash topically for inflammation, muscle pain, or ingested to help in lung ailments and coughs (Foster 1999). Leaf buds in hot water can be inhaled to decongest nasal passages (Weiner 1990). Concerning the bark, it contains salicin, a glycoside that breaks down into salicylic acid in the body during metabolism. This compound has anodyne (pain reliever), anti-inflammatory, and febrifuge (decreases fever) properties (Weiner 1990; Bown 2001). Tea from inner bark can be used for colds, coughs, lung problems, rheumatism, kidney and urinary problems (Erichsen-Brown 1989; Moerman 1998).

Figure 45: *Populus balsamifera* L. (Salicaceae)
(taken from : http://www.rook.org/earl/bwca/nature/trees/populusbal.html)

1.10.2.1 *In vitro* screening studies using *P. balsamifera* demonstrate antiobesity potential

The inner bark of *P. balsamifera* was used in the *in vitro* screening assays as well as in the *in vivo* studies of the present thesis. The phytochemical markers found in this part of the plant species, include salicins, salicortin, salireposide, and populosides (Harbilas et al. 2009), which are phenolic acid glycosides (Si et al. 2009). Once they are ingested, these compounds are eventually broken down into salicylic acid (Bown 2001; Kar 2003; Boeckler et al. 2011). There are two classes of phenolic acids, derivatives of benzoic acid to which the markers found in balsam poplar belong to, and derivatives of hydroxycinnamic acid.

Balsam poplar demonstrated a strong antiobesity potential in the *in vitro* screening studies (Harbilas et al. 2009; Martineau 2010a; Martineau 2010b). In fact, it completely inhibited triglyceride accumulation during adipogenesis in 3T3-L1 adipocytes (where cells undergo differentiation from pre-adipocyte into adipocytes accumulating TG in the process). The cells

treated with the plant species retained pre-adipocyte fibroblastic morphology and there was total absence of any visible lipid droplets (Martineau 2010a). This may, in part, be attributable to its PPARγ antagonist effect, as determined in an independent gene-reporter assay.

Using bioassay-guided fractionation, salicortin was identified as being the active principle responsible for the observed effects of balsam poplar on adipogenesis. Although it has been documented as having antiproliferative effects (cancer studies), its antiobesity potential is newly ascribed (Subramanian et al. 2006; Martineau 2010b). This compound is abundant in poplar, willow bark, and throughout the salicaceae family. Salicylates are well known for having anti-inflammatory properties, and for improving insulin sensitivity. Salicortin, which composes 10% of the whole plant extract, was produced through fractionation, isolation, purification of the plants' crude extract as previously described (Martineau 2010b; Martineau 2010a)(Figure 46). The structure of the purified compound was identified and confirmed by ^{1}H and ^{13}C NMR and by comparison with previously reported data.

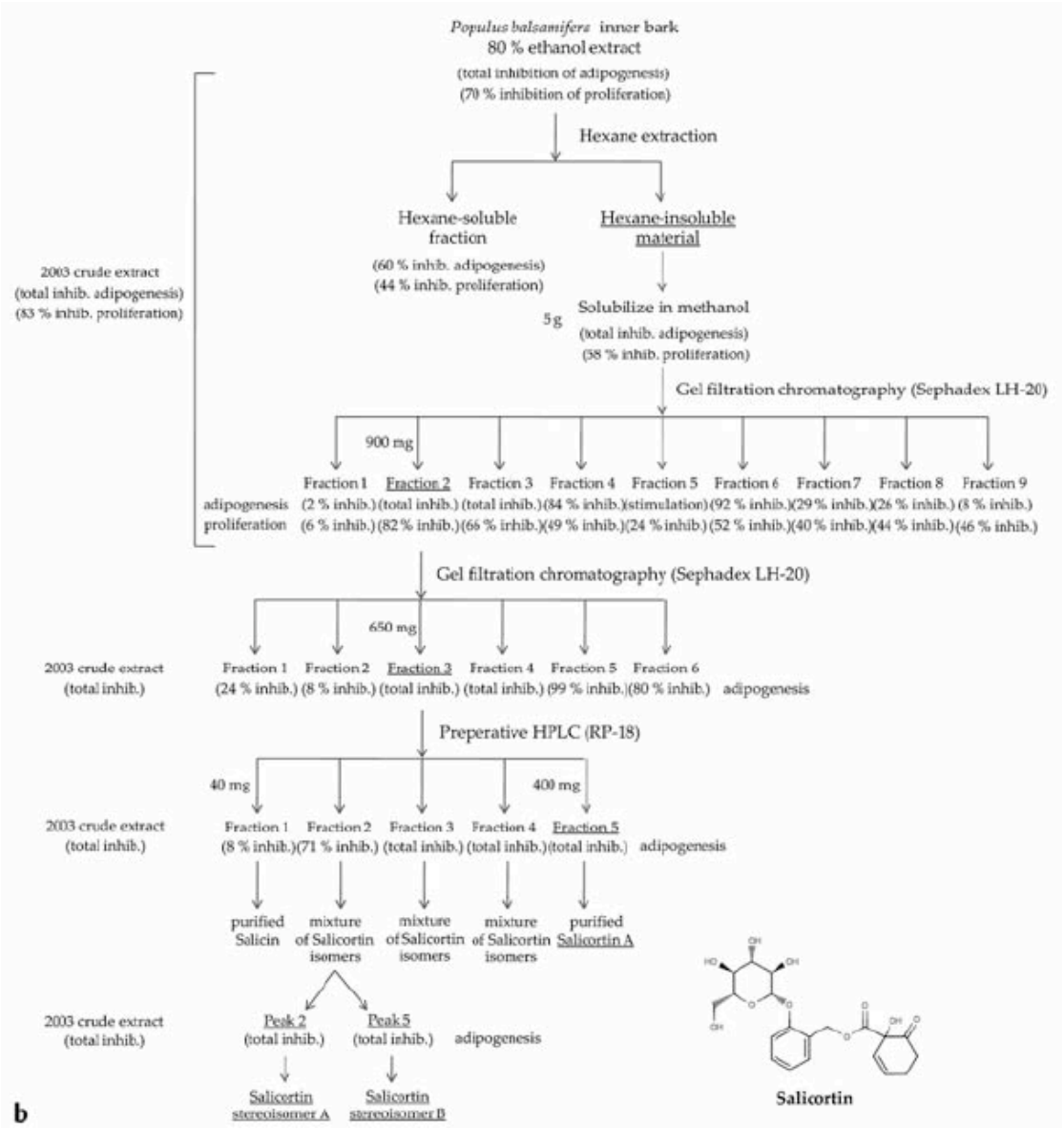


Figure 46: Fractionation, isolation and purification of salicortin from *P. balsamifera* crude extract (taken from:(Martineau 2010b)).

1.10.3 *Larix laricina* K. Koch (Pinaceae): traditional uses

Larix Laricina K. Koch, also referred to as tamarack, belongs to the family Pinaceae (Figure 47). It is a deciduous conifer tree, that needs full sun and moist to wet rich soil. It can

grow up to 18m mainly in the northern part of North America (from Labrador to Alaska, and North Eastern United States), up to the Arctic tree line (PFAF 2008a).

Figure 47: *Larix laricina* K. Koch (Pinaceae)
(taken from : http://wisplants.uwsp.edu/scripts/detail.asp?SpCode=LARLAR)

The branches, needles and bark have been used for many years by North American tribes, such as the CEI, under different preparation forms to treat a wide array of diseases. When the bark is prepared in tea form, it can be used as an alterative, diuretic, laxative, and tonic (Grieve 1971; Foster 1999), or in the treatment of jaundice, anaemia, rheumatism, colds and skin ailments (Moerman 1998; Foster 1999). It is useful for sore throats when gargled, and indicated as a poultice for infections stemming from wounds, sores, swellings and burns (Moerman 1998; Foster 1999). When bark is infused with buds, it is an expectorant (Moerman 1998), and with needles it can either be considered cough medicine, or as a disinfectant if applied as a poultice (Moerman 1998). The resin is chewed and used to treat indigestion (Foster 1999), or can even be beneficial for kidney or lung ailments, or as dressing for ulcers and burns (Lauriault 1989).

1.10.3.1 *In vitro* screening studies using *L. laricina* demonstrate antiobesity and antidiabetic potential

The inner bark of *L. laricina* was used in the *in vitro* screening assays (Spoor et al. 2006; Martineau et al. 2010) as well as in the *in vivo* assays of the present thesis. The phytochemical markers found in this part of the plant species include taxifolin, a flavanonol (type of flavanoid), and hydroxystilbenes (stilbenes). Compounds of these two phytochemical classes have been reported to have antiobesity and antidiabetic properties, as well as to protect against the associated complications (cardiovascular, peripheral nerve damage, etc.) (Haraguchi et al. 1997; Baur et al. 2006; Baur and Sinclair 2006; Peluso 2006; Kundu and Surh 2008; Pearson et al. 2008; Shabrova et al. 2011).

L. laricina demonstrated antidiabetic potential *in vitro* by increasing glucose uptake in C2C12 myotubes (Spoor et al. 2006), by acting on the AMPK pathway (no Akt activation) and increasing ACC phosphorylation (involved in FA oxidation)(Martineau et al. 2010). It also upregulated adipogenesis in the 3T3-L1 adipocyte cell line. The effects of the plant species were comparable to their positive controls, commonly used antidiabetic medications, i.e. Metformin for the glucose uptake assay and Rosiglitazone for the adipogenesis assay. *L. laricina* was observed to be one of the strongest uncouplers, disrupting mitochondrial function. Uncouplers are being closely studied in the scientific literature for their potential use as antiobesity agents, since fuel consumption/metabolic rate is increased to try and compensate for low ATP formation. During uncoupling, heat is produced instead of ATP formation. If this becomes excessive, fatal hyperthermia can arise. Uncoupling can also lead to lactic acidosis by promoting anaerobic glycolysis in order to compensate for decreased ATP production (Martineau et al. 2010). However, the safety profile of the plant extract was confirmed in an *in vitro* screening study (Martineau et al. 2010).

1.11 Animal models of obesity and diabetes

There are many animal models that are available to study diabetes and/or obesity: ob/ob and db/db mice, KKAy, streptozotocin models, pancreatectomy in mice or rats, Zucker diabetic fatty rats, transgenic or knockout models, or diet-induced obesity and diabetes in desert rats or C57BL/6 mice. The ob/ob (leptin deficiency) and db/db mice (leptin receptor defect), lead to hyperphagia, hyperinsulinemia, and insulin resistance (Sima 2005; Cefalu 2006a; Wolf 2006). KKAy mice are a cross between black KK glucose intolerant mice and yellow Ay mice. The Ay gene encodes agouti peptide, which is an antagonist of melanocortin 4 receptor (MC4R) in the hypothalamic melanocortin system inhibiting leptin activity (Barsh et al. 2000). Streptozotocin models lead to destruction of pancreatic β-cells in mice or rats, and therefore to impaired pancreatic function, insulin deficiency, hyperglycemia, and the development of diabetes (Cefalu 2006a). Pancreatectomy corresponds to a surgical removal of 90% of the pancreas, leading to hyperglycemia and diabetes (Rossetti et al. 1987). Zucker diabetic fatty rats are a model of type 2 diabetes that have a mutation in the leptin receptor. They become obese, hyperglycaemic and have an inability to increase β-cell mass. This results in insufficient insulin secretion to compensate for the state of obesity-induced insulin resistance (Cefalu 2006a). There also exists transgenic and knockout models, in which there are mutations in genes needed for insulin action or secretion (Glut4, Glut2, insulin receptor, IRS, etc.)(Nandi et al. 2004). Models that assess the impact of increased energy intake on type 2 diabetes include the spiny mice (Acomys chirinus), the desert gerbil (Psammomys obesus) as well as the diet-induced obese C57BL/6 mouse model (Cefalu 2006a).

In humans, the prevalence of T2D is closely related to lifestyle and caloric intake, with obesity being a major contributor to the development of T2D and the metabolic syndrome. It has been proven that the percentage of fat in the diet is positively correlated with percentage of body fat, is predictive of the subsequent increase in body mass index, and is independent of total energy intake (Dreon et al. 1988; Miller et al. 1990; Tucker and Kano 1992; Parekh et al. 1998). As a reference, in humans 20-35% of daily total calories should come from fat, and 45-65% from carbohydrates (focusing on complex carbs that have a higher fiber content and take longer for the body to break down into glucose, while avoiding refined carbs and foods with added sugar and low nutritional value) (CDC 2011b; CDC 2011a). Having taken all these factors into consideration, an animal model of choice to study the development of obesity and its resulting T2D is that of diet-induced obesity (DIO) in C57BL/6 mice. It relies on high-calorie diet and inactivity, without any genetic involvement. Increased dietary fat content has been shown to produce obesity in mice and rats (Schemmel et al. 1970; West et al. 1992; Parekh et al. 1998). The high-fat diet used in the studies composing this thesis is made up of 60% of the kcal coming from fat, as opposed to 5% in the standard laboratory chow diet (see Appendix A Figures 1 and 2). Mice fed a high-fat diet exhibit significant weight gain, hyperglycemia, hyperinsulinemia, and hyperlipidemia, reflecting the establishment of the metabolic syndrome and a pre-diabetic state (Jiang et al. 2005).

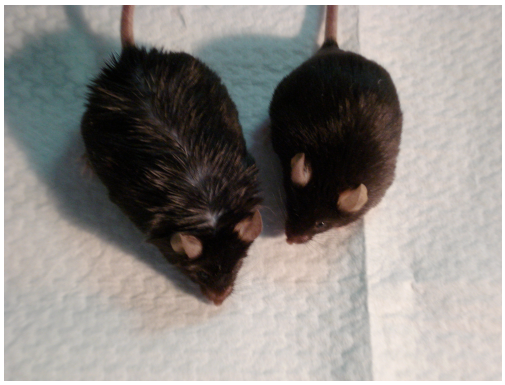
1.12 Aim and scope

Based on the *in vitro* data obtained thus far, *P. balsamifera* and its active principle salicortin possess strong antiobesity potential, by potently inhibiting adipogenesis and triglyceride accumulation. Concerning *L. laricina*, it can be beneficial in treating both obesity and diabetes by

acting as a mitochondrial uncoupler and exhibiting similar mechanisms of action to both the commonly used antidiabetic medications metformin and rosiglitazone. Therefore, the aim of this thesis is to investigate and validate the antiobesity and antidiabetic potential of *P. balsamifera* and its active principle salicortin, as well as that of *L. laricina*, in mitigating (preventing) or reducing (treating) the development of obesity and insulin resistance in C57BL/6 mice subjected to a high-fat diet for a period of 8 or 16 weeks, respectively (Figure 48; see Appendix A, figure 1 for standard laboratory chow diet composition, and figure 2, for high-fat diet composition). The plants extracts, as well as the active principle were each added (in powder form) into the high-fat diet, and mixed to ensure proper distribution throughout the food. An example of the calculation used for plant extracts or active principle dosing can be seen in Appendix A Figure 3. The calculation is based on assessment of average food consumption and body weight. Incorporation of the plant extracts or the active principle into the diet did not in any case alter its texture. Both balsam poplar and tamarack were administered at doses of 125 and 250 mg/kg of body weight. The choice of these doses is based on previous dose-searching experiments (doses ranging from 125 to 500 mg/kg) conducted by our team in various animal models using different plant species. In natural health product research, it is common to use a starting dose of 100 mg/kg of body weight for crude plant extracts (Haddad et al. 2012). It is to note that these doses do not bear a direct relationship with human therapeutic doses, and as is the case with conventional medications, they cannot be easily translated into human equivalents (Haddad et al. 2012). Salicortin was administered at 12.5 mg/kg of body weight, since it composes 10% of the whole plant extract, and the effective starting dose of balsam poplar is 125 mg/kg of body weight. Measurements were taken over time for parameters such as body weight, food intake and glycemia. The area under the curve (AUC) was calculated for these continuous measurements. The AUC simplifies statistical analysis, especially when the number of repeated measures is too

high, or if the interval between repeated measures is not identical (ANOVA for repeated measures is not able to adjust for this difference)(Bryant 1983; Matthews et al. 1990; Fekedulegn et al. 2007). The AUC also integrates the concept of trend/tendency, something that an ANOVA test on repeated measures does not do. This will allows us to determine the time-course of balsam poplar, salicortin and tamarack; whether treatments are effective at onset, later in time, throughout or even if they lose their efficacy with time. Finally, in order to determine the signaling pathways that are involved in regulating glucose and lipid metabolism, tissues such as the liver, muscle and adipose tissue, all implicated in obesity and the development of insulin resistance, were closely examined.

a)



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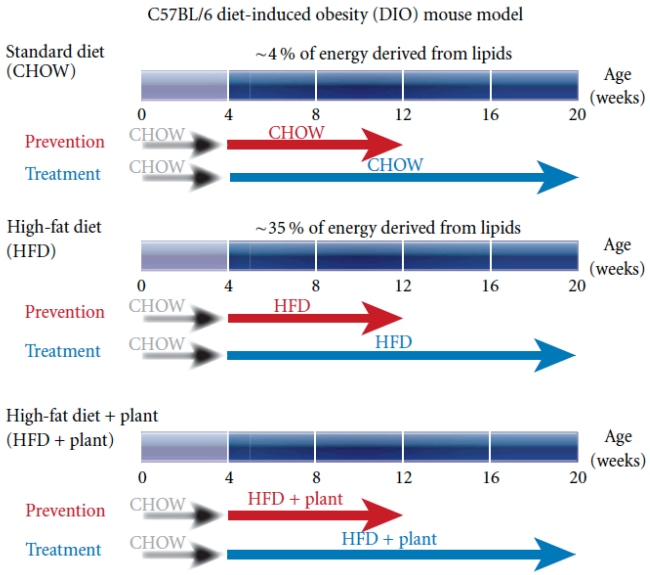

Figure 48: a) C57BL/6 mice: mouse on left fed a high-fat diet (DIO model) and mouse on right fed a standard laboratory chow diet (Chow control); b) Schematic representation of the DIO model submitted to a HFD for 8 weeks (prevention study) or 16 weeks (treatment study), where plant indicates administration of either *P. balsamifera*, or *L. laricina* or Salicortin (Haddad et al. 2012).

Chapter 2: Article 1

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Role of each co-author belonging to this manuscript

It is to note that this manuscript has been accepted for publication in the Journal of Ethnopharmacology. I the student Despina Harbilas did the great majority of the work in this manuscript, with the technical support of Antoine Brault and Diane Vallerand: treatment, care and sacrificing of the animals, as well as all post-sacrifice analysis on blood and tissue parameters, and finally all data and statistical analysis. Drs. Ammar Saleem and John T. Arnason are our collaborators, and contributed in the preparation and characterization of the plant species. Drs Lina Musallam and Louis C. Martineau assisted in correcting the article. Dr. Pierre S. Haddad, my supervisor, contributed conceptual, intellectual and moral input as well as correcting the article.

Title:

***Populus balsamifera* L. (Salicaceae) mitigates the development of obesity and improves insulin sensitivity in a diet-induced obese mouse model**

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Abstract

Ethnobotanical relevance: In previous *in vitro* bioassay studies, *Populus balsamifera* L. (Salicaceae), a medicinal plant ethnobotanically identified from the traditional pharmacopoeia of the Cree of Eeyou Istchee (Eastern James Bay area of Canada), exhibited a strong anti-obesity potential by potently inhibiting adipogenesis in 3T3-L1 adipocytes. Aim of the study: To evaluate the effectiveness of this plant extract in mitigating the development of obesity and the metabolic syndrome in diet-induced obese (DIO) C57BL/6 mice. Materials and methods: Mice were subjected for eight weeks to a standard diet (CHOW), a high fat diet (HFD; DIO group), or HFD to which *P. balsamifera* was incorporated at 125 and 250 mg/kg. Results: The results showed that *P. balsamifera* decreased in a dose-dependent manner the weight gain of whole body, retroperitoneal fat pad and liver as compared to DIO controls and reduced the severity of hepatic macrovesicular steatosis and triglyceride accumulation. This plant extract also decreased glycemia in the second half of the feeding period and improved insulin sensitivity by diminishing insulin levels and the leptin/adiponectin ratio, as well as augmenting adiponectin levels. These effects were associated with slightly but significantly reduced food intake with 250 mg/kg *P. balsamifera* as well as with an increase in energy expenditure (increase in skin temperature and increased expression of uncoupling protein-1; UCP-1). Data also suggest other mechanisms, such as inhibition of adipocyte differentiation, decrease of hepatic inflammatory state and potential increase in hepatic fatty acid oxidation. Conclusion: Taken together, these results confirm the potential of *P. balsamifera* as a culturally adapted therapeutic approach for the care and treatment of obesity and diabetes among the Cree.

List of abbreviations

ACC: acetyl-CoA carboxylase

AMPk: adenosine monophosphate-activated protein kinase

BAT: brown adipose tissue

C/EBP: CCAAT-enhancer binding proteins

CPT-1: carnitine palmitoyltransferase-I

DIO: diet-induce obese

ERK: extracellular signal-regulated kinase

FAS: fatty acid synthase

Glut4: glucose transporter 4

HFD: high-fat diet

IKK: IkB kinase

MAPk: mitogen-activated protein kinase

NAFLD: non-alcoholic fatty liver disease

NASH: non-alcoholic steatohepatitis

NF-κB: nuclear factor kappa light chain enhancer of activated B cells

P. balsamifera: *Populus balsamifera* L. (Salicaceae) or balsam poplar

PPAR: peroxisome proliferator-activated receptor

SOX: Sry-related HMG box

T2D: type 2 diabetes

UCP-1: uncoupling protein-1

WAT: white adipose tissue

2.1. Introduction

According to the World Health Organization (WHO), obesity is defined as excess fat accumulation that represents a risk to health (WHO, 2011). Sedentary lifestyle and a high-calorie diet are the most prominent factors involved in the development of obesity. Over time, this excess in body weight increases the risk of developing chronic health problems, such as type 2 diabetes (T2D), cardiovascular disease and cancer (WHO, 2011). Therefore, obesity constitutes a major public health problem worldwide with astounding human and economic consequences (Withrow and Alter, 2010).

Obesity contributes to around 55% of all cases of T2D by altering the body's response to insulin and creating a pro-inflammatory state (Eberhardt, 2004). Hence, taking into account the pathways that are involved in lipid and glucose homeostasis as well as inflammation in the main insulin-sensitive tissues, namely liver, muscle, and fat, is of great importance. Indeed, the liver plays a central role in systemic glucose and lipid homeostasis. The muscle is the principal tissue involved in postprandial glucose disposal, accounting for about 80% of glucose uptake through insulin- or exercise-sensitive glucose transporters, Glut4. Finally, adipose tissue is a key player in the development of insulin resistance and T2D. Abdominal fat (white adipose tissue; WAT) releases adipokines, such as adiponectin and leptin, which affect insulin resistance, satiety and energy expenditure. Brown adipose tissue (BAT) is implicated in thermal regulation (uncoupling protein-1; UCP-1) and fatty acid oxidation (CPT-1, PPARα). These represent potential targets of effective anti-obesity and ensuing anti-diabetic therapies.

The prevalence of obesity is increasing at an alarming rate worldwide, and has tripled in the past 25 years among diverse Canadian populations, reaching 25 % in recent statistics (PHAC, 2009; PHAC, 2011a; PHAC, 2011b; Statistics and Canada, 2005). In particular, Canadian

Aboriginal populations are highly affected by this disease, where the prevalence of obesity and overweight is 2-3 times higher than the rest of the Canadian population (Garriguet, 2008). Since this disease is linked to T2D, the prevalence of the latter is also especially high among these populations, where it reached 29% in 2009 among adults (>20 yrs) of the Cree of Eeyou Istchee (CEI) in the Eastern James Bay area of Quebec (Dannenbaum et al., 2008; Dannenbaum et al., 2010).

Due to the cultural disconnect of modern T2D therapies and in order to respond to the CEI primary health care needs, our team (CIHR-TAAM) conducted an extensive ethnobotanical study among the CEI Elders and Healers to identify culturally relevant anti-diabetic treatments (Leduc et al., 2006). Seventeen plant extracts were identified and further studied in a variety of *in vitro* bioassays for their anti-diabetic potential. One of these CEI plant extracts, *Populus balsamifera* L. (Salicaceae), also known as balsam poplar, contains a number of active components, such as salicylates and other phenolics, namely salicin, salicortin, salireposide, populoside. It displayed a potent anti-obesity potential *in vitro*. It effectively prevented 3T3-L1 adipogenesis by maintaining pre-adipocyte fibroblastic morphology and acting as a PPARγ antagonist during early processes of adipocyte differentiation (Harbilas et al., 2009; Martineau et al., 2010a; Martineau et al., 2010b).

These findings warrant further investigation of the anti-obesity potential of *P. balsamifera* and its ensuing preventive effect on the development of diabetes *in vivo*. Our hypothesis is that *Populus balsamifera* L. (Salicaceae) can mitigate weight gain and prevent or attenuate the development of insulin resistance in the diet-induced obese C57BL/6 mouse (DIO) model. This is an animal model of choice to study the development of obesity, which relies on high-calorie diet and inactivity to trigger obesity without any genetic involvement (Buettner et al., 2007; Collins et al., 2004). After 8 weeks on a high fat diet, DIO animals display raised body and abdominal fat

pad weights, generally exhibit mild hyperglycemia and hyperinsulinemia, alongside an increase in the leptin/adiponectin ratio and ectopic fat storage in insulin-sensitive tissues such as the liver and muscle. These parameters reflect the establishment of the metabolic syndrome and a pre-diabetic state. Indeed, the metabolic syndrome is characterized by the presence of three out of the five following symptoms: central obesity (increased waist circumference or waist-to-hip ratio), elevated triglycerides, lowered HDL, raised blood pressure, as well as high fasting glycemia levels or the use of medications in order to control hyperglycemia (Alberti, 2006). Other parameters that appear to be related to the metabolic syndrome, and that should be included in a research-related context, include body fat distribution with a particular focus on central fat distribution, leptin, adiponectin and insulin levels, as well as liver fat content (Alberti, 2006).

In this study, we concomitantly administered extracts of balsam poplar to mice fed the high fat diet and report that it significantly mitigates the development of obesity and attenuates the associated insulin resistance.

2.2 Materials and Methods

2.2.1 Plant extracts

Specimens of *Populus balsamifera* L., belonging to the Salicaceae family, were collected on the territories of the CEI of Northern Quebec, Canada. Dr. Alain Cuerrier, taxonomist at the Montreal Botanical Garden, confirmed the botanical identity of the plant species. Voucher specimens were deposited in the Marie-Victorin herbarium of the Montreal Botanical Garden in Montreal, Quebec, Canada (Mis03-49). The crude 80% ethanolic extract of *Populus balsamifera* L. (Salicaceae), which will be referred to as *P. balsamifera* or by its common name balsam poplar, was prepared as previously described (Harbilas et al., 2009).

2.2.2 Animals and Diets

Four-week old male non-diabetic C57BL/6 mice were purchased from Charles River Laboratories (Saint-Constant, QC, Canada). All mice had *ad libitum* access to food and water. They were housed in individual cages and maintained on a 12 h light-dark cycle in a temperature-controlled animal room. Following acclimatization, the mice were divided into groups of approximately 12 mice each before being treated for 8 weeks. The control groups consisted in a negative control group (Chow) receiving a standard diet (SD) purchased from Charles River (18% protein content, 4.5% crude fat, Charles River Animal rodent diet) and a positive control group (DIO) receiving a high fat diet (HFD) acquired from Bio-Serv (Bio-Ser Diet #F3282, Frenchtown, NJ, 60% fat by energy). The remaining groups received the HFD to which was incorporated the dried 80% crude ethanolic plant extract of *P. balsamifera* at levels adjusted to deliver 125 or 250 mg/kg body weight. Body weight, food and water intake, as well as glycemia were measured 3 times/week during the course of the study. These measurements were always

performed at the same time, day, in the same order and by the same person, every week for the entire duration of the protocol. Glycemia was measured by collecting blood from pricked tail vein and using a commercial glucometer (Accu-Check Roche, Montreal, QC, Canada). In order to determine the overall effect of treatment regimens on parameters measured continuously, we calculated the total area under the curve (AUC_T). We also fractionated such measurements into the first and second half of the feeding period corresponding to weeks 0-4 (F1) and weeks 4-8 (F2), respectively, in order to gain insight on the temporal (early/late) effects of the plants. All experimental protocols were approved by the animal experimentation ethics committee of the Université de Montréal and were carried out in full respect of the guidelines from the Canadian Council for the Care and Protection of Animals.

2.2.3 Surgical Procedure

At the end of the experimental protocol, the mice were anesthetized using 50 mg/kg pentobarbital intraperitoneally and then sacrificed by exsanguination via the inferior vena cava. Livers were flushed with a physiological saline solution and the median lobes were then dissected; one of the sections being placed immediately in a 10% formalin solution for subsequent histopathological analysis. The remaining dissected liver sections were immediately placed in liquid nitrogen, and then stored at -80°C until further use. During the sacrifice, various organs were removed, collected and weighed; notably, liver, muscle, white adipose tissue (WAT; epididymal and retroperitoneal fat pads), and subscapular brown fat (BAT). All were placed in liquid nitrogen and then stored at -80°C until further use.

2.2.4 Blood parameters

Plasma insulin, adiponectin and leptin were assessed by radioimmunoassay (RIA: Linco Research, St-Charles, MO) according to manufacturer's instructions. In order to avoid

interrupting the dietary plant treatment, mice were not fasting when their glycemia and insulin levels were measured. Plasma levels of circulating lipids (Triglycerides (TG), total cholesterol, HDL, LDL) were measured by the Department of Biochemistry of Sainte-Justine's Children Hospital (Montreal, QC, Canada).

2.2.5 Histological evaluation and tissue triglyceride measurement

The dissected liver sections embedded in paraffin were cut, mounted on glass slides and stained with hematoxylin phloxine saffron (HPS) by the Institut de recherche en immunovirologie et cancer (IRIC), Department of Histology (Université de Montréal, Montreal, QC, Canada). Each stained liver section was analyzed for the severity of lipid accumulation in the hepatocytes. The extent of hepatocyte lipid accumulation was then scored based on the percentage of hepatocytes that contained macrovesicular fat: namely, grade 0 (0-5%), grade 1(5-33%), grade 2 (33-66%), and grade 3 (66-100%) (Brunt et al., 1999; Kleiner et al., 2005). Part of the frozen liver and muscle sections were used to determine the extent of triglyceride content. Liver and muscle samples, 100 mg of each sample, were ground into powder under liquid nitrogen and then extracted using Folch's chloroform/methanol (2:1) method. Triglyceride content was then quantified using a commercial kit (Randox Laboratories Ltd., UK). Our research team has observed a strong correlation between quantified hepatic triglyceride content and stratification of liver lipid accumulation by histological analysis (Haddad et al., 2009; Haddad et al., 2011).

2.2.6 Skin temperature

In order to evaluate the temperature of the animals, a non-invasive and least stressful procedure was used by applying a probe on the external intercostal region of the animal for 2 minutes (Dallmann et al., 2006). The skin temperature was read and recorded with a digital thermometer (Cole-Parmer Instrument Company, USA) at weeks 4 and 8 of the feeding period.

2.2.7 Western Blot analysis

Frozen liver, WAT, and BAT were homogenized in RIPA lysis buffer (50mM Hepes, 150mM NaCl, 5mM EGTA, 2mM $MgCl_2$, 5% glycerol, 1% Triton X-100, 0.1% SDS, 1% Na-deoxycholate, pH 7.4) containing protease inhibitors (complete mini EDTA-free; Roche Diagnostics, Laval, QC) and phosphatase inhibitors (2mM PMSF, 10mM NaF, 100μM Na-orthovanadate, 1mM Na-pyrophosphate). In order to measure Glut4 expression in the muscle, samples were homogenized in sucrose buffer (Tris buffer pH 7.4, 20mM Tris-HCl, 255mM sucrose, 1mM EDTA), centrifuged (12000g for 12 min) and supernatant removed to carry out western blot analysis. Western blot analysis was performed on total liver, muscle, WAT and BAT homogenates by loading equal amounts of protein (50μg) on 10% SDS-polyacrylamide gels that were subjected to electrophoresis and then transferred to nitrocellulose membranes (BioRad Laboratories, ON, Canada). Membranes were probed with the following antibodies: p-Akt (Ser 473), Akt, phospho p44/42 MAPK, 44/42 MAPK, p-IKKαβ, β-actin (each at 1:1000 in blocking buffer incubated overnight at 4°C; Cell Signaling Tech Inc., Danvers, MA, USA). PPARα, PPARγ, UCP-1 were also measured using a 1:200 dilution in blocking buffer and incubated either at 1h room temperature (RT) or overnight at 4°C (Santa Cruz Biotechnology inc., Santa Cruz, CA, USA). The membranes were then washed and probed for 1h at RT in blocking buffer with HRP-conjugated secondary antibodies: anti-rabbit (1:10000; Jackson Immunoresearch Laboratories Inc., West Grove, PA), or anti-mouse (1:4000; Cell Signaling Tech Inc., Danvers, MA, USA), or anti-goat (1:5000; Santa Cruz Biotechnology inc., Santa Cruz, CA, USA). The immunoreactive proteins were detected by enhanced chemiluminescence method (GE Healthcare, Baie d'Urfé, QC, Canada). Densitometric analysis was performed using NIH Image J software (version 1.42q, NIH, USA).

2.2.8 Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), followed by Bonferonni post-hoc test, as appropriate using Sigma Stat software (Jandel Scientific, San Rafael, CA). Areas under the curve (AUC) were calculated by using PRISM software (GraphPad, San Diego, CA, USA). Data are expressed as mean ± SEM of the indicated number of determinations. Statistical significance was set at $p < 0.05$.

2.3 Results

2.3.1 DIO model: an obese and prediabetic state

As expected, C57BL/6 mice fed with HFD for 8 weeks displayed a typical DIO profile: **1)** increased whole body, liver and fat pad (WAT and BAT) weights (Figure 1 & Table 1); **2)** elevated blood lipid profile (total cholesterol, LDL, and HDL; Table 2); **3)** higher levels of grade 2 and 3 hepatic steatosis (Table 3); **4)** increased hepatic and muscle TG levels (Figure 2, panels A and B); and **5)** increased circulating Leptin/Adiponectin ratio, an indicator of insulin resistant state (Table 2) (Finucane et al., 2009). Taken together, these findings thus confirm the prediabetic and insulin resistant state of the DIO group. Of note, all these changes occurred without any significant change in energy intake (data not shown).

2.3.2 *P. balsamifera* significantly diminishes body weight gain and hepatic steatosis while improving insulin resistance of DIO mice

While control DIO mice gained about 70% of their weight during the second half of their feeding period (between weeks 4 to 8; F2; Table 1), *P. balsamifera*'s reduction of weight gain was immediate (weeks 0-4; F1) and was maintained throughout the entire feeding period (F1 and F2) with the same intensity. This was associated with smaller size WATs (retroperitoneal fat pad; by 12% - 17%; $p < 0.05$ Table 1) as compared to DIO congeners. However, the circulating lipid profile (LDL, HDL, and total cholesterol) was not significantly affected by administration of the plant extract (Table 2). Finally, *P. balsamifera* exhibited no toxicity as demonstrated by unaltered blood biochemical parameters and tissue histological examination (data not shown).

P. balsamifera also displayed a mild effect on glycemia. The highest dose significantly reduced the AUC of glycemia-vs-time by 10% as compared to DIO controls during the F2 period

($p<0.05$; Table 2). This is also the period when the DIO group glycemia increased most as compared to respective values in non-obese CHOW controls (increase of 24 and 13% for AUC_{F2} and AUC_{F1} of DIO versus CHOW; $p<0.05$; Table 2). Similarly, blood glycemia of plant fed animals was 17% lower than that of DIO control animals on the day of sacrifice, albeit this difference failed to reach statistical significance (Table 2). In parallel, insulin sensitivity was improved with the plant extract administration as evidenced by the tendency for insulin levels to be reduced (by 42-45%; NS; Table 2), the significant increase of adiponectin levels (by 40-62%; $p<0.05$; Table 2) and decrease of Leptin/Adiponectin ratio (by 43-49%; $p<0.05$; Table 2). Furthermore, liver weights were smaller by 14% - 22% ($p<0.05$; Table 1) and the prevalence of grade 2 and 3 steatotic livers was prevented by 40% when *P. balsamifera* was added to HFD (30% versus 50% for Plant and DIO groups respectively; Chi square $p<0.05$; Table 3). Hepatic (NS; Figure 2A) and muscle ($p<0.05$; Figure 2B) TG levels were lowered by approximately 40% in animals treated with *P. balsamifera* at 250 mg/kg, although the change was only statistically significant in muscle.

2.3.3 *P. balsamifera* has only a mild effect on energy intake, but significantly increases skin temperature and UCP-1 levels in BAT

Parameters of energy intake and energy expenditure were also assessed. Although overall energy intake was not significantly altered by experimental protocols (AUC_T for food intake expressed in kcal), a slight but significant decline was observed in the group fed with 250 mg/kg of the plant extract during the F2 period only as compared to DIO control group (8% reduction; $p<0.05$; data not illustrated).

We also used skin temperature as an indirect measure of energy expenditure, which was recorded at the midpoint (4 weeks) and at the end (8 weeks) of the feeding protocol. As figure 3 demonstrates, CHOW and DIO control animals exhibited similar superficial body temperatures after 4 and 8 weeks of treatment. Interestingly, animals fed with *P. balsamifera* experienced a significant increase in their skin temperature at 4 weeks as compared to that of the control DIO group and this was maintained at 8 weeks ($p < 0.05$). Notably, skin temperature of animals ingesting *P. balsamifera* for 8 weeks was increased by 1.3°C and 1.8°C at 125 mg/kg and 250 mg/kg doses respectively ($p<0.05$; Figure 3). These results correlated with a strong tendency for the protein levels of uncoupling protein-1 (UCP-1) to increase in BAT of *P. balsamifera* treated animals (22%-49%, NS; Figure 4A).

2.3.4 *P. balsamifera* activates the MAPK pathway in retroperitoneal adipose tissue

In order to establish the mechanisms of action of *P. balsamifera*, we then investigated key molecular events involved in the development of obesity and diabetes in the major insulin-responsive tissues, beginning with adipose tissue. Since *P. balsamifera* was previously shown to inhibit 3T3-L1 preadipocytes proliferation and differentiation *in vitro* (Martineau et al., 2010a; Martineau et al., 2010b), we investigated the ERK pathway, which has been shown to inactivate PPARγ transcriptional activity (Camp and Tafuri, 1997; Hu et al., 1996) during adipogenesis. As shown in Figure 4B 44/42 MAPK phosphorylation levels significantly increased with the 125 mg/kg dose of *P. balsamifera* (63% vs DIO; $p < 0.05$) in WAT. This was associated with a slight, albeit not statistically significant decrease in PPARγ expression levels (11% decrease, NS; Figure 4C).

2.3.5 *P. balsamifera* significantly decreases hepatic inflammation while improving hepatic lipid and glucose metabolism

When we probed liver tissue for levels of Akt phosphorylation, and therefore activation, no significant differences were observed (Figure 4D). However, DIO animals tended to display elevated values of the phosphorylated protein, consistent with the associated hyperinsulinemic state described above. Interestingly Akt phosphorylation in animals treated with *P. balsamifera* at 250 mg/kg returned to levels recorded in non-obese Chow controls (Figure 4D; N.S.). More dramatically, *P. balsamifera* significantly and dose-dependently decreased liver p44/42 MAPK phosphorylation levels (by 48% and 79% for 125 mg/kg and 250 mg/kg groups versus DIO; $p=0.086$ and $p < 0.05$, respectively; Figure 4E). Furthermore, hepatic IKK-αβ phosphorylation, a marker of inflammatory state (Arkan et al., 2005; Kim et al., 2001; Yuan et al., 2001), was doubled in the HFD as compared to Chow ($p= 0.093$; Figure 4F). Addition of *P. balsamifera* to HFD decreased IKK-αβ phosphorylation as compared to DIO controls, especially at the 250 mg/kg dose (by 45%, $p < 0.05$; Figure 4F). In contrast, PPARα expression did not display statistically significant differences between treatment groups; albeit values appeared to be increased in animals treated with the plant extract (by 20% and 31% for 125 mg/kg and 250 mg/kg groups, respectively Figure 4G). Finally, we probed skeletal muscle tissue for Glut4 protein and found no variation in levels of expression between the plant extract-treated and control DIO animals (data not shown).

2.4 Discussion

Our research team has developed a comprehensive and structured approach to identify Boreal forest plants stemming from the traditional pharmacopoeia of the CEI that exhibit promising potential for the treatment and care of diabetes and its complications. Seventeen plants were selected for study on the basis of their traditional use against a set of symptoms related to diabetes (Fraser et al., 2007; Harbilas et al., 2009; Leduc et al., 2006). Balsam poplar or *P. balsamifera* attracted our team's attention because it unexpectedly and potently prevented 3T3-L1 adipocyte differentiation in an *in vitro* bioassay designed to assess glitazone-like adipogenesis and insulin sensitizing biological activity (Harbilas et al., 2009). This inhibitory effect suggested that balsam poplar might have therapeutic potential against obesity. However, if inhibition of adipogenesis was the sole action of the plant, it could lead to redistribution of fat stores to other tissues such as the liver and muscle, thus contributing to insulin resistance. Therefore, the careful assessment of the plant's effect in the DIO mouse model of obesity and insulin resistance was warranted.

In the present study, concomitant administration of *P. balsamifera* with a HFD was employed to assess the potential of this plant extract to attenuate the development of obesity and the associated metabolic disturbances. Our results clearly indicate that *P. balsamifera* significantly mitigates whole body weight gain of mice subjected to a continuous hypercaloric fat-laden diet. They even suggest a dose-dependent effect on many of the measured parameters. This was also associated with a significant reduction in WAT (retroperitoneal fat pads) weight gain. It is worthy to note that retroperitoneal fat pads, as part of the visceral adipose tissue, have been involved in the negative effects of obesity in humans (Freedland, 2004; Oben et al., 2006), while epididymal fat does not exhibit such involvement (Morton et al., 2005) Therefore, *P.*

balsamifera's targeted effect on retroperitoneal fat pads heightens its therapeutic potential for the metabolic syndrome. This effect on WAT size could be the result of the observed increase in 44/42 MAPK activation. Indeed, such activation has been associated with an inhibition of adipocyte differentiation *in vitro* and *in vivo* (Donzelli et al., 2010) (Finucane et al., 2009) and has been shown to inhibit the transcription activity of PPARγ as well as to activate SOX9, which in turn decrease the expression of C/EBPβ and C/EBP δ (Sul, 2009). Although PPARγ levels did not vary significantly with ingestion of *P. balsamifera*, other effectors of adipogenesis could be involved and require further study.

Most significantly, changes in weight gain occurred without drastic effect on cumulative energy (hence caloric) intake, since only a slight decline was recorded with the higher dose of *P. balsamifera* during the second half of treatment (F2 period). However, the intensity of this reduction in caloric intake (8%) amounted to about one third of the observed decrease in weight gain during that same period (24%). Nevertheless, this apparent slight anorexic effect will require further investigation.

Another interesting observation is the increase in superficial body temperature induced by *P. balsamifera* treatment. This elevation occurred in an apparent dose-dependent manner after 8 weeks of treatment, therefore suggesting that increased energy expenditure in the form of heat could represent part of *P. balsamifera*'s mode of action to afford beneficial actions on obesity and the metabolic syndrome. These findings are corroborated by the tendency for *P. balsamifera* to increase brown adipose tissue expression of UCP-1, the mitochondrial protein used to generate heat by non-shivering thermogenesis in BAT. However, detailed analysis of energy expenditure in animals fed with this plant extract is required to confirm this interpretation.

Balsam poplar exhibited a mild effect on glycemia, decreased blood levels in the second half of feeding period (4 to 8 weeks) occurring only with the 250 mg/kg dose. This suggests that the plant's effect on glycemia is not immediate and might be the consequence of improved obesity and/or of the insulin resistant state. This is consistent with the lack of significant change in muscle Glut4 protein expression, which would have been expected to have a direct impact on glycemia.

Nevertheless, several lines of evidence highlight the capacity of *P. balsamifera* to mitigate the development of an insulin resistant state in DIO animals. Firstly, insulin levels in balsam poplar fed group were almost half of those of DIO group, although data variability precluded statistical significance. This variability could be partially explained by the fact that insulin measurements were conducted in non-fasting animals in order to respect the treatment protocols. Indeed, we needed to ensure an uninterrupted delivery of the plant preparations to the animals, extracts being incorporated into the diet. In addition, the primary goal of this study was to evaluate the anti-obesity effect of the plant in a diet-induced obesity model. Hence, fasting the animals would likely have resulted in a drop of food intake and potentially in body weight, which could have biased data interpretation. On the other hand, elevated circulating levels of adiponectin and low leptin/adiponectin ratio represented a second line of evidence to support plant-induced improvements in insulin resistance and this is consistent with the literature (Abbasi et al., 2004; Finucane et al., 2009; Tschritter et al., 2003; Weyer et al., 2001; Yamamoto et al., 2002). Thirdly, the increase of muscle TG levels observed with the DIO model was significantly attenuated with ingestion of *P. balsamifera*, thus contributing to the improvement of insulin sensitivity.

Furthermore, balsam poplar treatment diminished the development of liver steatosis by 40%, which shifted the severity of liver fat accumulation towards grades of weak and absent

steatosis as opposed to moderate and high steatosis in DIO controls. This correlated with a strong, albeit not significant, tendency to decrease hepatic triglyceride levels as compared to DIO group. In fact, DIO associated hepatic steatosis, as seen in human non-alcoholic fatty liver disease (NAFLD) and its more severe manifestation of non-alcoholic steatohepatitis (NASH), is recognized as being an important component of the metabolic syndrome and is involved in the development of insulin resistance in the liver (den Boer et al., 2004; Girard and Lafontan, 2008). Therefore, improvement of hepatic steatosis represents an important feature of any therapy directed against the metabolic syndrome, potentially such as *P. balsamifera.*

At the molecular level, the 250 mg/kg dose of *P. balsamifera* shows a tendency to normalize hepatic Akt levels. This is consistent with the concurrent decrease in insulin levels, itself indicative of an improvement in insulin sensitivity. At the same time, balsam poplar essentially prevented the DIO-related increase in hepatic p44/42 MAPK activity. This pathway has been associated with more intense pre-replicative phases of hepatocyte proliferation and hyperplasia leading to the development of NAFLD (Chavez-Tapia et al., 2009). It is therefore possible that reduced MAPK phosphorylation contributed, at least in part, to the observed decrease in hepatic steatosis and TG content. On the other hand, *P. balsamifera* had only a marginal impact on PPARα pathway, normally responsible for enhancing lipid β-oxidation. However, other components controlling TG synthesis (FAS) or β-oxidation (AMPK, ACC) could be involved and future studies will need to assess their implication.

Finally, the presence of a low-grade inflammatory state is another important component of obesity and the metabolic syndrome leading to diabetes. IKK-αβ, a protein kinase that is implicated in the stimulatory pathway of the pivotal pro-inflammatory transcription factor NF-κB (Arkan et al., 2005; Kim et al., 2001; Yuan et al., 2001), has been associated with insulin

resistance in several models of the metabolic syndrome. Our results show that the highest dose of *P. balsamifera* significantly prevented phosphorylation of hepatic IKK-αβ. This clearly indicates a reduced inflammatory state and completes several lines of evidence that converge in support of a significant positive impact of balsam poplar extracts on insulin sensitivity in an *in vivo* context of obesity and metabolic syndrome.

It is worthy to note that no changes in the behavior of mice treated with the plant extracts were observed. Moreover, *P. balsamifera* treatment yielded no significant liver toxicity. It must be stressed that balsam poplar has been used safely for generations by several Aboriginal peoples of the Northern hemisphere (Arnason et al., 1981; Leighton, 1985; Marles et al., 2000; Marles and Farnsworth, 1995; Moerman, 1998), including CEI populations. In addition, the inner bark of balsam poplar (the plant part used by the CEI and hence evaluated in the current studies) is considered as an emergency food (Leighton, 1985; Marles et al., 2000).

In summary, this study confirms that *P. balsamifera*, a Boreal forest medicinal plant identified through ethnobotanical surveys in CEI (Fraser et al., 2007; Harbilas et al., 2009; Leduc et al., 2006), exerts a significant anti-obesity and anti-diabetic potential in the DIO mouse model of metabolic syndrome. Results notably and clearly show that balsam poplar can attenuate the development of obesity by decreasing weight gain and significantly improving insulin sensitivity in normal mice continuously consuming a hypercaloric fat-laden diet. It is important to point out that *P. balsamifera*'s effect on final body weight gain (7% decrease compared to DIO controls at the end of our protocol) is in the same efficacy range as the anti-obesity treatments currently available on the market, which yield 5-10% decrease in body weight (Grundy, 2006). Furthermore, *P. balsamifera*'s ability to inhibit adipogenesis *in vitro* was corroborated by decreased WAT weight *in vivo* and, importantly, without fat redistribution to ectopic sites such as the liver and muscle, as evidenced by decreased steatosis and TG accumulation in those tissues.

In conclusion, these findings thereby bring solid evidence that *P. balsamifera* can mitigate the development of obesity and its ensuing negative metabolic impacts, thus validating its traditional use in the context of these diseases. Indeed, in view of the soaring rise in obesity and diabetes among Aboriginal Canadian populations, *P. balsamifera* offers an interesting option for the care and treatment of these diseases stemming from CEI Traditional Medicine. Therefore, our results provide strong impetus for the integration of *P. balsamifera* in culturally adapted clinical studies as well as care and treatment regimens for obesity and diabetes among the Cree, alongside lifestyle changes such as a healthy diet and exercise.

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Disclosure

The authors declare no conflict of interest.

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2.7 Figure Legends

Figure 1 : Cumulative change in body weight (CCBW) in C57BL/6 mice treated with either standard diet (CHOW), HFD (DIO), or HFD in combination with *P.balsamifera* at 125 or 250 mg/kg for a period of 8 weeks. All values are mean ± SEM (n=10 to 13). *denotes significantly different as compared to DIO group (one way ANOVA, Bonferonni post-hoc test; $p < 0.05$).

Figure 2: A) Hepatic triglyceride levels, and B) Muscle triglyceride levels of C57BL/6 mice treated with either standard diet (CHOW), HFD (DIO), or HFD in combination with *P. balsamifera* at 125 or 250 mg/kg for a period of 8 weeks. All values are mean ± SEM (n=10 to 13). *denotes significantly different as compared to DIO group (one way ANOVA, Bonferonni post-hoc test; $p < 0.05$).

Figure 3: Skin temperature after 4 weeks and 8 weeks of treatment, from mice fed standard diet (CHOW), HFD (DIO) and HFD with *P. balsamifera* at 125 and 250 mg/kg. All values are mean ± SEM from 10-13 animals in each group. *denotes significantly different as compared to DIO group (one way ANOVA, Bonferonni post-hoc test; $p < 0.05$).

Figure 4 : Samples of BAT, WAT and liver from mice fed a standard diet (CHOW), HFD (DIO) and HFD with *P. balsamifera* at 125 and 250 mg/kg were homogenized and analyzed by immunoblotting. Blots were quantified by densitometry and data are expressed as mean ± SEM from 10-13 animals in each group. Representative immunoblots and their quantification are shown for samples probed with A) UCP-1 in BAT, B) phospho p-44/42 MAPK in adipose tissue, C) PPARγ in adipose tissue, D) phospho Akt in liver, E) phospho p-44/42 MAPK in liver, F)

phospho IKKαβ in liver, G) PPARα in liver. *denotes significantly different as compared to DIO group (one way ANOVA, Bonferonni post-hoc test; $p < 0.05$).

Table 1: Effects of obesity and *P. balsamifera* treatments on body and organ weights

		CHOW	DIO	*P. balsamifera* 125 mg/kg	*P. balsamifera* 250 mg/kg
Parameters at sacrifice	**Body Weight** *(g)*	29.5± 0.6*	43.4 ± 0.8	41.6 ± 0.7	40.2 ± 1.0*
	Retroperitoneal Fat Pad *(g)*	0.41 ± 0.04*	1.39 ± 0.06	1.23 ± 0.04*	1.16 ± 0.05*
	Epididymal Fat Pad *(g)*	0.85 ± 0.05*	2.39 ± 0.09	2.57 ± 0.06	2.58 ± 0.09
	Brown Fat Pad *(g)*	0.15 ± 0.01*	0.31 ± 0.03	0.28 ± 0.02	0.26 ± 0.03
	Liver Weight *(g)*	1.60 ±0.04*	1.83 ± 0.10	1.57 ± 0.05*	1.43 ± 0.08*
Continuous measurements	**CCBW - AUC_T**	347.1 ± 16.1*	702.8 ± 30.3	603.0 ± 28.5*	529.8 ± 26.6*
	CCBW - AUC_{F1}	111.9 ± 6.0*	174.4 ± 8.5	147.1 ± 7.8*	130.2 ± 6.5*
	CCBW - AUC_{F2}	224.5 ± 10.5*	506.4 ± 21.3	435.6 ± 20.4*	382.0 ± 19.9*

Measurements were obtained after 8 weeks of treatment with either standard diet (Chow), HFD (DIO), and HFD in combination with *P. balsamifera* at 125 or 250 mg/ kg. All values represent the mean ± SEM (n=10 to 13). Total area under the curve (AUC_T) for CCBW-vs-time was calculated based on CCBW curve shown in Figure 1, and then fractionated into the first and second half of the feeding period corresponding to weeks 0-4 (F1) and weeks 4-8 (F2), respectively. * denotes that groups are significantly different as compared to DIO (one-way ANOVA, Bonferonni post-hoc test; $p < 0.05$).

Table 2: Effects of obesity and *P. balsamifera* treatments on systemic parameters

		CHOW	DIO	*P. balsamifera* 125 mg/kg	*P. balsamifera* 250 mg/kg
Parameters at sacrifice	**Glucose** *(mmol/L)*	7.1 ± 0.2*	10.3 ± 0.5	8.5 ± 0.2	8.5 ± 0.3
	Insulin *(ng/mL)*	1.27 ± 0.10*	10.11 ± 1.84	5.85 ± 0.61	5.53 ± 1.72
	TG *(mmol/L)*	0.75 ± 0.07*	0.53 ± 0.05	0.43 ± 0.03	0.43 ± 0.04
	LDL *(mmol/L)*	0.20 ± 0.05*	0.82 ± 0.06	0.89 ± 0.07	0.78 ± 0.06
	HDL *(mmol/L)*	0.83 ± 0.04*	1.22 ± 0.07	1.34 ± 0.04	1.34 ± 0.04
	Total cholesterol *(mmol/L)*	1.39 ± 0.07*	2.27 ± 0.10	2.43 ± 0.10	2.32 ± 0.07
	Leptin *(ng/mL)*	13.12 ± 2.08*	42.13 ± 4.30	35.42 ± 3.00	36.98 ± 2.47
	Adiponectin *(μg/mL)*	8.81 ± 0.63	8.62± 0.69	12.10± 0.55*	14.00 ± 0.58*
	Leptin/adiponectin ratio	1.46± 0.17*	5.39 ± 0.75	3.07 ± 0.37*	2.74 ± 0.25*
Continuous measurements	**Glycemia - AUC_T**	468.9 ± 6.7*	556.8 ± 9.0	553.2 ± 7.5	546.6 ± 12.6
	Glycemia - AUC_{F1}	220.4 ± 4.3*	249.5 ± 5.2	263.0 ± 6.4	268.2 ± 5.1
	Glycemia - AUC_{F2}	184.2 ± 2.8*	228.8 ± 6.3	213.5 ± 4.3	204.9 ± 6.9*

Measurements were obtained after 8 weeks of treatment with either standard diet (Chow), HFD (DIO), and HFD in combination with *P. balsamifera* at 125 or 250 mg/ kg. All values represent the mean ± SEM (n=10 to 13), and were taken at the end of the protocol on sacrifice day. Total area under the curve (AUC_T) for glycemia levels-vs-time was calculated, and then fractionated into the first and second half of the feeding period corresponding to weeks 0-4 (F1) and weeks 4-8 (F2), respectively. * denotes that groups are significantly different as compared to DIO (one-way ANOVA, Bonferonni post-hoc test; $p < 0.05$).

Table 3: Effect of *P. balsamifera* on histological analysis (scoring) of liver lipid accumulation (steatosis)

Histology	CHOW	DIO	*P. balsamifera* 125 mg/kg	*P. balsamifera* 250 mg/kg
Grade 0	10	1	3	5
Grade 1	0	5	6	4
Grade 2	0	5	3	2
Grade 3	0	1	1	2
n	10	12	13	13
Chi square	***P* < 0.05**			

Histological scoring of HPS-stained livers from C57BL/6 mice treated for a period of 8 weeks with either standard diet (Chow), HFD (DIO), or HFD in combination with *P. balsamifera* at 125 or 250 mg/kg. Percentage of hepatocytes containing macrovesicular steatosis, grade 0 : 0-5 %, grade 1 : 5-33 %, grade 2 : 33-66 %, grade 3 : more than 66 %.

Figure 1

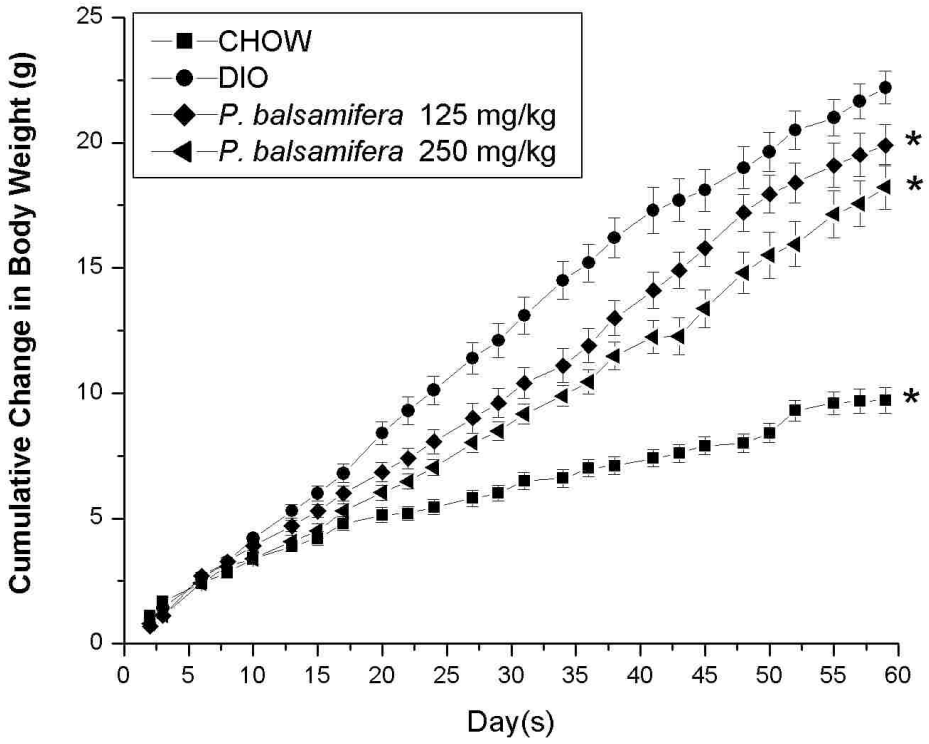

CHOW
DIO
P. balsamifera 125 mg/kg
P. balsamifera 250 mg/kg
Cumulative Change in Body Weight (g)
Day(s)
0
5
10
15
20
25
30
35
40
45
50
55
60
*
*
*

Figure 2

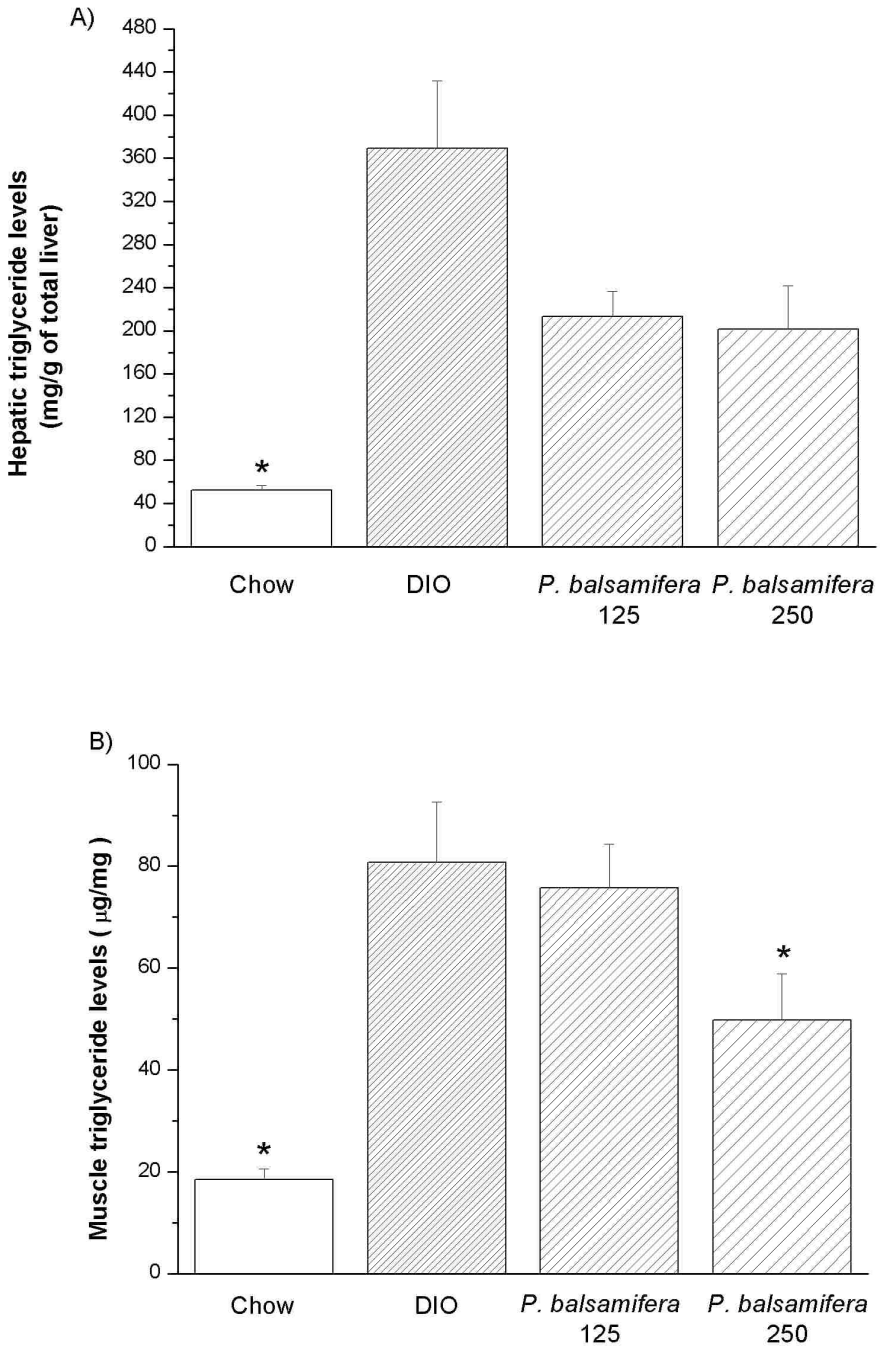

A)
Hepatic triglyceride levels (mg/g of total liver)
480
440
400
360
320
280
240
200
160
120
80
40
0
*
Chow
DIO
P. balsamifera 125
P. balsamifera 250
B)
Muscle triglyceride levels (μg/mg)
100
80
60
40
20
0
*
*
Chow
DIO
P. balsamifera 125
P. balsamifera 250

Figure 3

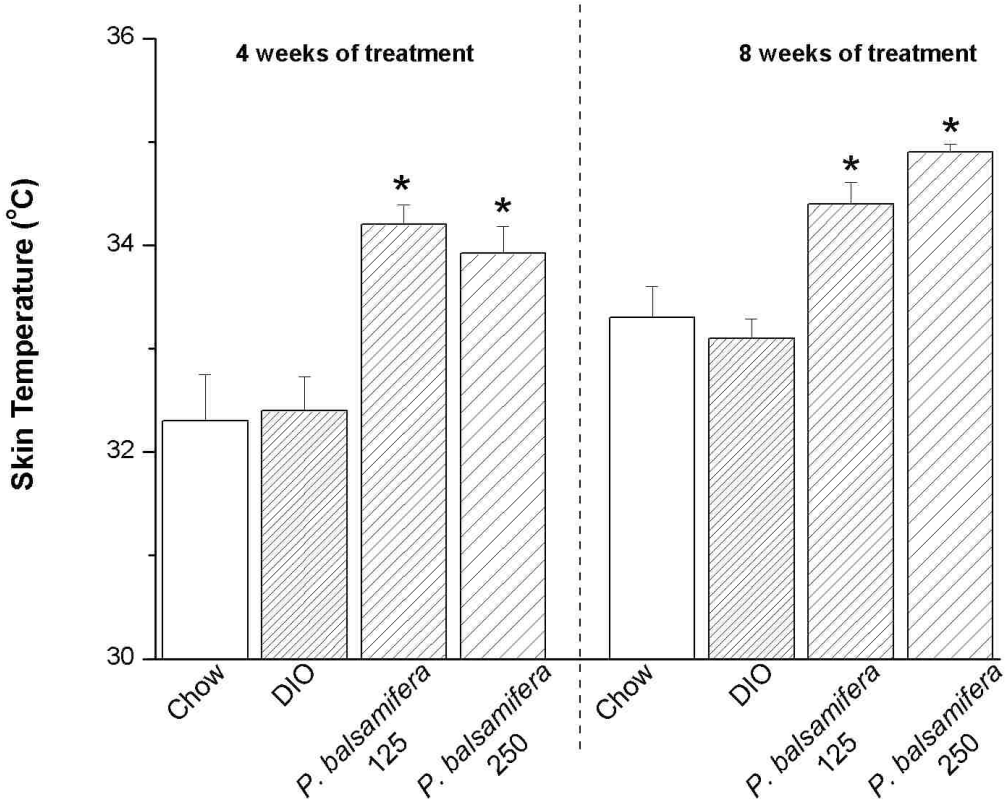

Skin Temperature (°C)
36
34
32
30
4 weeks of treatment
8 weeks of treatment
*
*
*
*
Chow
DIO
P. balsamifera 125
P. balsamifera 250
Chow
DIO
P. balsamifera 125
P. balsamifera 250

Figure 4

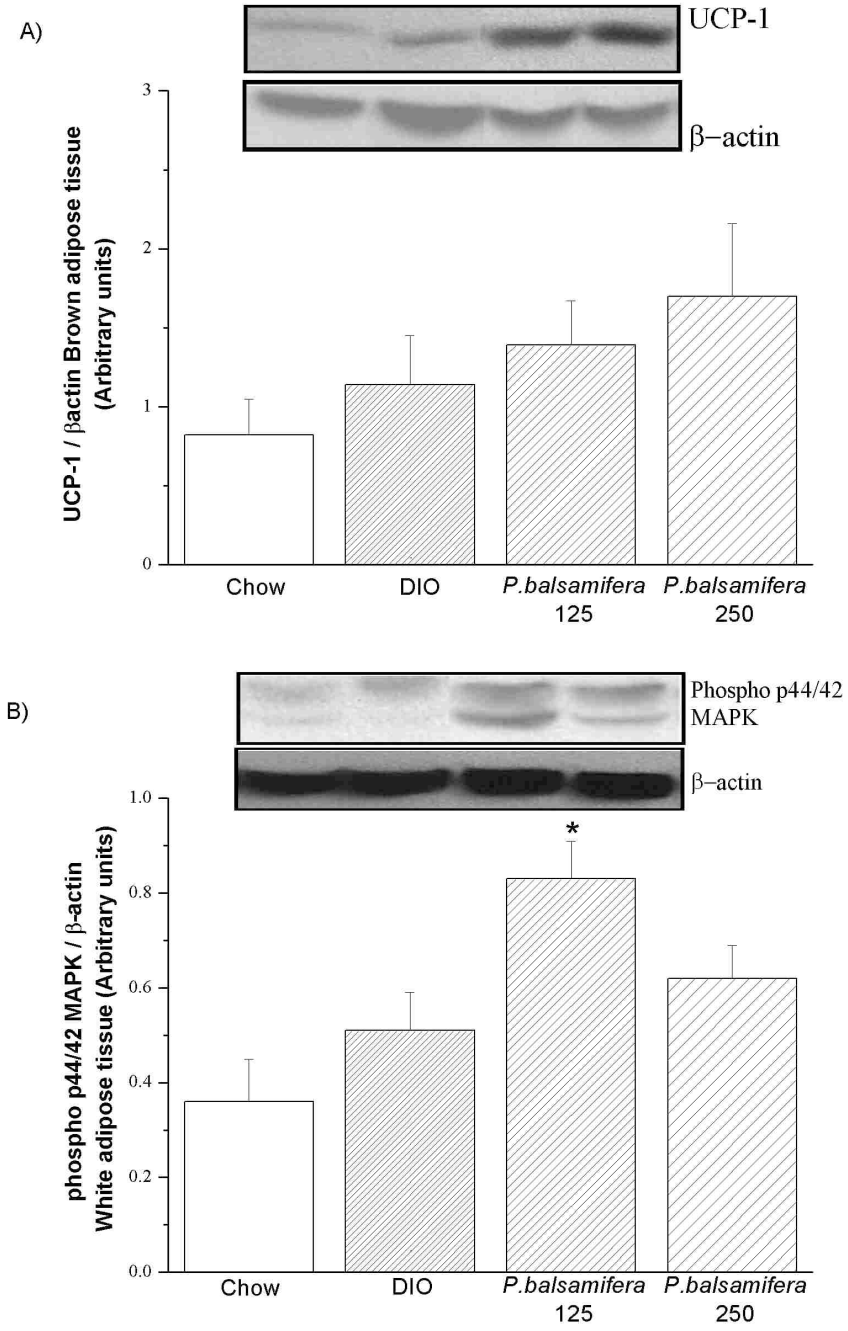

A)
UCP-1
β−actin
UCP-1 / βactin Brown adipose tissue (Arbitrary units)
3
2
1
0
Chow
DIO
P.balsamifera 125
P.balsamifera 250
B)
Phospho p44/42 MAPK
β−actin
phospho p44/42 MAPK / β-actin White adipose tissue (Arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
*
Chow
DIO
P.balsamifera 125
P.balsamifera 250

Figure 4

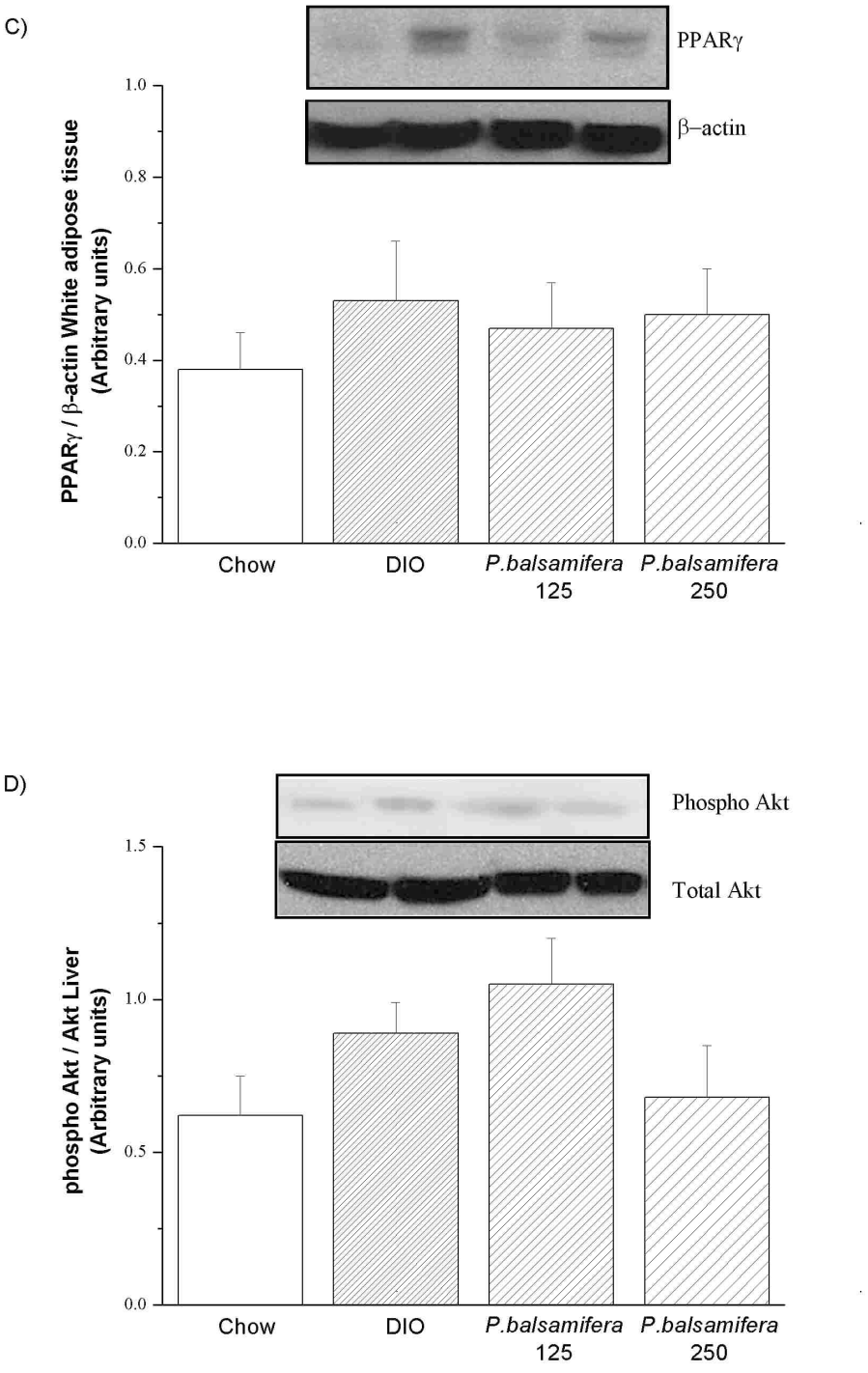

C)
PPARγ
β–actin
PPARγ / β-actin White adipose tissue (Arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
Chow
DIO
P.balsamifera 125
P.balsamifera 250
D)
Phospho Akt
Total Akt
phospho Akt / Akt Liver (Arbitrary units)
1.5
1.0
0.5
0.0
Chow
DIO
P.balsamifera 125
P.balsamifera 250

Figure 4

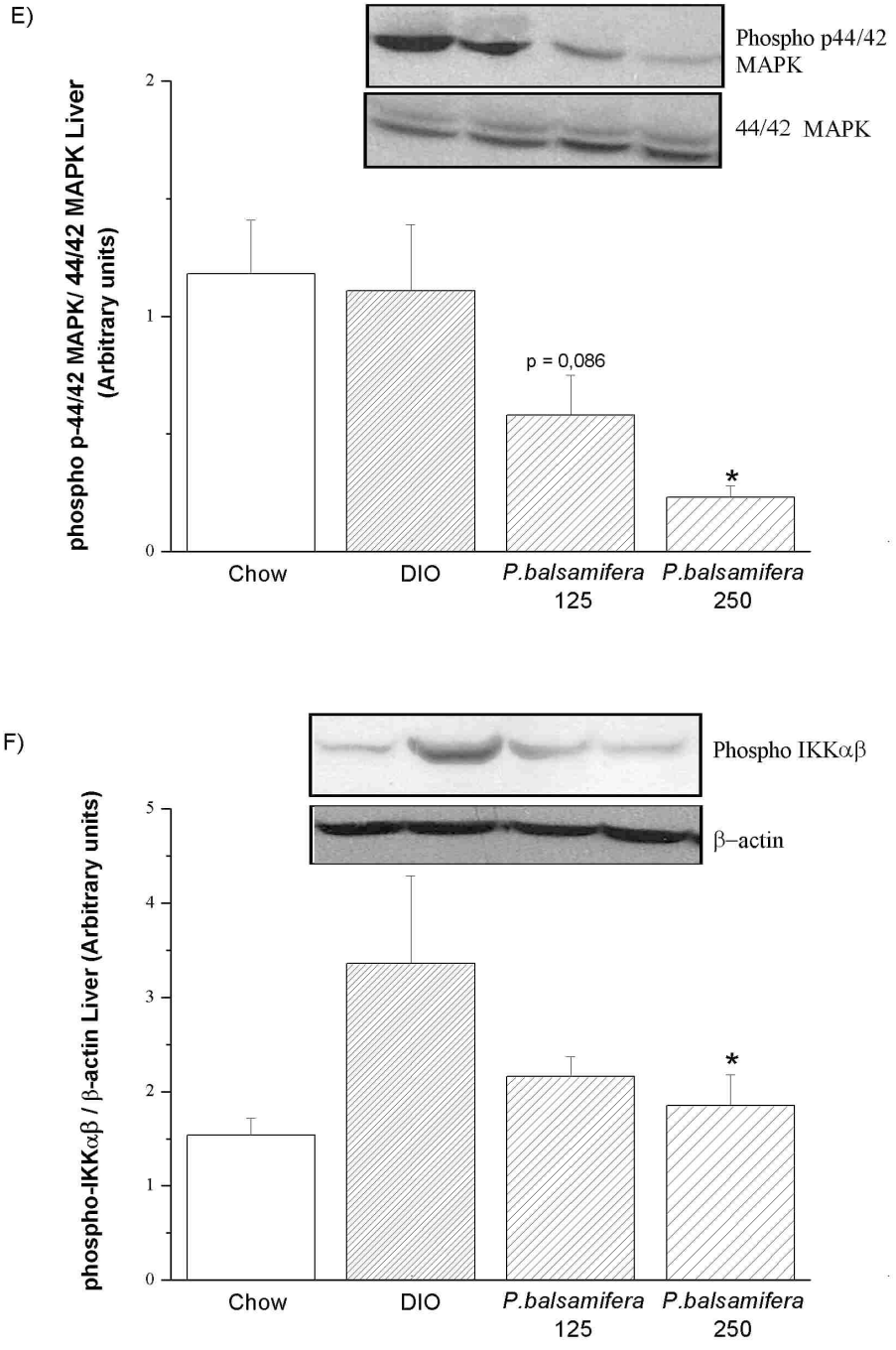
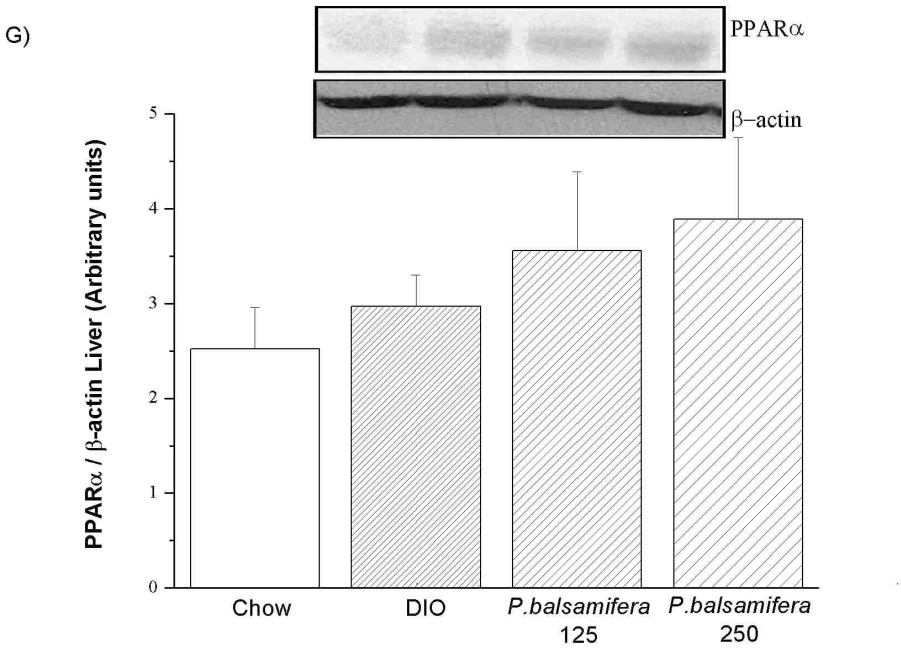

E)
Phospho p44/42 MAPK
44/42 MAPK
phospho p-44/42 MAPK/ 44/42 MAPK Liver (Arbitrary units)
2
1
0
p = 0,086
*
Chow
DIO
P.balsamifera 125
P.balsamifera 250
F)
Phospho IKKαβ
β–actin
phospho-IKKαβ / β-actin Liver (Arbitrary units)
5
4
3
2
1
0
*
Chow
DIO
P.balsamifera 125
P.balsamifera 250

Figure 4


G)
PPARα
β–actin
PPARα / β-actin Liver (Arbitrary units)
0
1
2
3
4
5
Chow
DIO
P.balsamifera 125
P.balsamifera 250

Chapter 3: Article 2

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Role of each co-author belonging to this manuscript

It is to note that this manuscript is ready for resubmission to the International Journal of Obesity. I the student Despina Harbilas did the great majority of the work in this manuscript, with the technical support of Antoine Brault and Diane Vallerand: treatment, care and sacrificing of the animals, as well as all post-sacrifice analysis on blood and tissue parameters, and finally all data and statistical analysis. Drs. Ammar Saleem and John T. Arnason are our collaborators, and contributed in the preparation and characterization of the plant species. Dre Lina Musallam assisted in correcting the article. Dr. Pierre S. Haddad, my supervisor, contributed conceptual, intellectual and moral input as well as correcting the article.

Title:

Populus balsamifera extract and its active component Salicortin reduce obesity and attenuate insulin resistance in a diet-induced obese mouse model

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Running title: **Balsam poplar counters obesity and diabetes**

Abstract

Background: The prevalence of the metabolic syndrome is on the rise among the Cree of Eeyou Istchee (CEI - Northern Quebec) as a result of increases in obesity and insulin resistance. Non-traditional diet and sedentary lifestyle along with cultural disconnect of modern type 2 diabetes (T2D) therapies are involved. Exploring treatments from within CEI traditional pharmacopeia represents a valuable alternative. *Populus balsamifera*, or Balsam poplar (BP), a CEI plant from the Canadian Boreal Forest, demonstrated anti-obesity properties *in vitro*. Its active component salicortin was identified through a bioassay-guided fractionation approach. **Aim:** The present study aimed to assess the potential effects of BP extract and salicortin at reducing obesity and attenuating insulin resistance in a mouse model of diet-induced obesity and T2D. **Materials and Methods:** C57Bl/6 mice were subjected to high fat diet (HFD) for eight weeks resulting in obesity, hyperinsulinemia and mild steady-state hyperglycemia. BP or salicortin was introduced in the HFD for an additional eight weeks. **Results:** BP and salicortin effectively reduced body weight, retroperitoneal fat pad weight, and lipid content of the liver, as compared to HFD controls. This was accompanied by a small yet statistically significant reduction in food intake only in animals treated with BP. Glycemia, insulinemia, leptin and adiponectin levels were also improved as compared to HFD controls. Key components of signaling pathways were assessed by Western blot analysis in the three main insulin-sensitive tissues, namely liver, muscle and adipose tissue. The pathways activated most potently by BP and slightly by salicortin are involved in glucose regulation and lipid oxidation. **Conclusion:** BP and salicortin thus reduce obesity while increasing insulin sensitivity and modulating key components of glucose and lipid metabolism. These results confirm the validity of the CEI traditional pharmacopeia as alternative and complementary antiobesity and antidiabetic therapies.

Keywords: Anti-obesity, antidiabetic, adipokines, C57BL/6 mice, Balsam poplar, Aboriginal Traditional Medicine.

3.1 Introduction

Obesity results from a variety of risk factors, including unhealthy dietary habits and a sedentary lifestyle, resulting in higher energy input than output [1]. It also increases the risk for other chronic illnesses such as type 2 diabetes (T2D) and insulin resistance (IR)[1]. Insulin resistance is characterized by a decreased ability of insulin sensitive tissues to respond to insulin action. Skeletal muscle is the principal tissue involved in glucose metabolism through insulin-dependent or exercise-sensitive glucose transport (Glut4)[2] implicating the Akt [3] and AMPK [4] pathways, respectively. These pathways are also implicated in glucose metabolism in the liver [5-7] and adipose tissue [3, 8-12]. The liver is considered to be the principal tissue involved in glucose storage and production [13]. Adipose tissue synthesizes and stores fatty acids and is recognized as an endocrine organ; releasing adipokines (leptin, adiponectin) that are implicated in glucose and lipid metabolism [14-20]. Obesity not only leads to excessive fat storage in adipose tissue, but also to ectopic fat storage in other insulin sensitive tissues such as the muscle and liver (non-alcoholic fatty liver disease; NAFLD). This, in part, contributes to the development of insulin resistance [21, 22].

Several metabolic and signaling pathways are involved in perpetrating the disturbances of obesity and insulin resistance in the three main insulin-sensitive tissues. PPARγ is involved in the differentiation of adipose tissue; inducing lipid accumulation [23]. Other pathways are involved in lipid entry (FAT/CD36, FABP4)[24-27], lipid metabolism (SREBP-1c and FAS)[28, 29], and oxidation (ACC, CPT-1, PPARα, UCP pathways) [30]. The ERK pathway, involved in cell proliferation and differentiation, also seems to play an important role in both the liver (leads to NAFLD)[31] and adipose tissue [32, 33]. The IKKαβ pathway is involved in the inflammatory response characteristic of obesity and indirectly mediates insulin resistance [27].

In Canada, the Cree of Eeyou Istchee (CEI) of Eastern James Bay have a prevalence of obesity and T2D that is respectively at least 1.5 [34, 35] and 4 times higher [36] than the general Canadian population. This may be the consequence of major lifestyle changes (decreased physical activity and gradual adoption of non-traditional diets), as well as cultural difficulty to comply with modern T2D treatments. Our team has been working with the CEI to identify plants stemming from their traditional pharmacopeia that could offer culturally adapted complementary and alternative treatments for obesity and T2D. As part of an ethnobotanical survey, *Populus balsamifera* L. (Salicaceae) (balsam poplar) was identified as a plant used by the CEI to treat a variety of symptoms associated with T2D. As part of an *in vitro* bioassay platform used to screen for the antidiabetic potential of CEI plants, the 3T3-L1 cell line was selected to assess glitazone-like activity and stimulation of adipogenesis. *Populus balsamifera* L., also known as balsam poplar, unexpectedly and potently inhibited the accumulation of intracellular triglycerides [37-39], suggesting potential anti-obesity activity. In subsequent studies conducted in the same cell line, a bioassay-guided fractionation approach identified salicortin, a salicylate glycoside, as the principal active component of P. balsamifera responsible for the observed inhibition of adipogenesis [38]. Salicortin is abundant in poplar, willow bark, and throughout the salicaceae family [38]. Although salicylates are well known for having anti-inflammatory properties, improving insulin sensitivity [40-42], and even having anti-proliferative effects [43], anti-adipogenic activity had never been ascribed prior to the studies conducted by our team . We thus introduced *P. balsamifera* extract, alongside a high-fat diet (HFD), to study the plant's ability to mitigate the development of obesity using the *in vivo* diet-induced obese (DIO) C57BL/6 mouse model. The results clearly demonstrated that the plant extract substantially attenuated weight gain and the development of insulin resistance [44].

In the present studies, we sought to evaluate the effectiveness of *P. balsamifera* as well as its active principle salicortin at treating obesity and insulin resistance once they have been established in the same model [45, 46]. As previously described by other researchers [47] and discussed further below, DIO mice respond in a stratified manner to the HFD; some animals being resistant to the HFD (low responders – LR) while others show the clearcut profile of metabolic disease (high responders – HR). *P. balsamifera* and salicortin were thus administered to the latter DIO mice in order to determine their potential effectiveness in countering obesity and insulin resistance.

3.2 Materials and Methods

3.2.1 Plant extracts

Specimens of *Populus balsamifera* L. (Salicaceae), were collected on CEI territory (Eastern James Bay, Quebec, Canada). Dr. Alain Cuerrier, taxonomist at the Montreal Botanical Garden, confirmed the botanical identity and voucher specimens were deposited in the Marie-Victorin herbarium of the Montreal Botanical Garden in Montreal, Canada (Mis03-49). A crude 80% ethanolic extract of *P. balsamifera* was prepared as previously described [37]. Salicortin, the active principle of *P. balsamifera*, was produced through fractionation, isolation and purification of the crude plant extract as previously described [38]. The structure of the purified compound was identified and confirmed by ^{1}H and ^{13}C NMR and by comparison with previously reported data. 1D- and 2D−NMR spectra were generated using an Avance 400 spectrometer (Bruker Biospin Corporation) [38].

3.2.2 Animals and Diets

Four-week old male non-diabetic C57BL/6 mice (Charles River Laboratories, Saint-Constant, QC, Canada) were housed in individual cages, maintained on a 12 h light-dark cycle in a temperature and humidity-controlled animal room and given free access to food and water. Following acclimatization, the mice were divided into groups of approximately 12 mice each. Chow controls received a standard diet (SD; 18% protein content, 4.5% crude fat; Charles River Animal rodent diet) for 16 weeks. Other groups were fed a high fat diet (HFD; Bio-Serv Diet #F3282; 60% energy from fat) for eight weeks. *P. balsamifera* at 125 or 250 mg/kg, and salicortin at 12.5 mg/kg were incorporated in the HFD and treatments continued for an additional 8 weeks (DIO controls receiving only HFD). Balsam poplar extract studies were initiated first

while the active compound was being identified, isolated or purified. Since, salicortin composes 10% of the whole plant extract, and that the most efficient dose of balsam poplar was 125 mg/kg, salicortin was administered at 12.5 mg/kg. Hence, experimental groups of plant extract and salicortin are compared to distinct CHOW non-obese and DIO controls. However, both experimental protocols were conducted in an identical manner, and DIO controls reacted in a fully comparable fashion relative to non-obese Chow congeners in both studies. Body weight, food and water intake, as well as glycemia were measured 3 times/week during the entire study. Glycemia was measured by pricking the tail vein and by using a commercial glucometer (Accu-Check Roche, Montreal, QC, Canada). Measurements were always performed at the same time/day, in the same order and by the same person. All experimental protocols were approved by the animal experimentation ethics committee of the Université de Montréal and were carried out in full respect of the guidelines from the Canadian Council for the Care and Protection of Animals.

3.2.3 Data and animal segregation

The area under the curve (AUC) was calculated for parameters measured in a continuous manner throughout the study. The total AUC was then separated into two parts: fraction 1 (F1), representing AUC between week 0 and 4 (first month of treatment), and fraction 2 (F2) corresponding to the AUC between week 4 and 8 of plant extract administration (second month of treatment). This segregation served to determine the plant extract's temporal course of action, i.e. whether early in onset (first 4 weeks), later (last 4 weeks) or present throughout the study. Once the experimental feeding protocol had been carried out, we became aware of the studies of Peyot and collaborators [47] discriminating low responders (LR) and high responders (HR) in the DIO mouse model. As discussed by these authors and observed in our own studies, pooling

animals with different characteristics, such as low weight gain, weak IR and near-normal glycemia, with animals with high weight gain, frank IR, and hyperglycemia, can yield misleading results [47]. Therefore, we segregated the DIO animals based on these published criteria and analyzed our data accordingly. As expected, low responder animals exhibited a near normal metabolic profile and treatment with the plant extract or its active component essentially had little if any effect. This is positive in the sense that *P. balsamifera* extract and salicortin may have a desirable safety margin by being active only in metabolically compromised animals. This also confirms the validity of the segregation. Hence, data are presented for the effects of plant extract and active principle in HR animals only. This segregation did however reduce our sample size, hence contributing to data variability.

3.2.4 Surgical Procedure

At the end of the experimental protocol, mice were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital and sacrificed by exsanguination. Livers were flushed with physiological saline, dissected, immediately placed in liquid nitrogen and stored until further use at -80°C. Soleus skeletal muscle, white adipose tissue (WAT; epididymal and retroperitoneal fat pads), subscapular brown adipose tissue (BAT), and kidneys were also collected, placed in liquid nitrogen and stored at -80°C until further use.

3.2.5 Blood parameters

Plasma insulin, adiponectin and leptin were assessed by radioimmunoassay (RIA: Linco Research, St-Charles, MO). To avoid interrupting dietary plant treatment and disturbing the HFD feeding pattern (hence affecting the DIO model), mice were not fasting when blood parameters were measured. Plasma levels of AST, ALT, LDH, creatinine, alkaline phosphatase and circulating lipids were measured by the Department of Biochemistry of Sainte-Justine's Children Hospital (Montreal, QC, Canada).

3.2.6 Tissue triglyceride measurement

Part of the frozen liver and muscle sections (around 100 mg of each sample) were ground into powder under liquid nitrogen and extracted using Folch's chloroform/methanol (2:1) method [48]. Triglyceride content was quantified using a commercial kit (Randox Laboratories Ltd., UK).

3.2.7 Western Blot analysis

Western blot analysis was performed on frozen liver, muscle, and WAT using the following antibodies: p-Akt (Ser 473), Akt, p-AMPK (Thr 172), AMPK, Glut4, p-ACC, ACC, FAS, FABP4, phospho p44/42 MAPK, p44/42 MAPK, p-IKKαβ, β-actin (each at 1:1000 in blocking buffer incubated overnight at 4°C; Cell Signaling Tech Inc., Danvers, MA, USA). PPARα, PPARγ, CPT-1, CD36, UCP-2, SREBP1-c were measured using a 1:200 dilution in blocking buffer and incubated either at 1h room temperature (RT) or overnight (Santa Cruz Biotechnology inc., Santa Cruz, CA, USA). The following HRP-conjugated secondary antibodies were used:

anti-rabbit (1:10000; Jackson Immunoresearch Laboratories Inc., West Grove, PA), anti-mouse (1:4000; Cell Signaling Tech Inc., Danvers, MA, USA), or anti-goat (1:5000; Santa Cruz Biotechnology inc., Santa Cruz, CA, USA). Immunoreactive proteins were detected by enhanced chemiluminescence method (GE Healthcare, Baie d'Urfé, QC, Canada). Densitometric analysis was performed using NIH Image J software (version 1.42q, NIH, USA).

3.2.8 Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Bonferroni post-hoc analysis, or by unpaired Student's t test (Sigma Stat software, Jandel Scientific, San Rafael, CA), as appropriate. Areas under the curve (AUC) were calculated with PRISM software (GraphPad, San Diego, CA, USA). Data are expressed as the mean ± SEM of the indicated number of determinations. Statistical significance was set at $p<0.05$.

3.3 Results

3.3.1 Metabolic profile of responders to the High Fat Diet (DIO controls)

As anticipated in relation to recent data by Peyot et al. [47], roughly half of the mice consuming the HFD became obese and insulin resistant. Compared to Chow controls, such DIO control animals gained body weight, increased liver, BAT and WAT weights (Table 1, $p<0.05$) and displayed hyperlipidemia (total cholesterol, LDL, HDL). These mice also exhibited increased plasma glucose and insulin levels, an enhanced leptin/adiponectin ratio (Table 2, $p<0.05$) as well as elevated hepatic and muscle triglyceride (TG) levels (Table 3; $p<0.05$ compared to Chow), thus confirming the presence of an insulin resistant state. Only mice displaying an altered metabolic profile at 8 weeks of HFD feeding were selected for the present study and randomized to receive *P. balsamifera* or its active component, salicortin, for an additional 8 weeks.

3.3.2 *P. balsamifera* and salicortin decrease body weight, liver weight and steatosis in DIO mice

Treatment with *P. balsamifera* (at 125 mg/kg) significantly decreased body weight. This decrease reached 13% at sacrifice when compared to DIO controls ($p<0.05$; Table 1). When taking into account continuous measurements, the area under the curve (AUC) of cumulative changes in body weight (CCBW) was lowered by 8% ($p<0.05$; Figure 1A) with 125 mg/kg of *P. balsamifera*. This effect was gradual, beginning within the first month (F1=6% reduction; N.S.; Figure 1B), but becoming more pronounced in the second month of the treatment (F2=10% decrease; $p<0.05$; Figure 1C). Animals receiving 250 mg/kg of *P. balsamifera* exhibited a similar pattern of effects, albeit without reaching statistical significance. Mice fed the active compound,

salicortin, also displayed a significant decrease in body weight amounting to an 8% drop at sacrifice ($p<0.05$; Table1) and a 15% decrease in total AUC of CCBW ($p<0.05$; Figure 1A). Interestingly, its effect was immediate with 18% decrease in the F1 AUC ($p<0.05$; Figure 1B) that continued into the second month, albeit slightly less prominently (F2=13%; $p<0.05$; Figure 1C).

Concomitantly, retroperitoneal fat pad weight was smaller when animals were treated with either dose of balsam poplar (9%-17% reduction) or with salicortin (10% decrease) than in those receiving HFD alone, although these changes failed to reach statistical significance (Table 1; N.S). In contrast, the drop in liver weight was significant in the three aforementioned treated groups (decrease by 27%-35%; $p<0.05$ vs DIO controls; Table 1). Consistent with these results, hepatic triglyceride (TG) content was also reduced by 44% to 53% in the treated animals in comparison to DIO controls ($p<0.05$; Table 3). Muscle triglycerides, however, were not significantly altered by any of treatments (N.S.; Table 3).

It must be noted that a weak anorexic effect was observed in animals receiving *P. balsamifera* at 125 mg/Kg dose. Indeed, the AUC of cumulative food intake of this group was significantly reduced by 6% as compared to DIO congers ($p<0.05$, data not shown). No such effect was observed with the higher dose of *P. balsamifera* or with salicortin.

3.3.3 *P. balsamifera* and salicortin improve insulin sensitivity, while only the active principle modulates lipidemia in DIO mice

Along with body weight changes, *P. balsamifera* (at 125 and 250 mg/kg) and salicortin improved insulin sensitivity, albeit with slightly different profiles. Firstly, continuous measurement of glycemia showed that both doses of whole plant extract as well as the active significantly reduced

total AUC by 17-%-18% and by 11% respectively. Although *P. balsamifera* and salicortin effects were rather rapid in onset, the effect of the whole plant was constant throughout the treatment period (F1=F2), whereas that of the active decreased with time (F1=14%, $p<0.05$ vs DIO controls; F2=9%, N.S.; Figure 1 panels B & C). At sacrifice, glycemia of the three treatment groups was reduced as compared to their respective controls, albeit not in a statistically significant manner (Table 2). Secondly, insulinemia diminished by 73% with balsam poplar at 125 mg/Kg ($p<0.05$) and by 85% with 250 mg/Kg ($p=0.052$) as well as with salicortin ($p<0.05$) in comparison to DIO controls (Table 2). Thirdly, the two doses of *P. balsamifera* decreased leptin/adiponectin ratio by 41%-54% as compared to congeners receiving HFD alone ($p<0.05$; Table 2). Salicortin also significantly decreased this ratio, although to a lesser extent (by 21%; $p<0.05$; Table 2). In terms of the circulating lipid profile, only the salicortin treated group exhibited significantly lowered total plasma cholesterol and LDL levels, which were reduced by 25% and 40% respectively (Table 2; $p < 0.05$) as compared to the DIO controls. Altogether, these findings illustrate an improvement in insulin sensitivity when balsam poplar or its active principle are added to the HFD.

Finally, *P. balsamifera* and salicortin tended to normalize several systemic parameters of toxicity, although this did not reach statistical significance, except in the case of AST levels for salicortin ($p<0.05$; Table 2) and LDH levels for *P. balsamifera* at 125 mg/Kg ($p<0.05$; Table 2).

3.3.4 *P. balsamifera* and salicortin do not affect skeletal muscle Glut4 or the Akt and AMPK pathways, yet the plant extract improves components related to muscle lipid oxidation

Despite the significant reduction of overall glycemia exerted by the plant extract and its active, analysis of protein components involved in muscle glucose homeostasis did not exhibit any statistically significant changes. There was a tendency for Glut4 expression to increase in animals treated with *P. balsamifera* at 125 mg/kg, (Table 4; N.S. balsam poplar vs corresponding DIO controls). Similarly, insulin-dependent Akt phosphorylation tended to increase in animals fed with salicortin, although data variability precluded any definitive interpretation of these results. The insulin-independent AMPK pathway remained more clearly unchanged.

In contrast, components involved in muscle lipid homeostasis showed evidence of improvement with balsam poplar treatment. Indeed, *P. balsamifera* at 125 mg/kg more than doubled muscle PPARα expression levels (Table 4; 137% increase compared to DIO $p<0.05$). When looking at components involved in muscle fatty acid oxidation and synthesis, again only the plant extract seemed to act on such pathways, by tending to increase phosphorylated ACC levels and to normalize FAS levels back down to Chow levels (Table 4; N.S. compared to DIO controls). The p44/42 MAPK pathway linked to exercise and insulin stimulation was significantly activated with salicortin (Table 4, $p < 0.05$), and showed a tendency to do so with the plant extract at 250 mg/kg (Table 4, N.S.).

3.3.5 The effects of *P. balsamifera* and salicortin on liver components of glucose and lipid homeostasis

Both doses of *P. balsamifera* significantly increased hepatic phosphorylated Akt in HFD-fed animals (Table 4; increases by 111% and 87% for 125 and 250 mg/kg groups, respectively; $p<0.05$ compared to DIO controls), while the active showed only a slight tendency to do so (22% increase). A number of parameters related to hepatic lipid homeostasis or inflammation showed interesting tendencies, but none of these effects reached statistical significance. In all cases, tendencies were more pronounced with the 125 than the 250 mg/kg dose of *P. balsamifera*. Notably, PPARα appeared to be increased by both balsam poplar and the active, while CPT-1 seemed to be increased only by the plant extract (Table 4, N.S.). As for IKKαβ it appeared to be affected only by the plant extract, exhibiting a decrease of 43% and 30% with 125 and 250 mg/kg doses, respectively (Table 4; N.S.).

3.3.6 The effect of *P. balsamifera* and salicortin on adipose tissue components of glucose and lipid homeostasis

P. balsamifera at 125 mg/kg showed a strong tendency to increase phosphorylated Akt levels in adipose tissue (Table 4; increase by 65%, $p=0.068$ compared to DIO controls). Likewise, CPT-1 expression in animals treated with the plant extract at 250 mg/kg exhibited a strong tendency to be enhanced (Table 4; increase by 47%, $p=0.079$ compared to DIO), whereas the active had a similar albeit much weaker effect on this parameter (Table 4; 11% increase; N.S.). In contrast, FABP4 was clearly and significantly increased by *P. balsamifera* at both doses (54% and 60% at 125 and 250 mg/kg respectively, Table 4, $p<0.05$ compared to DIO controls), while salicortin showed only a slight tendency to do so (16%, N.S., Table 4;). Salicortin and balsam poplar

showed a tendency to normalize PPARγ and phosphorylated p44/42 MAPK to levels similar to those observed in Chow animals (Table 4). Other components failed to show any significant changes in plant or active treated animals compared to their respective DIO controls.

3.4 Discussion

According to the World Health Organization (WHO), 75% of the world population still relies on traditional medicine for primary health care needs and this often involves crude preparations of medicinal plants [49]. In the Canadian province of Quebec, regional health authorities assigned to the CEI are currently considering the usefulness of Cree traditional medicine, notably its associated pharmacopoeia, to deal with several health concerns such as Type 2 diabetes; a condition that has reached epidemic proportions in the region [36]. Our group has been working since 2003 with communities and health authorities in CEI to build the scientific evidence base in support of this initiative. An ethnobotanical study was conducted in collaboration with CEI Elders and healers that identified several plants used to treat diabetes symptoms [37, 50, 51]. One of these was *Populus balsamifera* L. (Salicaceae) or balsam poplar. The plant did not demonstrate much anti-diabetic potential in *in vitro* bioassays; for instance, it had little effect on muscle glucose uptake [37]. However, the plant caught our attention by its complete inhibition of triglyceride accumulation and adipogenesis in the 3T3-L1 adipocyte cell line [37], suggesting potential therapeutic usefulness against obesity. Salicortin, a salicylate glycoside, abundant in poplar, willow bark, as well as throughout the Salicaceae family, was identified through bioassay-guided fractionation as the constituent of *P. balsamifera* having the most potential to inhibit adipogenesis in the 3T3-L1 cell line [38]. Prior to our studies, anti-adipogenic activity had never been ascribed to balsam poplar, to members of its botanical family, or to its known phytochemical constituents, such as salicortin [38]. The goal of the present study was to evaluate the effectiveness of balsam poplar and salicortin as anti-obesity, anti-adipogenic, and consequently anti-diabetic agents in an in vivo mouse model.

The DIO mouse model was used in this study. It closely mimics human metabolic syndrome (notably obesity and insulin resistance) and requires lesser quantities of plant extracts (also, more importantly, of active principles) than larger animals for long-term studies. Indeed, in this model, a period of 8 weeks is necessary to establish obesity and insulin resistance, as confirmed in the present studies. The plant was then incorporated into the high fat diet for a further 8 weeks to fully assess its potential to treat obesity and the associated metabolic disturbances. After 16 weeks on a high fat diet, control DIO animals develop obesity, mild hyperglycemia, hyperinsulinemia, hyperleptinemia, and increased ectopic fat storage (notably hepatic steatosis), all reflecting the establishment of the metabolic syndrome and an insulin resistant state. In previous studies, a less severe model was used whereby animals were subjected to a HFD for only 8 weeks; *P. balsamifera* being administered from the onset of the HFD feeding in order to evaluate its potential to prevent obesity and its associated insulin resistant state [44]. The plant extract effectively reduced body weight gain, retroperitoneal fat pad weight, liver lipid content, as well as circulating glucose, insulin and leptin levels. It also activated pathways that were involved with glucose and lipid oxidation, as well as thermoregulation. The onset of action of the plant extract was immediate and sustained throughout its course of administration.

The results of the current study clearly demonstrate that *P. balsamifera* as well as its active salicortin were able to significantly reduce the body weight of mice despite their being subjected to a continuous hypercaloric fat-laden diet; the plant's effect being more potent and statistically significant at 125 mg/kg than 250 mg/kg. Several anthropomorphic, systemic and tissue parameters were thus examined to circumscribe the possible mechanisms of action of the plant extract and its active principle, salicortin.

A first potentially important lead came from data on cumulative food intake. Indeed, the plant extract at 125 mg/kg slightly but significantly reduced energy intake, and this was mainly

visible in the second month of treatment (F2; data not shown). This correlated well with the plant's temporal action on body weight. This suggests that the plant may exhibit slight appetite-modifying effects that warrant further investigation. However, the reduction in caloric intake was weaker than the weight loss measured, roughly half to two-thirds as important when considering total or F2 AUC measurements of cumulative weight changes, respectively. On the other hand, the active salicortin decreased the overall AUC of CCBW more effectively than the plant extract, and did so without affecting cumulative energy intake, suggesting different profiles of biological activity.

On the other hand, although obesity was only partly countered by *P. balsamifera* and salicortin, systemic glucose homeostasis was more significantly improved. Indeed, continuous glycemia measurements showed that the plant and its active had an overall effect to reduce blood glucose variations toward normal values observed in Chow-fed controls. Even more telling was the dramatic decrease of insulinemia seen with *P. balsamifera* at 125 mg/kg and with salicortin. Likewise, the leptin-to-adiponectin ratio, also reflective of insulin resistance, was essentially halved with the plant extract, and decreased by 1.5 fold with the active. Interestingly, salicortin also significantly improved the blood lipid profile by decreasing LDL and total cholesterol levels, whereas the plant extract had no significant impact on systemic parameters of lipid homeostasis. This again points to variations in biological activity between the crude extract and the purified active principle.

Further analysis of the major insulin responsive tissues, notably skeletal muscle, liver and adipose tissue, yielded data that highlights potential mechanisms at several levels of metabolic control. Firstly, excessive skeletal muscle TG accumulation was not corrected by *P. balsamifera* or salicortin treatment. However, the crude plant extract did more than double the expression of PPARα, which could lead to increased fatty acid oxidation [30] and improved muscle insulin

sensitivity [52]. The latter was supported by the tendency for increased Glut4 expression and p44/42 MAPK phosphorylation observed in animals receiving 125 mg/kg of the plant. In contrast, salicortin treatment only significantly affected muscle p44/42 MAPK activation, again hinting at different actions of the plant extract and its active.

In contrast, in the liver, *P. balsamifera* and salicortin treatment more than halved the elevated levels of accumulated TGs. Since hepatic steatosis is increasingly recognized as a major contributor to systemic insulin resistance [53, 54], this action may have played a significant role in improving systemic glucose homeostasis and insulin sensitivity. Indeed, analysis of key tissue proteins indicated that *P. balsamifera* treatment induced a doubling of liver Akt phosphorylation. Since Akt is a major component of the insulin-signaling cascade, part of the effect of balsam poplar could involve improved hepatic insulin sensitivity. Indeed, Akt inhibits glucose production and promotes glycogen deposition in the liver [5, 6, 13]. In hepatic cell lines, our group recently found that *P. balsamifera* inhibits glucose-6-phosphatase [55]. Other components also tended to be modulated by *P. balsamifera* in the liver and suggest that the plant may favor salvaging lipid metabolism. Indeed, PPARα were increased by treatment with the plant and its active principle, this transcription factor being known to enhance fatty acid oxidation [30]. The tendency for a reduction of IKKαβ by the plant treatment, on the other hand, points to a potential improvement of inflammatory components known to be involved in non-alcoholic fatty liver disease and ensuing metabolic disturbances [27, 56]. Such effects of *P. balsamifera* and salicortin on liver lipid homeostasis and inflammation will require confirmation in future studies.

Despite large reductions in WAT weight at sacrifice consistent with the significant reduction in body weight, such changes induced by *P. balsamifera* and salicortin failed to reach statistical significance due to data variability. Nonetheless, analysis of adipose tissue components

yielded a number of insightful results. Firstly, the tendency for *P. balsamifera* and salicortin to reduce the p44/42 ERK MAP kinase is consistent with the parallel tendency for WAT weight reductions. Indeed, the ERK pathway is involved in adipogenesis and insulin resistance [32, 57] and our group observed that *P. balsamifera* inhibits clonal expansion in 3T3-L1 adipocytes [39]. On the other hand, FABP-4, a lipid chaperone carrying fatty acids to cellular pathways of oxidation, was significantly increased by plant treatment. As in liver, adipose tissue Akt and CPT-1 expression also showed a strong tendency to be increased, supporting the notion that *P. balsamifera* can enhance insulin-dependent lipid oxidative pathways. The active showed much weaker actions on adipose tissue components, notably mild tendencies to increase FABP-4 and CPT-1 expression.

The effects of both the crude plant extract and of the active principle salicortin occurred without any overt sign of toxicity Indeed, unaltered liver and renal functional parameters confirm that the plant and its active salicortin are innocuous. Indeed, products of this tree have been used safely for generations by several Aboriginal peoples of the Northern hemisphere [58, 59]. The inner bark of *P. balsamifera* (from which the plant extract used in the current studies was derived) is even documented as a survival food [59, 60].

Interestingly, the majority of metabolically and statistically significant changes were obtained with the lower dose of 125 mg/kg of *P. balsamifera*, whereas the larger 250 mg/kg dose exerted lesser or no effects. Such a counterintuitive dose-response relationship is not uncommon with polymolecular drug mixtures that crude plant extracts represent. Indeed, synergistic and antagonistic interactions between phytochemical components can yield such response profiles [61]. This is also supported by the aforementioned differences in the biological activity profile between the crude plant extract and salicortin. It is conceivable that other components in the crude extract may complement salicortin’s activity.

Indeed, the action of the active principle alone on continuously measured parameters (body weight and blood glucose) appeared to wane with time, since effects were more pronounced in the first month of administration (F1) than in the second (F2). This may limit the use of the active at this dose and may have contributed to mask effects on protein components in insulin-sensitive tissues. Further studies need to be conducted in order to determine if this apparent time-dependent decline in activity develops at any dosage, and if so, with what time course. Nevertheless, salicortin has a sufficiently promising biological profile in DIO mice to warrant further studies potentially leading to clinical assessments.

In summary, *P. balsamifera* and salicortin exerted significant weight-reducing properties in obese, insulin resistant mice in the face of continued HFD feeding. Part of the plant extract's effect appears to emanate from a putative weak anorexic effect that will need to be defined. The plant and the active had even more profound beneficial effects on systemic glucose homeostasis and indirect indices of insulin sensitivity. Analysis of tissue components involved in glucose and lipid homeostasis uncovered several potential lead mechanisms in key insulin responsive organs such as skeletal muscle, liver and adipose tissue. Generally, components involved in insulin-dependent lipid oxidative pathways were most prominently and coordinately modulated in animals treated with the plant extract. Such actions would favor the "wastage" of energy derived from excess lipids consumed through the HFD, thereby reducing the negative metabolic impact of obesity. This is highly relevant for Aboriginal populations like the CEI whose rapid changes in dietary habits over the last decades also involves a higher intake of lipid-enriched calorie-dense foods. It is noteworthy that salicortin did not always activate the same pathways and to the same degree as the plant extract, suggesting that other plant constituents in the crude extract may also participate in beneficial biological activity toward metabolic disease.

In conclusion, the present studies confirm the high potential of *P. balsamifera* as a complementary treatment derived from CEI traditional medicine, which can help combat the devastating effects of obesity, often leading to Type 2 diabetes. Having identified salicortin as an important active principle *in vitro* – its anti-obesity and mild anti-diabetic effects having also being validated by the present *in vivo* treatment study – it can now be considered as a valuable tool to ensure the quality and efficacy of *P. balsamifera* preparations. Salicortin can also serve as a template to develop novel therapeutic agents for the treatment of obesity and type 2 diabetes. Additional studies should further clarify the mode of action of the plant and its active. This will pave the way toward clinical studies designed to determine if *P. balsamifera* and salicortin can be used in a safe and efficacious manner, alongside conventional medical treatments, for the treatment of metabolic diseases.

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Disclosure

The authors declare no conflict of interest.

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3.7 Figure Legends

Figure 1: Area under the curve (AUC) of cumulative changes in body weight (CCBW) for A) Total 8-week treatment period, B) First 4 weeks of treatment (F1), C) Second 4 weeks of treatment (F2). C57BL/6 mice were administered either standard diet (Chow), HFD (DIO), and for the last 8 of the 16 weeks a HFD in combination with *P. balsamifera* at 125 or 250 mg/ kg, or with Salicortin 12.5 mg/kg. All values are mean ± SEM. Fraction 1 (F1) consists in the AUC between week 0 and 4, and fraction 2 (F2) corresponds to the AUC between week 4 and 8 of administration of the plant extract. The number of animals for the crude plant extract protocol was: CHOW (n=12), DIO (n=8), *P. balsamifera* 125 (n=5) and *P. balsamifera* 250 (n=7); and for the Salicortin protocol: CHOW (n=12), DIO (n=7), Salicortin (n=9). †denotes DIO significantly different as compared to Chow (unpaired Student's t test; $p < 0.05$). *denotes significantly different as compared to respective DIO (one way ANOVA; $p < 0.05$). §denotes significantly different as compared to respective DIO (unpaired Student's t test; $p < 0.05$).

Figure 2: Area under the curve (AUC) of cumulative changes in blood glucose levels for A) Total 8-week treatment period, B) First 4 weeks of treatment (F1), C) Second 4 weeks of treatment (F2). C57BL/6 mice were administered either standard diet (Chow), HFD (DIO), and for the last 8 of the 16 weeks a HFD in combination with *P. balsamifera* at 125 or 250 mg/ kg, or with Salicortin 12.5 mg/kg. All values are mean ± SEM. Fraction 1 (F1) consists in the AUC between week 0 and 4, and fraction 2 (F2) corresponds to the AUC between week 4 and 8 of administration of the plant extract. The number of animals for the crude plant extract protocol was: CHOW (n=12), DIO (n=8), *P. balsamifera* 125 (n=5) and *P. balsamifera* 250 (n=7); and for

the Salicortin protocol: CHOW (n=12), DIO (n=7), Salicortin (n=9). †denotes DIO significantly different as compared to Chow (unpaired Student's t test; $p < 0.05$). *denotes significantly different as compared to respective DIO (one way ANOVA; $p < 0.05$). §denotes significantly different as compared to respective DIO (unpaired Student's t test; $p < 0.05$).

Table 1: Effects of obesity, *P. balsamifera*, and salicortin treatments on body and organ weights at sacrifice

	DIO	*P. balsamifera* 125 mg/kg	*P. balsamifera* 250 mg/kg	DIO	Salicortin 12.5 mg/kg
Body Weight	138 ± 1†	120 ± 7*	131 ± 3	142 ± 2†	130 ± 3§
Retroperitoneal Fat Pad	229 ± 12†	190 ± 28	209 ± 20	242 ± 13†	218 ± 10
Epididymal Fat Pad	77 ± 3†	98 ± 17	103 ± 5*	97 ± 2	149 ± 11§
Brown Fat Pad	189 ± 14†	136 ± 22 p=0.057	168 ± 14	200 ± 6†	170 ± 11§
Liver Weight	167 ± 6†	108 ± 12*	122 ± 10*	166 ± 9†	118 ± 7§
Total Kidney	111 ± 4†	93 ± 3*	104 ± 4	104 ± 2	101 ± 3

Measurements were obtained after 16 weeks of treatment with either standard diet (Chow), HFD (DIO), and for the last 8 of the 16 weeks with HFD in combination with *P. balsamifera* at 125 or 250 mg/ kg, or with the active Salicortin at 12.5 mg/kg. All values are expressed as a percentage of respective Chow controls (reference set at 100%) and represent the mean ± SEM. The number of animals for each group for the *P. balsamifera* protocol were: CHOW (n=12); DIO (n=8); *P. balsamifera* 125 (n=5); *P. balsamifera* 250 (n=7); and for the salicortin protocol: CHOW (n=12); DIO (n=7); Salicortin (n=9). † denotes DIO significantly different as compared to Chow (unpaired Student's t test; $p < 0.05$). * denotes significantly different as compared to respective DIO (one way ANOVA, Bonferonni *post hoc* test; $p < 0.05$). § denotes significantly different as compared to respective DIO (unpaired Student's t test; $p < 0.05$).

Table 2: Effects of obesity, *P. balsamifera*, and salicortin treatments on systemic parameters at sacrifice

	DIO	*P. balsamifera* 125 mg/kg	*P. balsamifera* 250 mg/kg	DIO	Salicortin 12.5 mg/kg
Glucose (mmol/L)	135 ± 14†	105 ± 8	121 ± 10	121 ± 6†	114 ± 6
Insulin (ng/mL)	3056 ± 1074†	450 ± 238*	832 ± 423 (p = 0.052)	1035 ± 150†	272 ± 62§
Leptin (ng/mL)	211 ± 28†	108 ± 18*	145 ± 9 (p = 0.051)	246 ± 19†	197 ± 13§
Adiponectin (µg/mL)	70 ± 3†	78 ± 10	82 ± 5 (p = 0.054)	97 ± 5	101 ± 6
Leptin/adiponectin ratio	304 ± 37†	138 ± 16*	181 ± 16*	248 ± 12†	196 ± 18§
TG (mmol/L)	99 ± 8	80 ± 10	82 ± 8	118 ± 10	106 ± 14
LDL (mmol/L)	391 ± 31†	355 ± 37	307 ± 42	344 ± 20†	207 ± 22§
HDL (mmol/L)	141 ± 8†	112 ± 17	125 ± 8	157 ± 10†	137 ± 7
Total cholesterol (mmol/L)	180 ± 9†	152 ± 14	151 ± 12	203 ± 11†	151 ± 7§
ALT (U/L)	281 ± 39†	229 ± 47	271 ± 77	341 ± 123†	157 ± 27
AST (U/L)	172 ± 30†	153 ± 22	137 ± 22	163 ± 27†	86 ± 5§
Creatinine (U/L)	184 ± 50	491 ± 160	557 ± 138	276 ± 113	136 ± 30
Alkaline phosphatase (U/L)	115 ± 20	85 ± 15	100 ± 9	106 ± 9	88 ± 17
LDH (U/L)	341 ± 83†	137 ± 16§	193 ± 61	376 ± 156†	154 ± 38

Measurements were obtained after 16 weeks of treatment with either standard diet (Chow), HFD (DIO), and for the last 8 of the 16 weeks with HFD in combination with *P. balsamifera* at 125 or 250 mg/ kg, or with the active Salicortin at 12.5 mg/kg. All values are expressed as a percentage of their respective Chow controls (reference set at 100%) and represent the mean ± SEM. The number of animals for each group for the *P. balsamifera* protocol were: CHOW (n=12); DIO (n=8); *P. balsamifera* 125 (n=5); *P. balsamifera* 250 (n=7); and for the salicortin protocol: CHOW (n=12); DIO (n=7); Salicortin (n=9). †denotes DIO significantly different as compared to Chow (unpaired Student's t test; $p < 0.05$). *denotes significantly different as compared to

respective DIO (one way ANOVA, Bonferonni *post hoc* test; $p < 0.05$). [§]denotes significantly different as compared to respective DIO (unpaired Student's t test; $p < 0.05$).

Table 3: Effects of obesity as well as *P. balsamifera* and salicortin treatments on hepatic and muscular triglyceride accumulation

	DIO	*P. balsamifera* 125 mg/kg	*P. balsamifera* 250 mg/kg	DIO	Salicortin 12.5 mg/kg
Liver TG Levels (mg/g total liver)	$930 \pm 65^{\dagger}$	$436 \pm 146^{*}$	$521 \pm 116^{*}$	$1084 \pm 180^{\dagger}$	$559 \pm 93^{\S}$
Muscle TG levels (μg/mg)	$223 \pm 54^{\dagger}$	342 ± 81	267 ± 38	$230 \pm 32^{\dagger}$	219 ± 24

The colorimetric dosage of TG levels in both the liver and muscle was determined using a commercial kit (Randox Laboratories ltd). Measurements were obtained after 16 weeks of treatment with either standard diet (Chow), HFD (DIO), and for the last 8 of the 16 weeks with HFD in combination with *P. balsamifera* at 125 or 250 mg/ kg, or with the active Salicortin at 12.5 mg/kg. All values are expressed as percentage of respective Chow (reference set at 100%) and represent the mean ± SEM. The number of animals for each group for the *P. balsamifera* protocol were: CHOW (n=12); DIO (n=8); *P. balsamifera* 125 (n=5); *P. balsamifera* 250 (n=7); and for the salicortin protocol: CHOW (n=12); DIO (n=7); Salicortin (n=9). † denotes DIO significantly different as compared to Chow (unpaired Student's t test; $p < 0.05$). *denotes significantly different as compared to respective DIO (one way ANOVA, Bonferonni *post hoc* test; $p < 0.05$). §denotes significantly different as compared to respective DIO (unpaired Student's t test; $p < 0.05$).

Table 4: Effects of obesity as well as *P. balsamifera* and salicortin treatments on tissue components involved in glucose and lipid homeostasis

	DIO	*P. balsamifera* 125 mg/kg	*P. balsamifera* 250 mg/kg	DIO	Salicortin 12.5 mg/kg
Muscle					
Glut4	150 ± 63	321 ± 174	151 ± 52	70 ± 33	59 ± 7
pAkt/Akt	214 ± 59†	195 ± 47	267 ± 82	96 ± 23	120 ± 29
phospho p44/42 Mapk/ 44/42Mapk	178 ± 83	99 ± 15	273 ± 84	47 ± 11	146 ± 34§
pAMPk/AMPk	138 ± 43	79 ± 10	97 ± 22	108 ± 16	108 ± 22
pACC/ACC	122 ± 28	171 ± 56	157 ± 38	106 ± 22	89 ± 26
PPARα/β−actine	97 ± 23	229 ± 50§	143 ± 44	195 ± 90	162 ± 69
FAS/β-actine	118 ± 30	100 ± 39	77 ± 18	114 ± 16	143 ± 29
Liver					
pAkt/Akt	66 ± 14	139 ± 18*	124 ± 17*	106 ± 28	129 ± 37
phospho p44/42 Mapk/ 44/42Mapk	68 ± 21	48 ± 21	66 ± 14	109 ± 35	96 ± 44
pACC/ACC	105 ± 41	117 ± 74	114 ± 62	71 ± 26	77 ± 31
PPARα/β-actine	63 ± 6†	88 ± 20	75 ± 13	107 ± 26	147 ± 26
UCP-2/β-actine	94 ± 19	107 ± 29	88 ± 26	151 ± 32	149 ± 27
CPT-1/β-actine	84 ± 12	100 ± 8	86 ± 9	86 ± 6	83 ± 9
FAS/β-actine	69 ± 17	69 ± 22	59 ± 25	86 ± 27	74 ± 15
SREBP1-c/β-actine	88 ± 16	112 ± 27	121 ± 37	109 ± 14	109 ± 17
CD36/β-actine	63 ± 7†	93 ± 19	64 ± 3	69 ± 8†	67 ± 6
pIKKαβ/β-actine	108 ± 23	62 ± 31	75 ± 12	114 ± 21	129 ± 21
Adipose tissue					
pAkt/Akt	112 ± 13	186 ± 37 (p=0.068)	110 ± 15	138 ± 18	109 ± 14
phospho p44/42 Mapk/ 44/42Mapk	133 ± 31	103 ± 12	79 ± 10	156 ± 23†	128 ± 21
PPARγ/β-actine	73 ± 17	74 ± 15	102 ± 30	85 ± 12	116 ± 15
pACC/ACC	139 ± 46	119 ± 66	157 ± 70	95 ± 30	86 ± 20
CPT-1/β-actine	81 ± 9	86 ± 10	119 ± 19 (p=0.079)	89 ± 11	99 ± 14
FABP4/β-actine	85 ± 11	131 ± 11§	136 ± 19§	71 ± 6	83 ± 10
FAS/β-actine	40 ± 5†	33 ± 8	56 ± 11	49 ± 7†	45 ± 7
SREBP-1c/β-actine	77 ± 6	88 ± 9	96 ± 19	86 ± 7	87 ± 11

Samples of muscle, liver, and WAT were obtained after 16 weeks of treatment with either standard diet (Chow), HFD (DIO), and for the last 8 of the 16 weeks with HFD in combination with *P. balsamifera* at 125 or 250 mg/ kg, or with the active Salicortin at 12.5 mg/kg. The samples were homogenized and analyzed by immunoblotting. Blots were quantified by densitometry. All values are expressed as percentage of respective Chow (reference set at 100%) and represent the mean ± SEM. The number of animals for each group for the *P. balsamifera*

protocol were: CHOW (n=12); DIO (n=8); *P. balsamifera* 125 (n=5); *P. balsamifera* 250 (n=7); and for the salicortin protocol: CHOW (n=12); DIO (n=7); Salicortin (n=9). † denotes DIO significantly different as compared to Chow (unpaired Student's t test; $p < 0.05$). *denotes significantly different as compared to respective DIO (one way ANOVA, Bonferonni *post hoc* test; $p < 0.05$). §denotes significantly different as compared to respective DIO (unpaired Student's t test; $p < 0.05$).

Figure 1

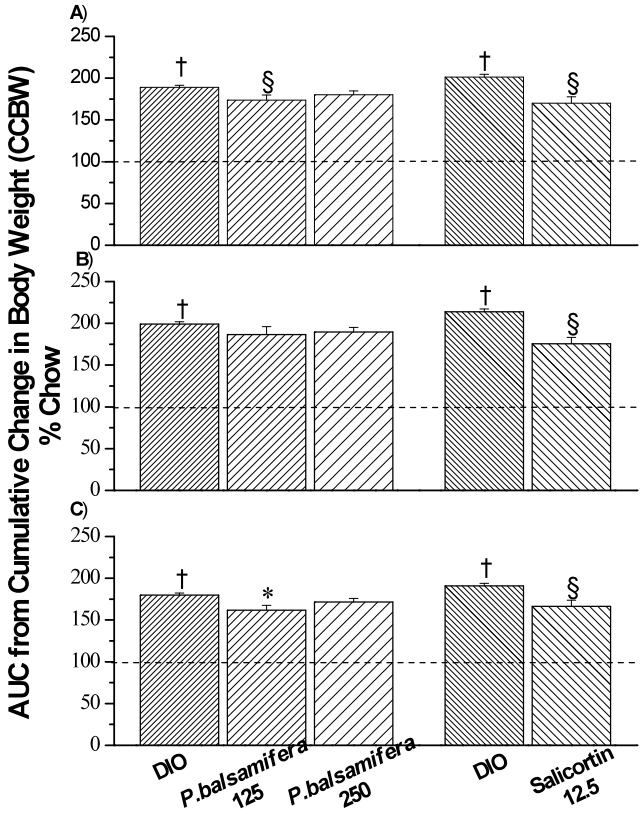
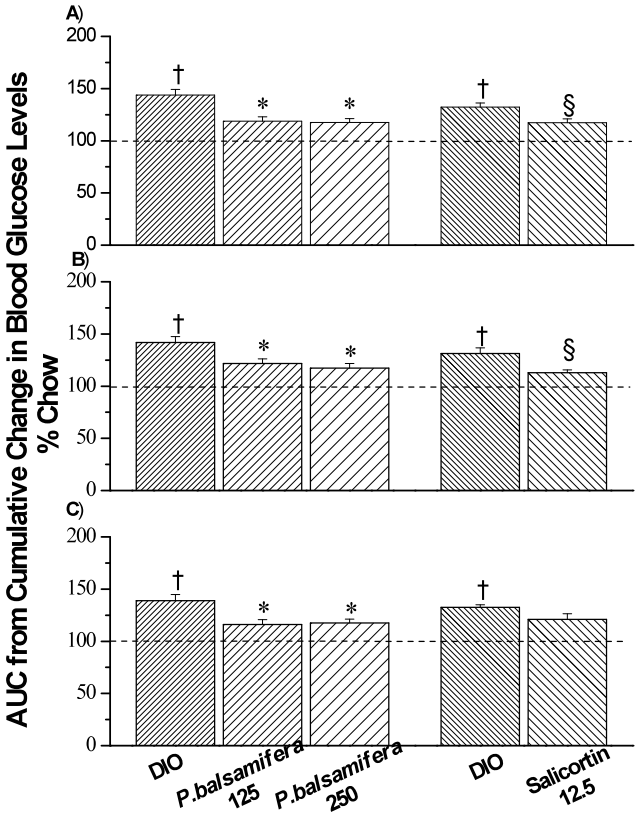

A)
B)
C)
AUC from Cumulative Change in Body Weight (CCBW)
% Chow
250
200
150
100
50
0
†
§
*
DIO
P.balsamifera 125
P.balsamifera 250
DIO
Salicortin 12.5

Figure 2


A)
B)
C)
AUC from Cumulative Change in Blood Glucose Levels
% Chow
200
150
100
50
0
†
*
§
DIO
P.balsamifera 125
P.balsamifera 250
DIO
Salicortin 12.5

Chapter 4: Article 3

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Role of each co-author belonging to this manuscript

It is to note that this manuscript has been submitted to the journal of Evidence-Based Complementary and Alternative Medicine. I the student Despina Harbilas did the great majority of the work in this manuscript, with the technical support of Antoine Brault and Diane Vallerand: treatment, care and sacrificing of the animals, as well as all post-sacrifice analysis on blood and tissue parameters, and finally all data and statistical analysis. Drs. Ammar Saleem and John T. Arnason are our collaborators, and contributed in the preparation and characterization of the plant species. Dre Lina Musallam assisted in correcting the article. Dr. Pierre S. Haddad, my supervisor, contributed conceptual, intellectual and moral input as well as correcting the article.

Title:

***Larix laricina*, an anti-diabetic alternative treatment from the Cree of Northern Quebec pharmacopoeia, decreases glycemia and improves insulin sensitivity in vivo**

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Running title: *Larix laricina* alternative anti-diabetic treatment

Keywords: Antiobesity, antidiabetic, adipokines, C57BL/6 mice, tamarack, skin temperature, mitochondrial respiration

Disclosure: The authors declare no conflict of interest.

Abstract

Larix laricina K.Koch is a medicinal plant belonging to traditional pharmacopoeia of the Cree of Eeyou Istchee (Eastern James Bay area of Canada). *In vitro* screening studies revealed that, like metformin and rosiglitazone, it increases glucose uptake, adipogenesis, activates AMPK, and uncouples mitochondrial function. The objective of this study was to evaluate the anti-diabetic and anti-obesity potential of *L. laricina* in diet-induced obese (DIO) C57BL/6 mice. Mice were subjected for eight or sixteen weeks to a high fat diet (HFD), or HFD to which *L. laricina* was incorporated at 125 and 250 mg/kg either at onset (prevention study), or in the last 8 of the 16 weeks of administration of the HFD (treatment study). *L. laricina* effectively decreased glycemia levels, improved insulin resistance and slightly decreased abdominal fat pad and body weights. This occurred in conjuction with increased energy expenditure as demonstrated by elevated skin temperature in the prevention study, and suggested improvement in mitochondrial function and ATP synthesis in the treatment protocol. *L. laricina* is thus a promising alternative and complementary therapeutic approach for the treatment and care of obesity and diabetes among the Cree.

4.1 Introduction

The prevalence of obesity and type 2 diabetes (T2D) has reached epidemic proportions worldwide. In Canada alone, obesity and T2D affect 25% [1-3] and roughly 6% [4] of the population respectively. The incidence of these diseases is worsened among the aboriginal population of Canada. The Cree of Eeyou Istchee (CEI) of the Northern James Bay Area of Quebec are particularly affected where 21% of adults over the age of 20 are diagnosed as diabetic and 38% as obese [5-10]. This increased prevalence might be caused by their adoption of more westernized way of living : sedentary lifestyle, non-traditional diet (with increased consumption of carbohydrates and saturated fats) in addition to a low-compliance to modern T2D therapies [11-13]. The implementation of educational programs on lifestyle intervention (diet and exercise) has come to no avail, wherefrom the importance to identify alternative and culturally-adapted treatments or solutions for obesity and T2D [6, 9-11, 13, 14].

Currently, around 70-95% of the population in the world relies on alternative and complementary medicine in order to respond to their primary health care needs. Since the CEI possess a rich traditional pharmacopoeia, we conducted an ethnobotanical survey to identify plant species with potential to treat symptoms related to T2D [15]. These plant species were screened for their anti-diabetic potential in extensive *in vitro* studies [16-24]. Of the 17 plants identified, *Larix laricina* K.Koch, belonging to the Pinaceae family, demonstrated anti-diabetic potential by increasing glucose uptake and phosphorylation levels of AMPK and ACC in C2C12 myotubes to levels almost comparable to those of metformin [20, 24]. It also potentiated adipogenesis in 3T3-L1 adipocytes, thus acting like the commonly used thiazolidinedione (TZD), rosiglitazone [24]. In addition, we showed that *L. laricina* was one of the strongest uncouplers by severely disrupting mitochondrial function and decreasing ATP production [20]. Uncouplers increase metabolic rate

and therefore fuel consumption in order to compensate for decreased ATP, which makes them potential anti-obesity agents [20]. Obesity contributes to 55% of all cases of T2D, therefore affecting one or the other might have therapeutic potential for both diseases [25].

There are a variety of medications available on the market that help in the management of T2D. Some of them, such as the antidiabetic medications metformin (biguanide) and exenatide (GLP-1 analogue), have been reported to produce a minimal amount of weight loss, albeit not strong enough to be considered as an anti-obesity agent [26, 27]. The diet-induced obese (DIO) mice is an excellent model to study the well-documented relation of losing weight and improving insulin resistance. It relies on high-calorie diet and inactivity (no genetic involvement) to induce significant weight gain, hyperglycemia and hyperinsulinemia, thus reflecting the establishment of the metabolic syndrome and a pre-diabetic state. Therefore, we wanted to study the anti-diabetic and anti-obesity potential of *L. laricina* using DIO mouse model using two different administration regiments. Animals either received the plant concomitantly with high fat diet (prevention study), or received the plant after becoming obese and pre-diabetic (treatment study).

4.2 Materials and Methods

4.2.1 Plant extracts

Specimens of the plant species used in this study, *L. laricina* K. Koch, of the Pinaceae family, were collected in 2004 on the territories of the CEI of Northern Quebec, Canada. Dr. Alain Cuerrier, taxonomist at the Montreal Botanical Garden, confirmed the botanical identity of the plant species. Voucher specimens of the plant species were deposited in the Marie-Victorin herbarium of the Montreal Botanical Garden in Montreal, Quebec, Canada (Whap04-11, Mis03-12, Mis03-47). Crude 80% ethanolic extract of *L. laricina* was prepared as previously described [24].

4.2.2 Animals

Four-week old male non-diabetic C57BL/6 mice were purchased from Charles River Laboratories (Saint-Constant, QC, Canada). All mice had *ad libitum* access to food and water. They were housed in individual cages and maintained on a 12 h light-dark cycle in a temperature-controlled animal room. All experimental protocols were approved by the animal experimentation ethics committee of the Université de Montréal and were carried out in full respect of the guidelines from the Canadian Council for the Care and Protection of Animals.

4.2.3 Prevention Protocol

Following acclimatization, the four-week old male non-diabetic C57BL/6 mice were divided into four groups of approximately 12 mice each, where they were monitored for 8 weeks. The control groups consisted in administering to one group (Chow) a standard diet purchased from Charles River (18% protein content, 4.5% crude fat, Charles River Animal rodent diet) and to another

group (DIO) a high fat diet (HFD) acquired from Bio-Serv (Bio-Ser Diet #F3282, Frenchtown, NJ, 60% fat by energy). The remaining groups received the HFD to which was incorporated the dried 80% crude ethanolic plant extract of *L. laricina* at levels adjusted to deliver 125 or 250 mg/kg body weight.

4.2.4 Treatment protocol

Following acclimatization, the four-week old male non-diabetic C57BL/6 mice were divided into four groups of approximately 12 mice each. Chow controls received a standard diet (18% protein content, 4.5% crude fat; Charles River Animal rodent diet) for 16 weeks. Other groups were fed a HFD (Bio-Serv Diet #F3282; 60% energy from fat) for 8 weeks. *L. laricina* at 125 or 250 mg/kg was incorporated in the HFD and treatments continued for an additional 8 weeks, with DIO controls receiving only HFD. Based on published observations and criteria, the animals fed a HFD were segregated into low responders (LR) and high responders (HR) (roughly 50/50) according to the data just prior to plant administration (at 8 weeks). Indeed, it has been reported that pooling animals with a more normal metabolic profile even when fed with HFD (such as low weight gain, weak IR and near-normal glycemia; LR) with animals displaying overt obesity and insulin resistant state when fed HFD (such as high weight gain, frank IR, and hyperglycemia; HR) can yield misleading results [28]. For simplicity reasons, LR fed with the HFD in the presence or absence of the plant extract will not be depicted since they portrayed an almost normal metabolic profile.

The data representing the effect of a HFD on C57BL/6 mice as compared to their CHOW fed congeners will not be discussed since the DIO mouse model is well established. However, our model (in both the prevention and treatment studies) follows the expected and published data. In

addition, the CHOW group was used as a non-obese control to insure that the model is functional and therefore the results of this group are not presented. Therefore, the effects of the plant extract, *L. laricina*, will be compared to their respective HFD controls for all the stated parameters, for both the prevention and treatment protocols.

4.2.5 Continuous physiological and morphological parameters

In both protocols body weight, food and water intake, as well as glycemia were measured 3 times/week during the course of the study, consistently at the same time, day, in the same order by the same person, throughout the entire duration of the protocols. In order to assess blood glucose levels, blood was collected from puncturing the tail vein and measured using a commercial glucometer (Accu-Check Roche, Montreal, QC, Canada). The area under the curve (AUC) was calculated for these parameters, and the total AUC was then separated into two parts: fraction 1 (F1), representing AUC between weeks 0 and 4, and fraction 2 (F2) corresponding to the AUC between weeks 4 and 8 of plant extract administration. This segregation served to determine the temporal course of action of *L. laricina* in both the prevention and treatment protocols, i.e. whether it was effective early in onset (first 4 weeks), later (last 4 weeks) or throughout the study.

4.2.6 Surgical Procedure

At the end of each experimental protocol, the mice were anesthetized using 50 mg/kg pentobarbital intraperitoneally, and then sacrificed by exsanguination via the inferior vena cava. During the sacrifice, various organs were removed, collected and weighed; notably, liver, muscle, white adipose tissue (WAT; epididymal and retroperitoneal fat pads), and subscapular brown fat (BAT). All were placed in liquid nitrogen and then stored at -80°C until further use. As for the

livers, they were flushed with a physiological saline solution, weighed and the median lobes were then dissected, immediately placed in liquid nitrogen, and then stored at -80°C until further use.

4.2.7 Blood parameters

In order to ensure uninterrupted delivery of plant extract, and to avoid the complications of interrupting the dietary plant treatment (ex. drop in food intake and body weight caused by fasting the animals), glycemia, insulin, adipokines, correspond to non-fasting measurements. Plasma insulin, adiponectin and leptin were assessed by radioimmunoassay (RIA: Linco Research, St-Charles, MO) according to manufacturer's instructions.

4.2.8 Tissue triglyceride measurement

Tissue triglyceride content was measured by grinding up into powder, under liquid nitrogen, around 100 mg of each of the collected liver and muscle samples, and then using Folch's chloroform/methanol (2:1) extraction method [29]. Triglyceride content was then quantified using a commercial kit (Randox Laboratories Ltd., UK).

4.2.9 Skin temperature

In the prevention protocol, after 4 and 8 weeks of treatment, the temperature of the animals was read and recorded with a digital thermometer (Cole-Parmer Instrument Company, USA) by placing a probe on the external intercostal region of the animal for 2 minutes. This procedure is non-invasive and the least stressful for the animals [30].

4.2.10 Isolation of Mitochondria and Measurement of Respiration

Following anesthetization, the liver of mice from the treatment protocol were flushed with Krebs-

Henseleit buffer (pH 7.4, 22°C). Mitochondria were then isolated following the method of Johnson and Lardy, as previously described [31-33].

4.2.11 Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), followed by post-hoc analysis (Bonferroni-Dunn test or Holm-Sidak) as appropriate using Sigma Stat software (Jandel Scientific, San Rafael, CA). Areas under the curve (AUC) were calculated by using PRISM software (GraphPad, San Diego, CA, USA). Data are expressed as mean ± SEM of the indicated number of determinations. Statistical significance was set at $p < 0.05$.

4.3 Results

4.3.1 L. laricina significantly improved glycemia in the treatment protocol

Glycemia levels, which increased with HFD as compared to CHOW (by 19%; data not shown), were not significantly altered when *L. laricina* was added concomitantly with the HFD (prevention study; NS; Figures 1A-C). However, HFD-induced hyperglycemia (32% as compared to CHOW congeners; data not shown) significantly decreased in the group receiving the plant as treatment (following 8 weeks on HFD; Figures 1A-C). Indeed, blood glucose levels, as measured by the area under the curve (AUC) of glycemia versus time, decreased in a dose dependent manner by 10% and 12% with 125 mg/kg and 250 mg/kg doses respectively (AUC_T; $p<0.05$; Figure 1A). In order to determine the temporal aspect of this anti-hyperglycemic effect, we fractionated the AUC between the first month of treatment (weeks 0-4 : AUC_{F1}; Figure 1B) and second month (weeks 4-8: AUC_{F2}; Figure 1C). Our findings show that *L. laricina* lowered glycemia levels from the onset of the treatment (by 11-13%; $p<0.05$; Figure 1B) and this was maintained throughout the treatment, albeit remained significant with highest dose only (13% at 250 mg/kg; $p<0.05$; Figure 1C).

4.3.2 L. laricina significantly decreased insulin levels in the treatment protocol only, while decreasing leptin/adiponectin ratio in both protocols

Administration of either dose of *L. laricina* tended to lower insulin levels by 25% to 35% in the prevention study, but failed to reach statistical significance (NS; Table 1). In the treatment protocol however, *L. laricina* induced-decrease of insulinemia levels reached 72% with the

250 mg/kg dose ($p<0.05$; Table 1), suggesting improvement of insulin resistance state and coinciding with the plant's highest effect on glycemia as mentioned above.

Other indicators were measured to confirm the re-establishment of insulin sensitivity. Adipose tissue is considered an endocrine organ, releasing into circulation adipokines, such as leptin and adiponectin, involved in the development of insulin resistance. A decrease of leptin/adiponectin ratio is thus considered as a marker of improved insulin sensitivity. In both prevention and treatment protocols, *L. laricina* tended to increase adiponectin levels by 16-26%, however reaching statistical significance with the 125 mg/kg dose in the prevention protocol only ($p<0.05$; Table 1). In parallel, Leptin levels were reduced with *L. laricina* administration by 4-16% in the prevention study and by 21-30% in the treatment study, without however being statistically significant (NS; Table 1). Overall, these changes in adipokines levels resulted in significant decrease of the leptin/adiponectin ratio. Indeed, administration of *L. laricina* significantly lowered this ratio by 37% at 250 mg/kg in the prevention study ($p<0.05$; Table 1) and by 29-31% with both doses in the treatment study ($p<0.05$; Table 1). Therefore, *L. laricina* seems to decrease systemic insulin resistance.

Furthermore, since accumulation of lipids in the liver and skeletal muscle have been implicated in insulin resistance, we measured hepatic and muscular triglyceride levels (TG). Despite *L. laricina* decreasing systemic insulin resistance, as suggested by improvement in the aforementioned parameters, hepatic or muscular triglyceride levels were not significantly altered (NS, Table 2).

4.3.3 L. laricina diminished body weight gain in both the prevention and treatment protocols, while decreasing fat pad weight in the prevention protocol only

Continuous measurements of cumulative change in body weight (CCBW), represented as the area under the curve (AUC), revealed that while the effect of *L. laricina* on body weight gain was immediate in the prevention protocol for both doses (10% for AUC_{F1}; NS; Figure 2B), it only reached significant proportions in the second half of the protocol (AUC_{F2}) with the highest dose, decreasing it by 14% as compared to DIO controls (AUC_{F2}; $p<0.05$; Figure 2C). In contrast, in the treatment protocol, *L. laricina* produced its strongest and most significant effect in the first half of administration lowering AUC_{F1}-CCBW by 10% at 250 mg/kg as compared to DIO congeners (AUC_{F1}; $p<0.05$; Figure 2B). However, its anti-obesity effect was not maintained; *L. laricina* reduced AUC_{F2} by only 4% at 250 mg/kg (NS; Figure 2C).

Consistent with the observed decrease in body weight gain, *L. laricina* significantly lowered retroperitoneal/abdominal fat pad weight in the prevention study by 15% at 250 mg/kg ($p<0.05$; Table 3) as compared to DIO controls. As for the treatment, it corresponded to a slight decrease with both doses (5%-11%; NS; Table 3).

It is interesting to note that, in both protocols, mice administered *L. laricina* maintained similar food intake to their DIO controls (NS; Figure 2D-F), while being less prone to gaining weight. Finally, *L. laricina* exhibited no toxicity as demonstrated by unaltered blood biochemical parameters and tissue histological examination (data not shown).

4.3.4 L. laricina improved mitochondrial function

Regulation of body temperature requires regulating both heat production and heat loss. Mitochondria metabolism is an important source of heat production. For the most part, variations in the rate of electron transport are directly related to the demand by the cells for ATP. However, exogenous substances, which uncouple mitochondria, can lead to the disruption of oxidative phosphorylation, decreasing ATP synthesis and increasing heat production. Previous screening studies have shown that *L. laricina* uncoupled mitochondrial function in isolated Wistar rat hepatocytes [20]. Therefore, we used skin temperature as an indirect measure of energy expenditure and possible mitochondrial uncoupling in the prevention study. After 4 and 8 weeks of treatment, we observed a gradual and dose dependent increase in skin temperature with *L. laricina* administration ($p<0.05$; Figure 3A & B).

Having perfected the isolation of mitochondria in mice, we opted to directly evaluate mitochondrial function in the treatment study in plant-treated mice compared to DIO controls. As expected DIO mice, which have increased fatty acid deposition in the liver, exhibited a lower respiratory control ratio (RCR) accompanied by decreased ATP production, in comparison to CHOW animals, although data variability precluded statistical significance (Table 4; N.S.). Despite a small sample, animals treated with 250 mg/Kg of *L. laricina* seemed to restore mitochondrial function and capacity to the level of chow values, as observed by an increase in RCR and ATP synthesis (Table 4; N.S.).

4.4 Discussion

According to International Diabetes Federation latest figures, the number of people living with diabetes will rise from 366 millions in 2011 to 552 millions by 2030 [34]. The magnitude and impact of this disease dictate the urgent need for action. Although several drugs exist on the market to treat diabetes, the need to discover novel therapeutic options is warranted, especially in aboriginal context, such as the CEI [6, 9-11, 13, 14]. Indeed, recent lifestyle changes and non-compliance with western medicines could account for the staggering diabetes prevalence of 29% in adults over 20 years old in this community [5, 6, 11-13].

In our quest to discover a culturally adapted diabetes treatment, we identified *L. laricina* from the CEI pharmacopoeia as potential anti-diabetic medicinal plant. Initial *in vitro* screening showed that this plant stimulated glucose uptake, potentiated adipogenesis [24], activated AMPK and acted as mitochondrial uncoupler/inhibitor (on normal isolated mitochondria) [20]. These effects are reminiscent of the action of Metformin, which partially mediates its action through inhibition of mitochondrial respiration, activation of AMPK and upregulation of glucose uptake as well [20, 35-39]. It was therefore interesting to test the antidiabetic effect of *L. laricina* in a mouse model mimicking type 2 diabetes as a consequence of obesity by subjecting C57BL/6 mice to HFD (Diet-induced-Obesity model; DIO). We tested the plant in two different regiments: 1) *L. laricina* was administered concomitantly with HFD for 8 weeks in order to confirm its capacity to attenuate the development of obesity, diabetes and the associated insulin resistance (prevention study); 2) *L. laricina* was administered to obese and insulin resistant C57BL mice (already on HFD for 8 weeks) for 8 weeks to test its ability to reverse the establishment of both of these states (treatment study).

Typically, the DIO model is characterized by increased body weight gain, hyperglycemia and establishment of insulin resistant state (hyperinsulinemia, increase of the leptin/adiponectin ratio, ectopic fat storage in the liver and muscle)[40, 41]. We therefore examined these parameters to determine the effect of *L. laricina in vivo.*

The results of the present studies confirm that *L. laricina* holds great promise as an anti-diabetic medicinal plant. Although this plant had no effect of glycemia when administered concomitantly with HFD (prevention study), on the contrary, it significantly decreased glycemia in the treatment study, in a suggestive dose-dependent manner. These findings correlate well with our in vitro data where this plant extract increased glucose uptake in skeletal muscle cells and adipocytes [24], which accounts for 85% of postprandial glucose disposal, [42] and increased AMPK activity in C2C12 muscle cells [20]. It is worthy to note that glycemia levels of animals receiving HFD in the treatment study are higher than those in the prevention study (32% vs 19% respectively, compared to CHOW). One could suggest that *L. laricina* exert its anti-hyperglycemic effect better when disease processes are more pronounced, thus explaining the observed difference in the plant's effect between the prevention and the treatment study.

Insulin resistance parameters were also modulated with administration of *L. laricina* in both treatment regiments. While strong tendencies are apparent in the prevention study, insulinemia and leptin/adiponectin ratio were significantly decreased in the treatment study (especially with the highest dose of *L. laricina*), suggesting improvement of systemic insulin resistance. Intriguingly, *L. laricina* failed to decrease hepatic and muscle triglycerides in both studies. Several lines of evidence suggest that hepatic triglyceride accumulation lead to insulin defective signaling in the liver with increased hepatic glucose output. However, Buettner *et al* have shown that TG accumulation in the liver is not always sufficient to impair insulin signaling [43, 44]. In fact, they argue that systemic factors (such as adipokines, free fatty acids, pro-

inflammatory cytokines) may play an important role in the regulation of hepatic glucose output and insulin sensitivity in vivo [43, 44]. Hence, our data on the lack of depletion in intrahepatic and intramuscular triglyceride levels needs to be evaluated in further detail. Indeed, continued administration of HFD along side *L. laricina* could make elimination of steatosis difficult. Another possibility could be that since oxidation pathways are saturated with fatty acids being mobilized from the adipose tissue (decrease in adipose tissue weight due to probable hormone-sensitive lipase activity), this could consequently hinder any decrease in tissue triglyceride stores [45]. In all cases, since adiponectin levels tended to increase and leptin/adiponectin ratio (an indicator of insulin resistance) [46-50] significantly decreased with ingestion of *L. laricina*, pro-insulin resistant systemic factors seem to be decreased and insulin sensitivity improved. Interestingly, we have shown that treatment of hepatic cells *in vitro L. laricina* inhibits the activity of enzymes implicated in hepatic gluconeogenesis, such as glucose-6-phosphatase and activates those promoting glycogen formation, such as glycogen synthase (GS), thus directly modulating hepatic glucose output [51].

This plant showed slight decrease of body weight with both studies, which was significant if continuous measurements were taking into account for the first and second month of administration. These changes occurred while the animals were on a continuous hypercaloric/fat-laden diet, and without any observed change in energy intake. This could represent an indirect modulation of body weight as a consequence of *L. laricina* anti-diabetic activity, which in some cases is similar to Metformin.

L. laricina administration also decreased retroperitoneal fat pad weight significantly in the prevention study, and showed a tendency to do so in the treatment study. This represents an important action in the fight against insulin resistance since visceral adipose tissue has been implicated in the detrimental effects of obesity and insulin resistance [52]. Hence, modulation of

this tissue would influence adipokine secretion and contribute to the improvement of insulin sensitivity, as can be seen in our plant-treated mice.

On the molecular level, we have shown that *L. laricina* activates AMPK in C2C12 myotubes [20] and H4IIE hepatic cell line [51]. This activation may be secondary to a variety of factors, including adiponectin or metabolic stress induced by the disruption of mitochondrial energy transduction [53-56]. In the literature, it has been reported that animals (mice or rat) fed a high-fat diet exhibit a decreased mitochondrial respiratory capacity (state 3/state 4), as was observed in this treatment protocol in mice administered a HFD [57]. Increased consumption of dietary fat may lead to alterations in mitochondrial membrane composition, increased ROS production and peroxidation of fatty acids, which could damage mitochondrial structures, all affecting mitochondrial function [57]. Uncoupling agents are beneficial in alleviating the mitochondrial stress induced by a HFD, by increasing fatty acid oxidation, and decreasing ROS production [58]. In the treatment study, *L. laricina* at 250 mg/kg showed a tendency to improve mitochondrial capacity and ATP production to levels comparable to those observed in animals fed a standard Chow diet. As demonstrated in previous in vitro screening studies, the uncoupling effect of *L. laricina* is short-lived, and is followed by a prolonged activation of AMPK and an overshoot phenomenon occurring to restore energy homeostasis, where ATP production is greatly increased, through raised carbohydrate and lipid oxidation [20]. Other benefits of increased AMPK activity include protecting cells from further damage by potentiating mitochondrial biogenesis [20, 59, 60]. Therefore, it seems that in the current animal treatment protocol, the long-term effect of *L. laricina* improved mitochondrial capacity, and most probably through AMPK activation regulated glucose homeostasis. Of note, uncoupling agents usually lead to increased heat production due to increased energy expenditure. *L. laricina* treated animals in the

prevention study exhibited elevated skin temperature, thus confirming its uncoupling activity *in vivo*.

In conclusion, this study confirms the anti-diabetic activity of *L. laricina* in the context of diet-induced obesity in a mouse model. The results clearly show that *L. laricina* decreased hyperglycemia, insulin resistance and improved mitochondrial function in the treatment study, while partially modulating parameters involved in insulin sensitivity in the prevention one. It also had a slight effect on body weight gain in both studies. The exact mechanisms of action of *L. laricina* remain to be identified, but results point toward possible activation of AMPK and its downstream effectors. In view of the soaring increase in both obesity and diabetes among aboriginal populations and in particular the CEI, *L. laricina* represents a valuable alternative, and culturally-adapted treatment for both these diseases.

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Disclosure

The authors declare no conflict of interest.

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4.7 Figure Legends

Figure 1 : Area under the curve (AUC) of non-fasting glycemia levels, in C57BL/6 mice treated with either HFD (DIO), or *L. laricina* at 125 or 250 mg/ kg, which was incorporated in the HFD for 8 weeks in the prevention protocol and for the last 8 of 16 weeks in the treatment protocol. Total AUC for blood glucose levels-vs-time (A) was calculated and then fractionated into the first and second half of the feeding period corresponding to weeks 0-4 (AUC_{F1}; B) and weeks 4-8 (AUC_{F2}; C), respectively. All values are mean ± SEM. Number of animals/group for prevention protocol DIO = 11; *L. laricina* 125 = 13; *L. laricina* 250 = 13, and for the treatment protocol DIO = 7; *L. laricina* 125 = 5; *L. laricina* 250 = 8). *denotes significantly different as compared to DIO group (one way ANOVA; post-hoc analysis Holm-Sidak or Bonferroni-Dunn test; $p < 0.05$).

Figure 2: Area under the curve (AUC) of cumulative change in body weight (CCBW; panels A-C) and cumulative change in energy intake (CCEI; panels D-F) in C57BL/6 mice treated with either HFD (DIO), or *L. laricina* at 125 or 250 mg/ kg, which was incorporated in the HFD for 8 weeks in the prevention protocol and for the last 8 of 16 weeks in the treatment protocol. Total AUC for CCBW-vs-time (A) or CCEI-vs-time (D) was calculated and then fractionated into the first and second half of the feeding period corresponding to weeks 0-4 (AUC_{F1}; B or E) and weeks 4-8 (AUC_{F2}; C or F), respectively. All values are mean ± SEM. Number of animals/group for prevention protocol DIO = 11; *L. laricina* 125 = 13; *L. laricina* 250 = 13, and for the treatment protocol DIO = 7; *L. laricina* 125 = 5; *L. laricina* 250 = 8). *denotes significantly different as compared to DIO group (one way ANOVA; post-hoc analysis Holm-Sidak or Bonferroni-Dunn test; $p < 0.05$).

Figure 3: Skin temperature was measured in the prevention study after 4 weeks (A) and after 8 weeks (B) of treatment, from mice treated with either HFD (DIO), or *L. laricina* at 125 or 250 mg/ kg, which was incorporated in the HFD for 8 weeks. All values are mean ± SEM. Number of animals/group for prevention protocol DIO = 11; *L. laricina* 125 = 13; *L. laricina* 250 = 13. *denotes significantly different as compared to DIO group (one way ANOVA; post-hoc analysis Holm-Sidak or Bonferroni-Dunn test; $p < 0.05$).

Table 1: Effects of HFD and *L. laricina* administration on systemic parameters at sacrifice

	Prevention Protocol			**Treatment Protocol**		
	DIO	***L. laricina* 125 mg/kg**	***L. laricina* 250 mg/kg**	**DIO**	***L. laricina* 125 mg/kg**	***L. laricina* 250 mg/kg**
Insulin (ng/mL)	9.2 ± 1.8	6.9 ± 1.1	6.0 ± 0.9	39.9 ± 5.8	29.4 ± 9.1	11.0 ± 1.9*
Leptin (ng/mL)	39.3 ± 3.5	37.7 ± 3.2	33.1 ± 2.0	39.0 ± 3.0	27.1 ± 5.4	30.9 ± 2.1
Adiponectin (μg/mL)	8.8 ± 0.7	11.1 ± 0.8*	10.8 ± 0.4	11.1 ± 0.6	10.9 ± 0.7	12.8 ± 0.6
Leptin/adiponectin ratio	4.9 ± 0.6	3.7 ± 0.5	3.1 ± 0.2*	3.5 ± 0.2	2.5 ± 0.5*	2.4 ± 0.2*

Measurements were obtained after 8 weeks (prevention) or 16 weeks (treatment) of administration with either HFD (DIO), or *L. laricina* at 125 or 250 mg/ kg, which was incorporated in the HFD for 8 weeks in the prevention protocol and for the last 8 of 16 weeks in the treatment protocol. All values represent the mean ± SEM (prevention protocol DIO = 11; *L. laricina* 125 = 13; *L. laricina* 250 = 13, and for the treatment protocol DIO = 7; *L. laricina* 125 = 5; *L. laricina* 250 = 8). ***** denotes that treated groups are significantly different as compared to DIO (one-way ANOVA; post-hoc analysis Holm-Sidak or Bonferroni-Dunn test; $p < 0.05$).

Table 2: Effects of HFD and *L. laricina* administration on hepatic and muscular triglyceride accumulation

	Prevention Protocol			**Treatment Protocol**		
	DIO	***L. laricina* 125 mg/kg**	***L. laricina* 250 mg/kg**	**DIO**	***L. laricina* 125 mg/kg**	***L. laricina* 250 mg/kg**
Liver TG levels (mg/g total liver)	331 ± 54	407 ± 47	374 ± 52	1041 ± 173	919 ± 240	1138 ± 118
Muscle TG levels (µg/mg)	84 ± 12	60 ± 6	65 ± 8	212 ± 29	224 ± 80	255 ± 33

The colorimetric dosage of TG levels in both the liver and muscle was determined using a commercial kit (as described in detail in Material and Methods). Measurements were obtained after 8 (prevention) or 16 (treatment) weeks of administration with either HFD (DIO), or *L. laricina* at 125 or 250 mg/ kg, which was incorporated in the HFD for 8 weeks in the prevention protocol and for the last 8 of 16 weeks in the treatment protocol. All values represent the mean ± SEM (prevention protocol DIO = 11; *L. laricina* 125 = 13; *L. laricina* 250 = 13, and for the treatment protocol DIO = 7; *L. laricina* 125 = 5; *L. laricina* 250 = 8). * denotes that treated groups are significantly different as compared to DIO (one-way ANOVA; post-hoc analysis Holm-Sidak or Bonferroni-Dunn test; $p < 0.05$).

Table 3: Effects of HFD and *L. laricina* administration on body and organ weights at sacrifice

	Prevention Protocol			Treatment Protocol		
	DIO	***L. laricina* 125 mg/kg**	***L. laricina* 250 mg/kg**	**DIO**	***L. laricina* 125 mg/kg**	***L. laricina* 250 mg/kg**
Retroperitoneal Fat Pad (g)	1.34 ± 0.05	1.26 ± 0.04	1.14 ± 0.05*	1.51 ± 0.08	1.35 ± 0.24	1.44 ± 0.04
Epididymal Fat Pad (g)	2.40 ± 1.00	2.56 ± 0.08	2.56 ± 0.07	1.21 ± 0.03†	1.32 ± 0.18	1.85 ± 0.12*
Brown Fat Pad (g)	0.30 ± 0.03	0.33 ± 0.01	0.28 ± 0.02	0.44 ± 0.01	0.37 ± 0.06	0.42 ± 0.02
Liver Weight (g)	1.77 ± 0.09	1.85 ± 0.05	1.80 ± 0.07	2.62 ± 0.15	2.44 ± 0.38	2.57 ± 0.12
Liver index	410 ± 2	429 ± 1	437 ± 1	551 ± 3	539 ± 5	555 ± 2

Measurements were obtained after 8 weeks (prevention) or 16 weeks (treatment) of administration with either HFD (DIO), or *L. laricina* at 125 or 250 mg/ kg, which was incorporated in the HFD for 8 weeks in the prevention protocol and for the last 8 of 16 weeks in the treatment protocol. The liver index corresponds to: liver weight (mg) / body weight (mg). All values represent the mean ± SEM (prevention protocol DIO = 11; *L. laricina* 125 = 13; *L. laricina* 250 = 13, and for the treatment protocol DIO = 7; *L. laricina* 125 = 5; *L. laricina* 250 = 8). * denotes that treated groups are significantly different as compared to DIO (one-way ANOVA; post-hoc analysis Holm-Sidak or Bonferroni-Dunn test; $p < 0.05$).

Table 4: Effects of obesity as well as *L. laricina* administration on hepatic mitochondrial function

	Treatment Protocol			
	CHOW	**DIO**	***L. laricina* 125 mg/kg**	***L. laricina* 250 mg/kg**
State 3	62.90 ± 4.50	59.81 ± 8.34	51.41 ± 6.91	62.91 ± 3.34
State 4	18.23 ± 1.05	17.43 ± 0.89	16.20 ± 1.04	16.29 ± 0.85
RCR	3.45 ± 0.08	3.37 ± 0.32	3.18 ± 0.35	3.87 ± 0.14
ATP synthesis	3.53 ± 0.53	3.20 ± 0.52	2.73 ± 0.40	3.52 ± 0.25

Mitochondrial function was measured as described in detail in Materials and Methods, after 16 weeks of administration with either standard diet (CHOW), HFD (DIO), or *L. laricina* at 125 or 250 mg/ kg, which was incorporated in the last 8 of 16 weeks in the treatment protocol. State 3 represents the rate of oxygen consumed during oxidative phosphorylation; state 4 represents the rate of oxygen consumption obtained after oxidative phosphorylation; RCR (respiratory control ratio) represents the ratio between state 3 and state 4. All values represent the mean ± SEM (for the treatment protocol CHOW = 4; DIO = 5; *L. laricina* 125 = 4*; L. laricina* 250 = 4).

Figure 1

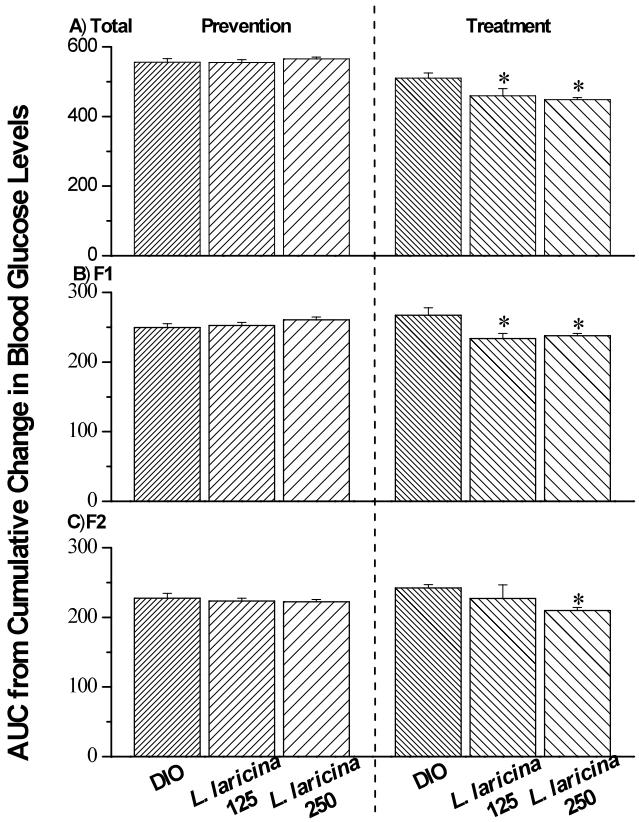

A) Total
Prevention
Treatment
B) F1
C)F2
AUC from Cumulative Change in Blood Glucose Levels
600
400
200
0
300
200
100
0
*
DIO
L. laricina 125
L. laricina 250

Figure 2

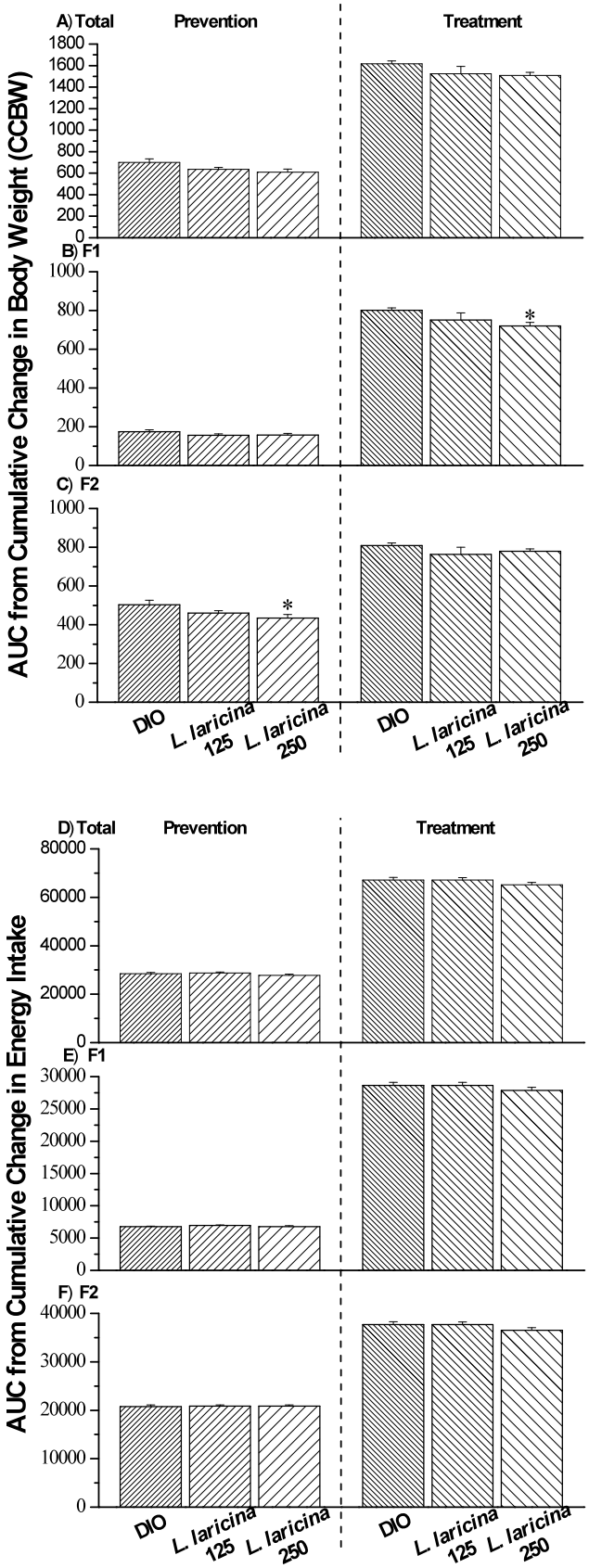
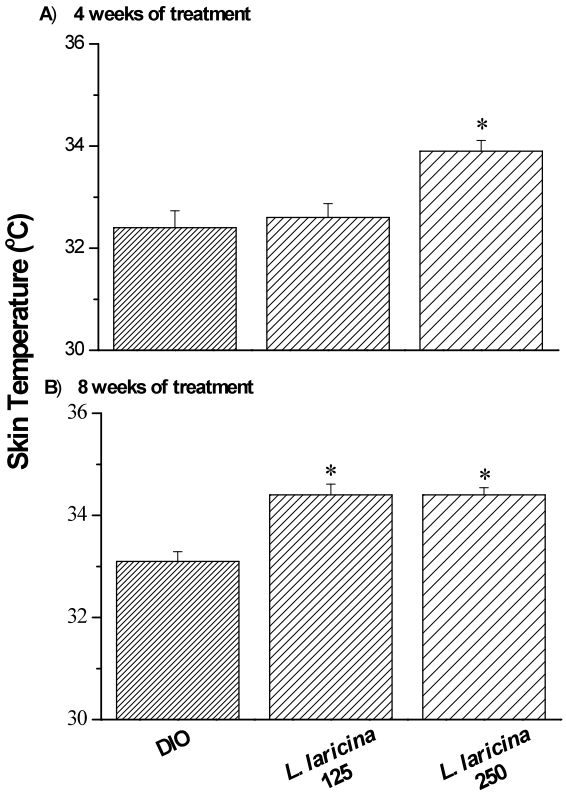

A) Total
Prevention
Treatment
B) F1
C) F2
D) Total
E) F1
F) F2
AUC from Cumulative Change in Body Weight (CCBW)
AUC from Cumulative Change in Energy Intake
DIO
L. laricina 125
L. laricina 250

Figure 3


A) 4 weeks of treatment
B) 8 weeks of treatment
Skin Temperature (°C)
36
34
32
30
*
DIO
L. laricina 125
L. laricina 250

Chapter 5: General Discussion

5.1 General Summary

Obesity and type 2 diabetes (T2D) have reached epidemic proportions worldwide, affecting both genders, as well as all age groups and ethnicities, with a predominance in the past decade especially among children and young adults. These morbid diseases not only result in devastating complications, whether they be physical or even psychological, but they also lead to a tremendous economical burden in countries around the world (Figure 49).

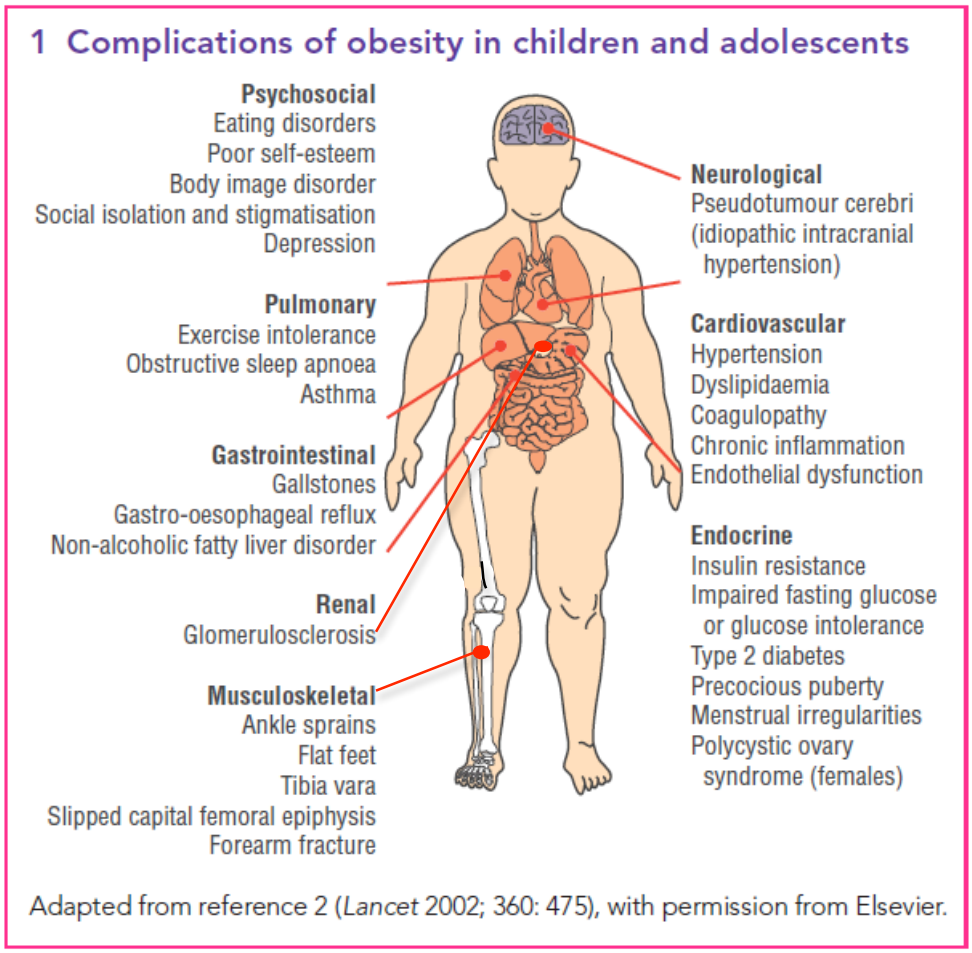


Figure 49: Complications arising from childhood obesity (adapted from:(Ebbeling et al. 2002; Batch and Baur 2005)).

In Canada, the aboriginal population is particularly affected, with the rate of obesity and T2D, being around 1.5 fold and 4-5 times higher, respectively than the rest of the population. The aboriginal population that our team focused on are the Cree of Eeyou Istchee (CEI) of the Northern James Bay Area of Quebec, which fall within the aforementioned prevalence of both these diseases. The high incidence of obesity and T2D among the CEI may be due to a change in their dietary habits. They had a long-standing tradition of being hunter-gatherers, which included fishing, eating green and root vegetables, fruits and berries. That lifestyle provided them with protective properties against diabetes (PHAC 2011a). They have now switched to a more western diet high in calories, saturated fats and simple sugars (Young 2000; Shah 2003; Kuhnlein et al. 2004). Other factors that may be involved, include a sedentary lifestyle (decreased physical activity), and genetic predisposition, with reference to the thrifty gene, which predisposes to conserve calories. This can be useful in times of famine, but detrimental with current food availability, lifestyle, and quality of diet (Neel 1962; Hegele et al. 1999; Neel 1999; Young 2000; Hegele 2001; Shah 2003). There are many other environmental factors that can have a negative impact on the health of the aboriginal population and therefore in the development of T2D. These social determinants of health include poor housing conditions, low level of education, unemployment, limited access to health care due to geographical location but also language barriers, and lack of culturally appropriate services, which also lead to low compliance to modern medications (Hales and Barker 2001; FNIGC 2007; Reading 2009; Southam et al. 2009; Carriere 2010).

Despite educational and dietary intervention programs that have been instituted with the objective of minimizing the incidence of obesity and T2D, both these disease continue to be on the rise among all aboriginal populations including the CEI. It has been noticed that in order for the CEI to have more compliance to any form of therapy, there needs to be an increase in

sensitivity to their culture and traditional medicines (Rideout and Menzies 1994). Wherefrom stems the necessity in reinforcing their traditional way of living, i.e. promoting healthy living and eating by going back to their hunter-gatherer lifestyle, as well as identifying and validating alternative treatments that are culturally relevant, and stemming from the CEI's own traditional pharmacopoeia (Boston 1997; Berman 1999; Gray-Donald 2000; Young 2000; Légaré 2004a; Légaré 2004b).

Our team conducted an ethnobotanical study among the Elders and healers of six communities, Mistissini, Whapmagoostui, Nemaska, Waskaganish, Wemindji, and Oujé-Bougoumou based on 15 symptoms associated with T2D (Leduc et al. 2006; Fraser et al. 2007). There were over 30 plants that were identified, with 17 of the most promising ones having been screened *in vitro* for their antidiabetic potential, by using cell-based screening assays that look at primary defects and secondary complications (Spoor et al. 2006; Harbilas et al. 2009; Martineau et al. 2010). These assays determined the ability of the plants to increase glucose uptake in C2C12 myotubes and 3T3-L1 pre-adipocytes, adipogenesis in 3T3-L1 pre-adipocytes, insulin secretion and β-cell proliferation, as well as their impact on protecting PC12-AC (neuronal precursor) cells against glucose toxicity and deprivation, and their anti-oxidant potential. The mechanisms of action of some of these plants were also examined *in vitro*, such as inhibition of Glut-2 or SGLT-1, activation of AMPK, ACC, ATP production, and their potential to uncouple mitochondrial respiration (Eid et al. 2010a; Eid et al. 2010b; Martineau et al. 2010; Nistor Baldea et al. 2010). Based on these studies, roughly half of the plant species revealed antidiabetic potential. Some plants also demonstrated a strong antiobesity potential. The two plant species that were of particular interest were *Populus balsamifera* L. (Salicaceae) and *Larix laricina* K. Koch (Pinaceae).

P. balsamifera was the only plant among the 17 to completely inhibit triglyceride accumulation (no visible lipid droplets) during adipogenesis in 3T3-L1 pre-adipocytes. Cells retained pre-adipocytic fibroblastic morphology (Harbilas et al. 2009; Martineau 2010a). This may be due to the fact that the crude plant extract exhibits PPAR antagonist action, as determined by gene-reporter assay (Martineau 2010a). The phytochemical compound, salicortin (salicylate glycoside) was identified through bioassay-guided fractionation in the adipogenesis assay, and revealed itself as the principal active component (10% of crude extract) of *P. balsamifera* responsible for the observed inhibition of adipogenesis (Martineau 2010b). It is of great importance that, prior to the *in vitro* studies conducted by our team and the *in vivo* studies that compose this thesis, the antiobesity potential had not been ascribed to *P. balsamifera*, or any of its known phytochemical constituents such as salicortin (Harbilas et al. 2009; Martineau 2010a; Martineau 2010b). Salicortin was however known for its anti-inflammatory and anti-proliferative effects (Subramanian et al. 2006).

Concerning *L. laricina*, it demonstrated antidiabetic potential by increasing glucose uptake and phosphorylation of AMPK and ACC (no activation of the Akt pathway) in C2C12 myotubes. Its action was comparable to levels comparable to the commonly prescribed antidiabetic medication Metformin (Spoor et al. 2006; Martineau et al. 2010). It also potentiated adipogenesis in 3T3-L1 adipocytes acting like the thiazolidinedione drug rosiglitazone, which is another class of antidiabetic medication (Spoor et al. 2006). In addition, hepatocytes isolated from Wistar rats, *L. laricina* was identified as one of the strongest uncouplers, severely disrupting mitochondrial function and ATP production (Martineau et al. 2010). Uncouplers are known to increase metabolic rate and fuel consumption, as a compensatory mechanism for decreased ATP production, suggesting that they may also serve as potential antiobesity treatments.

These results prompted further research in terms of validating the antiobesity and antidiabetic potential of *P. balsamifera* and its active salicortin, as well as that of *L. laricina* in an *in vivo* mouse model. The choice of the animal model, C57BL/6 diet-induced obese mice, was based on the fact that more than half of the cases of T2D are due to obesity, a combination of a hypercaloric high-fat diet and physical inactivity, and by reason of the incredibly high incidence of both these diseases among the CEI. The C57BL/6 mice are a widely used model that rely on a high-calorie diet and inactivity, without genetic involvement, to induce significant body and adipose tissue weight gain. This results in alterations in adipokine secretion, hyperglycemia, hyperinsulinemia, and hyperlipidemia, all reflecting the establishment of the metabolic syndrome and a pre-diabetic state (Jiang et al. 2005; Cefalu 2006a). Two distinct experimental protocols were used in the *in vivo* studies that make up this thesis and they were termed prevention and treatment protocols. The objective of the prevention protocol was to evaluate the potential of the plants to attenuate the development of obesity and its associated metabolic disturbances by administering either *P. balsamifera* or *L. laricina* concomitantly with the high-fat diet (HFD) for a period of 8 weeks. Conversely, the treatment protocol consisted in administering the HFD for a period of 8 weeks, allowing obesity and a state of metabolic syndrome to become established. Thereafter, over another 8 week period, *P. balsamifera* or its active principle salicortin, or *L. laricina* were incorporated to the HFD. This enabled to determine their respective potential at inhibiting further weight gain and even partially reversing the established state of obesity and metabolic syndrome.

In order to better highlight the results of the plant species and the active principle, it is important to briefly summarize the effects of the HFD on the model. The positive control group was termed DIO (diet-induced obesity), whereby a HFD was administered for either 8 weeks (prevention) or 16 weeks (treatment). DIO animals develop (to various degrees depending on the

protocol) significant body weight gain, increased retroperitoneal (abdominal) fat pad weight, dysregulation of adipokines secretion (adiponectin and leptin), upregulation of leptin/adiponectin ratio, increased blood glucose levels (hyperglycemia), hyperinsulinemia, and increased ectopic fat storage in the liver and muscle.

5.2 Summary of article 1 (chapter 2)

In the prevention study, *P. balsamifera* significantly attenuated body weight gain in mice continuously fed a hypercaloric fat laden diet, with results suggesting a dose-dependent effect. *P. balsamifera's* expected capacity to inhibit adipogenesis *in vivo* was corroborated by a decrease in retroperitoneal fat pad weight. *P. balsamifera* appeared to modulate the 44/42 MAPk pathway, increasing activation in the adipose tissue. The ERK pathway is associated with inhibition of adipogenesis *in vitro* and *in vivo*. It is known to inhibit transcriptional activity of PPARγ (activating SOX9 which in turn decreases expression of C/EBPβ and C/EBPδ)(Rosen et al. 2002; Sul 2009). Balsam poplar treatments produced a slight variation in PPARγ levels, however there may have been other adipogenesis effectors that may have been more strongly impacted and require further study.

As mentioned, the abdominal adipose tissue is associated with the negative effects of obesity, releasing adipokines and inflammatory cytokines that contribute to the onset of insulin resistance and secondary complications. *P. balsamifera*, not only altered abdominal weight but adipokine secretion as well. It increased adiponectin levels, and decreased the leptin/adiponectin ratio. Balsam poplar therefore contributed to the overall improvement of insulin sensitivity, which was further confirmed with its strong tendency to decrease glycemia and insulinemia levels.

Non-alcoholic fatty liver disease (NAFLD) and its more severe form of non-alcoholic steatohepatitis (NASH) is also recognized as being an important component of the metabolic syndrome and is involved in the development of insulin resistance in the liver (den Boer et al. 2004; Girard and Lafontan 2008). Therefore, improvement of hepatic steatosis represents an important feature of any therapy directed against the metabolic syndrome. There have been studies that have shown that increased ERK has been linked to more intense pre-replicative phase hepatocyte proliferation, hyperplasia and NAFLD (Chavez-Tapia et al. 2009). However, *P. balsamifera* decreased ERK (44/42 MAPk) in the liver. This may in part explain the decrease in hepatic steatosis observed in these animals. Balsam poplar shifted the severity of liver fat accumulation towards grades of weak and absent steatosis, as opposed to moderate and high steatosis in DIO controls. This finding correlated well with the strong tendency to reduce hepatic TG levels and increase PPARα levels (promotes fatty acid oxidation). Balsam poplar at its highest dose showed a tendency to normalize Akt levels, which is consistent with decreased insulinemia levels, while concomitantly being indicative of improvement in insulin sensitivity. IKK-αβ, a marker of inflammatory state and associated with insulin resistance in several models of the metabolic syndrome (Kim et al. 2001; Yuan et al. 2001; Arkan et al. 2005), was decreased in the liver of animals treated with *P. balsamifera* indicating a reduced inflammatory state. This completed several lines of evidence that support that balsam poplar improved insulin sensitivity by decreasing inflammation and ectopic fat storage (reduction in hepatic and skeletal muscle triglycerides).

Modifying either energy intake or energy expenditure, or even both can also regulate obesity. *P. balsamifera* decreased caloric intake at its highest dose, and may in part explain decrease in body weight (decrease in food intake amounts to 1/3 of observed decrease in body

weight). This slight anorexic effect requires further investigation. Balsam poplar also increased superficial body temperature, with results suggesting a dose-dependent effect. Superficial body temperature is an indication of energy expenditure in the form of heat. This finding correlated with balsam poplars' strong tendency to increase UCP-1 expression in BAT (associated with non-shivering thermogenesis).

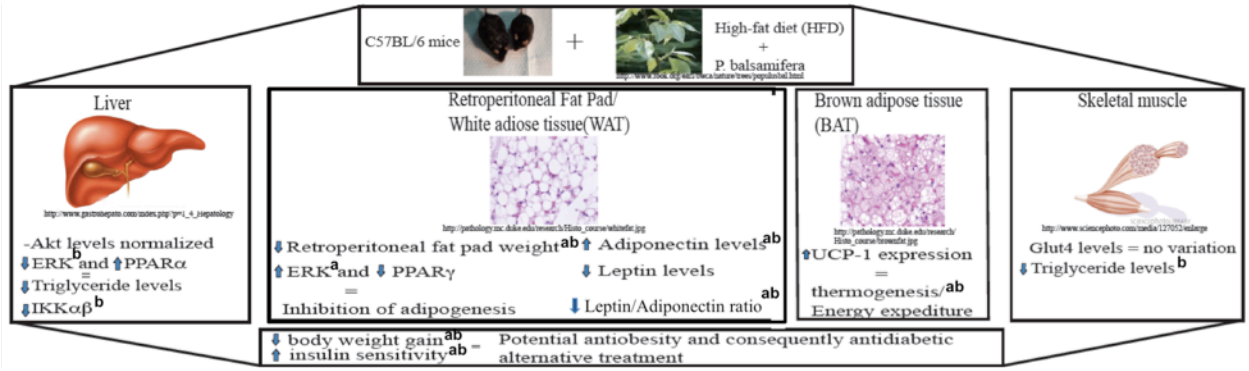


Figure 50: Schematic representation of the effects of *P. balsamifera* in the prevention protocol (article 1/chapter 2). In this figure the symbols used represent: [a] the dose of 125 mg/kg is significantly different as compared to DIO controls (see appropriate section for details on statistical analysis); [b] the dose of 250 mg/kg is significantly different as compared to DIO controls (see appropriate section for details on statistical analysis); [ab] both the 125 and 250 mg/kg doses are significantly different as opposed to DIO controls (see appropriate section for details on statistical analysis). No symbol next to the stated parameters indicates that the plant extract did produce an effect, however without reaching statistical significance.

5.3 Summary of article 2 (chapter 3)

In the treatment protocol, both *P. balsamifera*, more potent at 125 mg/kg than 250 mg/kg, and its active principle salicortin effectively modulated obesity and diabetes parameters. The

plant species reduced energy intake at 125 mg/kg, which may have contributed to its action on body weight. It is noteworthy, that the reduction in caloric intake was weaker than the weight loss measured. Nevertheless, this slight appetite-modifying effect does warrant further investigation. On the other hand, salicortin decreased body without affecting energy intake, suggesting different profiles of biological activity. It is to note that salicortin was identified as being the active principle in balsam poplar, based only on its ability to inhibit adipogenesis *in vitro*. It was not evaluated *in vitro* for any other beneficial effects that it may possess. Therefore, the current *in vivo* data suggests that there are certainly other phytochemical components (excluding salicortin) present within balsam poplar that may contribute to its appetite-modifying effect.

Glucose homeostasis was almost completely restored with *P. balsamifera* and salicortin. Glycemia levels returned to almost normal levels, and insulinemia was decreased by around 7 and 4 fold respectively. Likewise, the leptin-to-adiponectin ratio, also reflective of insulin resistance, was decreased with the plant extract and the active principle. Only salicortin significantly improved blood lipid profile (LDL and total cholesterol levels). Once again this points to variations in biological activity between the crude extract and the purified active principle.

The liver, adipose tissue, and skeletal muscle are all important players in the development of obesity, insulin resistance, and the metabolic syndrome. Therefore, a variety of effectors involved in glucose metabolism and fatty acid oxidation were evaluated in these tissues. As mentioned, hepatic steatosis is an important contributor to the development of insulin resistance and the metabolic syndrome, wherefrom the importance in decreasing hepatic TG, as was observed with both balsam poplar and salicortin. Both the plant extract and the active principle modulated pathways associated to hepatic lipid metabolism. They both showed tendencies at increasing levels of PPARα or CPT-1, favoring fatty acid oxidation, and decreasing ERK

phosphorylation. Hepatic IKKαβ, inflammatory component involved with NAFLD, also tended to be decreased by *P. balsamifera* (Arkan et al. 2005; Tilg and Moschen 2010). *P. balsamifera* also increased Akt phosphorylation in the liver (active principle demonstrated mild tendency) converging in the several lines of evidence that hepatic insulin sensitivity is improved.

Consistent with the observed reduction in body weight, both balsam poplar and salicortin greatly reduced retroperitoneal fat pad weight. However due to data variability, it failed to reach significant proportions. Nonetheless, analysis of a variety of adipose tissue components involved in glucose and lipid metabolism yielded a number of insightful results. Once more, the variations observed in the ERK pathway suggest that this may be the target pathway of both *P. balsamifera* and salicortin. A variety of factors are implicated in the regulation of the ERK pathway rendering interpretation of the data complex. In the treatment protocol, balsam poplar and the active principle showed tendencies in decreasing phosphorylated ERK levels, correlating with slight tendencies to increase PPARγ levels (see section 5.5.2 for possible explanations on ERK regulation). The plant species, and to lesser extent the active principle, seemed to upregulate factors associated with lipid metabolism, such as FABP4, a lipid chaperone that carries FA to cellular pathways of oxidation, and CPT-1 (strong tendency). Balsam poplar demonstrated a strong tendency at increasing Akt phosphorylation/activation, once again converging in the lines of evidence that insulin sensitivity was improved with plant species administration. Akt activation leads to Glut4 translocation and increased glucose uptake, as well as inhibition of lipolysis consequently decreasing TG being circulated to tissues such as the liver. In general, the active principle exhibited a weaker effect on the adipose tissue parameters measured herein.

Finally, in the muscle excessive muscle TG levels were not altered with *P. balsamifera* or salicortin treatment. However, the crude plant extract did seem to increase PPARα, which can

lead to increased fatty acid oxidation, potentially favoring increased muscle sensitivity to insulin. This was supported by *P. balsamifera* at 125 mg/kg increasing Glut4 expression and MAPK44/42 phosphorylation, and salicortin activating only the latter of the two pathways, highlighting once more their different biological activities.

In summary, analysis of key insulin responsive organs, such as liver, adipose tissue and skeletal muscle, uncovered potential lead mechanisms that are involved in glucose and lipid metabolism, and which were activated by balsam poplar and salicortin. In general, *P. balsamifera* at 125 mg/kg was more efficient overall in improving parameters associated to obesity and insulin resistance. In addition it was the only treatment condition that decreased food intake. Such counterintuitive dose-response relationships are not uncommon with polymolecular drug mixture, as is the case with the crude extract. Synergistic and antagonistic interactions occur between the phytochemical components, yielding conflicting response profiles, which is supported by aforementioned differences in biological activities between the plant extract and the active principle. It is therefore conceivable that there are other components in the crude extract that complement salicortin's activity. It is to note, that despite the active principle improving physiological (body and organ weights) or systemic parameters associated with obesity or insulin resistance, its effectiveness waned with time. This limits the use of the active principle at this dosage, and may have even contributed to masking effects on protein components responsible for glucose or lipid metabolism in insulin-responsive tissues.

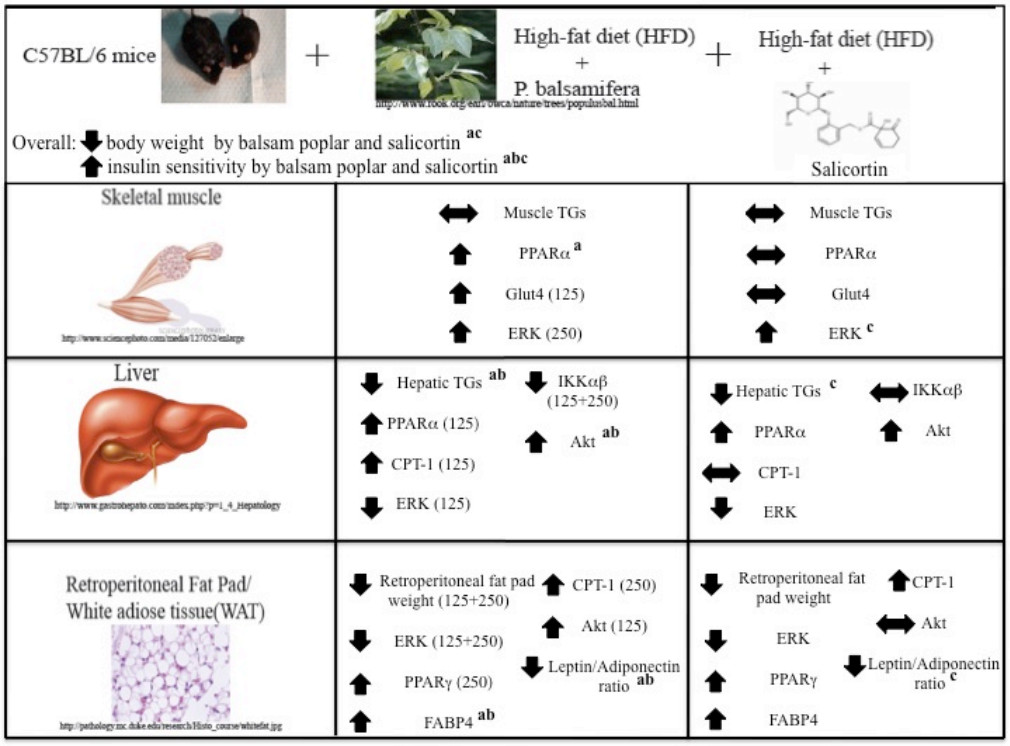


Figure 51: Schematic representation of the effects of *P. balsamifera* and salicortin in the treatment protocol (article 2/chapter 3). In this figure the symbols used represent: [a] the dose of 125 mg/kg is significantly different as compared to DIO controls (see appropriate section for details on statistical analysis); [b] the dose of 250 mg/kg is significantly different as compared to DIO controls (see appropriate section for details on statistical analysis); [ab] both the 125 and 250 mg/kg doses are significantly different as opposed to DIO controls (see appropriate section for details on statistical analysis). [abc] balsam poplar at 125 and 250 mg/kg, as well as salicortin are significantly different when compared to their respective DIO controls. [c] salicortin is significantly different as compared to DIO controls (see appropriate section for details on statistical analysis). The numbers indicated in the parentheses represent the doses at which balsam poplar showed a strong tendency to alter the parameter in question. No symbol next to the stated parameters indicates that the effect produced by the plant extract or the active principle did not reach statistical significance.

5.4 Summary of article 3 (chapter 4)

L. laricina was not only of interest by reason of its strong antidiabetic and antiobesity potential, but also because it is widely used and preferred by the CEI Elders and healers. *In vitro* data revealed that *L. laricina* increased glucose uptake in skeletal C2C12 myotubes, and 3T3-L1 adipocytes, potentiated adipogenesis, activated AMPK, and acted as a mitochondrial uncoupler (Spoor et al. 2006; Martineau et al. 2010). Both these tissues account for 85% of postprandial glucose disposal (DeFronzo 2004). *In vivo* data confirmed that *L. laricina* effectively modulated insulin resistance parameters, such as glycemia, insulinemia, and leptin/adiponectin ratio, showing strong tendencies in the prevention study and reaching significant proportions in the treatment protocol, especially at its highest dose of 250 mg/kg. This could suggest that *L. laricina* exerts its anti-hyperglycemic effect better when disease processes are more pronounced, explaining in part the difference in effect between the prevention and treatment study.

L. laricina did not alter muscle or hepatic TG levels neither in the prevention or treatment protocols. Ectopic fat storage is an important factor in insulin resistance leading to impaired insulin receptor function or signaling in the liver and muscle, as well increased hepatic glucose output. However, other systemic factors are involved such as those released from visceral adipose tissue, for example adipokines, free fatty acids, pro-inflammatory cytokines (TNF-, IL-1, IL-6, resistin, etc.)(Buettner et al. 2004). The lack of effect on hepatic or muscle TG needs to be evaluated in more detail, but can be explained by continuous administration of HFD in plant-treated animals, which could make elimination of steatosis more difficult. Another possibility is that oxidative pathways are being saturated with FA mobilized from adipose tissue (decrease in adipose tissue weight and probable hormone-sensitive lipase (HSL) activity), hindering in the process any decrease in tissue TG stores.

It is believed by reason of the data obtained at a molecular level in C2C12 myotubes (Martineau et al. 2010), and H4IIE hepatic cell lines (Nachar 2011), that *L. laricina* exerts its beneficial effects on glucose and lipid metabolism regulation through AMPK activation, and inhibition of enzymes involved in hepatic gluconeogenesis, for example glucose-6-phosphatase, GSK-3β, promoting regulation of glycogen formation such as glycogen synthase (Nachar 2011). Activators of AMPK include adiponectin and metabolic stress induced by disruption of the mitochondrial energy transduction. Mice on a HFD have a decreased mitochondrial respiratory control ratio state 3/4 (as was observed in the treatment protocol), and this may be due to the fact that an increase in dietary fat creates alterations in mitochondrial function. State 3 represents the rate of oxygen consumption during oxidative phosphorylation. It is controlled mainly by activity of ATP turnover (adenine nucleotide translocate, ATP synthase, etc.), and substrate oxidation (substrate uptake, processing enzymes, electron transport chain, etc.). State 4 represents the basal rate of oxygen consumption, and is influenced by proton leak and any ATPases (recycle ATP into ADP). A change in any aspect implicated in controlling state 3 or 4 leads to a change in respiratory control ratio (RCR). This implies that RCR is a useful measure of mitochondrial function: a high RCR indicates good function, while a decrease/low RCR is indicative of dysfunction (Brand and Nicholls 2011). Therefore, a combination of an increase in high fatty acid supply, as seen with HFD administration, an increase in the proton gradient (which slows-down electron transport chain), and a decrease in oxidative capacity, all lead to increased ROS production and peroxidation of FA, which amount to mitochondrial damage (Hesselink 2007; Crescenzo et al. 2011). Uncoupling agents alleviate mitochondrial stress induced by a HFD by decreasing proton gradient (through the flip/flop mechanism described in section 1.6.3), FA peroxidation, ROS production (the flip/flop mechanism allows for export of formed peroxides or fatty acid anions before they become peroxidized), and increasing fatty acid oxidation (Hamilton

and Kamp 1999; Schrauwen and Hesselink 2004; Hesselink 2007; Crescenzo et al. 2011). *L. laricina* in the treatment protocol showed tendencies of improvement in mitochondrial capacity and ATP production, reaching levels comparable to those observed in animals fed a standard Chow diet. In previous *in vitro* screening studies, the uncoupling effect of *L. laricina* was reported to be short lived, followed by a prolonged activation of AMPK and an overshoot phenomenon, which restores energy homeostasis and increases ATP production through increased carbohydrate and lipid metabolism. AMPK activation has additional benefits, such as protecting mitochondria from further damage by potentiating mitochondrial biogenesis. Uncoupling usually leads to increased heat production, due to increased energy expenditure. This was observed among animals in the prevention protocol as an indirect measure of uncoupling.

L. laricina also had an impact on body weight in both studies, producing significant effects when looking at continuous measures, and this occurred while animals were on an uninterrupted hypercaloric-fat-laden diet, without altering energy intake. Retroperitoneal/abdominal fat pad weight was decreased as well with plant extract administration, altering adipokine secretion (adiponectin and leptin), therefore improving insulin sensitivity. The impact of *L. laricina* on body weight parameters can be an indirect consequence of its antidiabetic activity, such as occurs with use of the commonly prescribed antidiabetic medication Metformin.

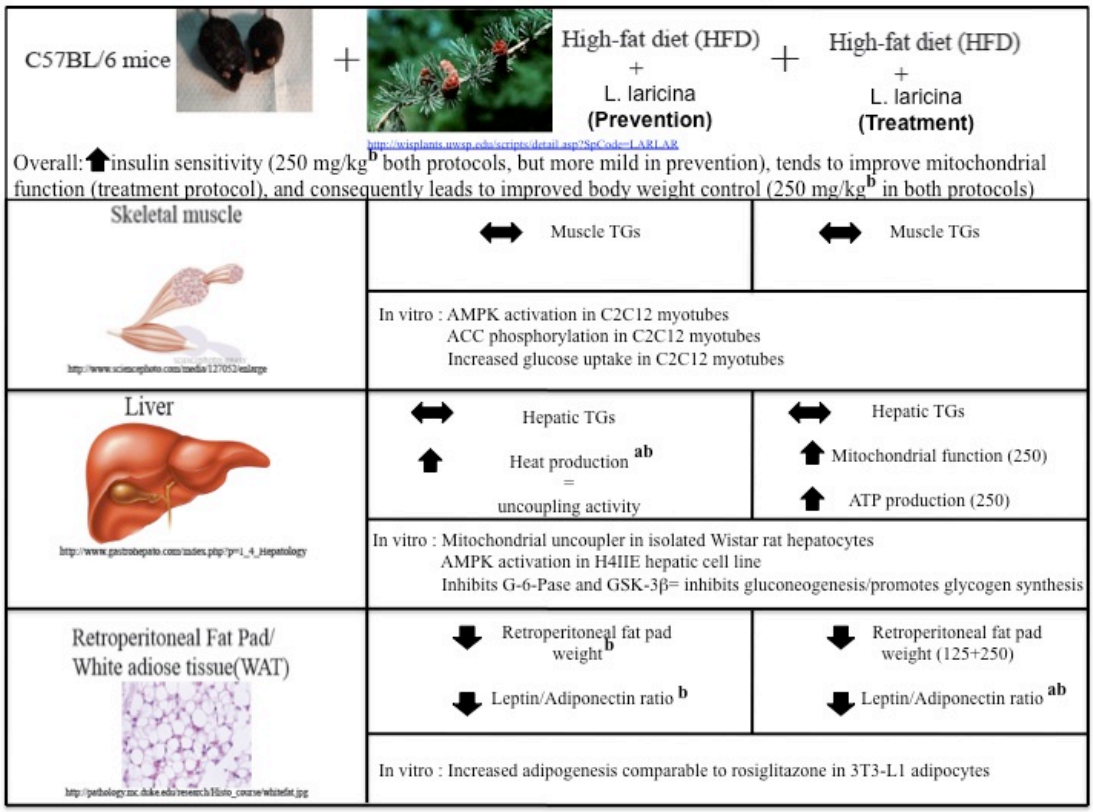


Figure 52: Schematic representation of the effects of *L. laricina* in the prevention and treatment protocol (article 3/chapter 4). In this figure the symbols used represent: [a] the dose of 125 mg/kg is significantly different as compared to DIO controls (see appropriate section for details on statistical analysis); [b] the dose of 250 mg/kg is significantly different as compared to DIO controls (see appropriate section for details on statistical analysis); [ab] both the 125 and 250 mg/kg doses are significantly different as opposed to DIO controls (see appropriate section for details on statistical analysis). The numbers indicated in the parentheses represent the doses at which tamarack showed a strong tendency to alter the parameter in question. No symbol next to the stated parameters indicates that the effect produced by the plant extract did not reach statistical significance.

5.5 Pespectives

5.5.1 Perspectives article 1

Many effectors, in addition to the ones evaluated in this article, may be involved in regulating adipogenesis, adiponectin expression, as well as fatty acid synthesis and oxidation in both the liver and muscle.

In this article, balsam poplar showed a tendency to decrease PPARγ levels, explaining in part downregulation of adipogenesis and a decrease in retroperitoneal fat pad weight. Other effectors involved in regulating adipogenesis include cyclooxygenase-2 (COX2), which plays an important role in early adipocyte differentiation (i.e. inhibition of COX2 attenuates adipocyte differentiation) (Fajas et al. 2003; Yan et al. 2003). Matrix metalloproteinase 2 and 9 (MMP-2 and MMP-9), two key enzymes involved in modulation of extracellular matrix (ECM), regulate adipose tissue remodeling which occurs with obesity (hypertrophy, hyperplasia, angiogenesis)(Bouloumie et al. 2001). The timely regulated expression p38 MAPk is also crucial, since its activity is high during initial stage of differentiation but decreases as fibroblasts undergo terminal differentiation into adipocytes (Engelman et al. 1999). AMPK stimulation is also implicated in adipocyte maturation by inhibiting cell proliferation (Hwang et al. 2005). SREBP-1c enhances adipose tissue conversion by inducing PPARγ expression (Feve 2005). Further studies are required to determine the implication of each one of the aforementioned effectors.

Although it is counterintuitive that decreased PPARγ expression is associated with increased adiponectin levels, it is to note that, once again, it is not the only transcription factor that regulates expression of adiponectin, these include C/EBPα, C/EBPβ, FOXO1, SIRT1, etc (Swarbrick and Havel 2008). We can hypothesize that since balsam poplar produced slight yet significant decrease in food intake, this effect might mimic a certain level of caloric restriction

with an effect on SIRT1 that could have increased adiponectin expression. Indeed SIRT1 decreases PPARγ expression, and deacetylates FOXO1, which enhances the interaction between FOXO1 and CEBPα, as well as that of these two transcription factors with the adiponectin promoter. This results in increased transcription of adiponectin gene in adipocytes (Liang et al. 2009). This hypothesis is purely speculative and requires further study.

Balsam poplar decreased hepatic and muscle TGs, reaching significance only in the latter. This therefore warrants further investigation into the pathways involved in fatty acid synthesis and oxidation in both these organs, such as FAS, SREBP-1c, AMPK and ACC, CPT-1, and PPARα in the muscle, just to name a few.

5.5.2 Perspectives article 2

Based on the data presented within this article, *P. balsamifera* and its active salicortin modulate the ERK pathway, which is involved in cellular proliferation and differentiation as well as insulin sensitivity. This pathway has also been reported to play an important role in adipogenesis and the development of NAFLD. Depending on the stimuli, the ERK pathway in the adipose tissue can either have proliferative or differentiating effect. It has been reported that activators such as insulin favor adipogenesis through increased MAPk activation (Aubert et al. 1999); this was observed with the control animals fed a HFD that were also considered hyperinsulinemic. The early versus late phases of differentiation can also impact ERK phosphorylation: increased during early stage of adipocyte maturation allowing for proliferation and inhibiting adipogenesis (decreased PPARγ levels); decreased in late phase allowing for adipocyte differentiation and adipogenesis (increased PPARγ levels)(Aubert et al. 1999). In the treatment protocol, balsam poplar and the active principle decreased phosphorylated ERK levels,

hinting towards stimulation of adipogenesis, correlating with slight tendency to increase PPARγ levels. These findings coincide with previous *in vitro* data. *P. balsamifera* added during early phase (comparable to prevention protocol) of 3T3-L1 adipocyte differentiation inhibits adipogenesis (corresponding *in vivo* to increased ERK levels and decreased PPARγ). However, when balsam poplar was added in the late phase (comparable to treatment protocol) it partially inhibits adipogenesis in the 3T3-L1 cell line, giving rise to adipocytes with normal morphology and to a small extent visible lipid droplets (corresponding *in vivo* to decreased ERK and increased PPARγ). This reasoning can possibly explain the lack of significance in retroperitoneal fat pad weight observed in the treatment protocol. Nevertheless, lipid metabolism in the adipose tissue may be regulated by other pathways that may be of interest for future studies: TG esterification (ex. glycerol-3-phosphate acyltransferase/GPAT), lipolysis (hormone-sensitive lipase or lipoprotein lipase), TG secretion from the adipose tissue, other effectors involved in adipogenesis such as COX-2, C/EBPβ or δ or α.

P. balsamifera and salicortin alter body weight, adipose tissue weight, adipokine secretion, glycemia and insulinemia levels, decrease hepatic TG, and improve glucose and lipid metabolism by modulating intracellular pathways in key insulin-sensitive tissues such as muscle, liver and adipose tissue (mostly insulin/Akt dependent pathways and their downstream effectors).

It is to note that there was more difficulty in identifying a specific mechanism of action with salicortin as opposed to balsam poplar. This may be attributed to the apparent time-course dependent tolerance present at the dosage used. Further dose searching experiments are required. They will aid in determining if this apparent time-dependent change in activity develops with any dosage of salicortin, and if so with what time course. This approach will aid in unmasking the

outcome of the active principle on protein components responsible for the observed beneficial effects.

P. balsamifera improved insulin sensitivity by activating the Akt pathway in the liver and in the adipose tissue (regulating glucose uptake, lipogenesis, and inhibiting lipolysis). Hepatic Akt activation leads to inhibition of glucose production and favors glycogen formation. *In vitro* screening studies on hepatic H4IIE cell lines indicate that *P. balsamifera* activated the Akt pathway and inhibited glucose-6-phosphatase (Nachar 2011). Future studies on *in vivo* livers are required to evaluate the effect of *P. balsamifera* and salicortin on effectors involved in hepatic glucose production (such as G-6-Pase, PEPCK, fructose-1,6-biphosphatase) and glycogen formation (such as GSK-3). Lipid oxidation pathways, such as PPARα and CPT-1, seemed to be activated by either the plant extract or the active principle. However other pathways may be involved in regulating lipid metabolism and would be of interest in future studies. Such examples include hepatic lipid export (incorporation in lipoproteins such as VLDL)(Begriche et al. 2006), lipid mobilization in the adipose tissue (lipogenesis, lipolysis, lipoprotein lipase, lipid oxidation), and fat absorption in the intestine (transported by chylomicrons to liver), as well as measuring lipid fecal content.

Finally, animal segregation into high and low responders reduced sample size, limiting interpretation of the results. In the future, increasing initial sample size prior to segregation may resolve this issue.

5.5.3 Perspectives Article 3

The exact mechanism of action for *L. laricina* remains to be identified, but results point towards possible activation of AMPK and downstream effectors (need to be investigated in future

in vivo studies). The beneficial effects of *L. laricina* can also be attributed to its ability at increasing mitochondria uncoupling, which is associated with heat production (need to evaluate UCPs expression in liver, muscle, BAT) and stimulation of the AMPK pathway.

L. laricina improved diabetes and obesity parameters in both the prevention and treatment protocols, while decreasing abdominal adipose tissue weight. *In vitro* data suggesting that *L. laricina* is a PPARγ agonist requires confirmation with a specific gene-reporter assay. PPARγ agonists such as rosiglitazone increase adipogenesis and intracellular triglyceride accumulation. They are also associated with an improvement in insulin sensitivity, increasing adiponectin levels (as observed in these studies) and activating AMPK pathways that influence downstream effectors involved in glucose (inhibiting hepatic glucose production) or lipid metabolism (increasing fatty acid oxidation). PPARγ agonists are usually associated with increased body weight and adiposity, while improving insulin sensitivity by shifting triglyceride accumulation from insulin-sensitive tissues, such as the liver and muscle, to fat tissue depots, mostly subcutaneous fat tissue (Larsen et al. 2003). In our studies, there was no observed increase in abdominal adipose tissue weight, or body weight (subcutaneous tissue not measured). This may suggest that instead of *L. laricina* acting as a pure PPARγ agonist, it may have dual PPARγ/PPARα properties. In other words, it increases lipid uptake while concomitantly increasing fatty acid oxidation (reports of PPARα expression in white adipose tissue). However this hypothesis remains to be investigated (Ferreira et al. 2006; Walker et al. 2007; Laurent et al. 2009).

In addition to these, other pathways involved in the regulation of glucose (insulin pathway, glucose transporters, glucose production and glycogen storage, etc.) and lipid metabolism (FAS, SREBP1-c, CPT-1, PPARα, PPARγ, lipolysis, lipogenesis, lipid absorption,

lipoproteins, etc.) need to be evaluated in all insulin-sensitive tissues (liver, muscle, adipose tissue). For instance, *in vitro* studies on hepatic H4IIE cell lines have indicated that *L. laricina* inhibits hepatic glucose production while promoting glycogen storage. The effectors involved in hepatic glucose production (such as G-6-Pase, PEPCK, fructose-1,6-biphosphatase) and glycogen formation (such as GSK-3) require further investigation in an *in vivo* context. One of these pathways may be responsible for the improvement in glucose metabolism observed in animals treated with *L. laricina*.

5.5.4 General perspectives

It is of the outmost importance to control both weight gain and glycemia levels, since both lead to devastating complications that contribute to the morbidity and mortality associated with obesity and T2D. Obesity, especially visceral, leads to low-grade inflammation, releasing into circulation pro-inflammatory cytokines that contribute to the development of insulin resistance and diabetes. Therefore it would be of interest to evaluate the effect of the plant extracts and the active principle on these parameters (TNF-α, IL-1β, IL-6, resistin, C-reactive protein, and so on). Obesity also leads to hepatic steatosis (NAFLD and NASH), dyslipidemia, insulin resistance and diabetes. It increases the risk of cerebrovascular (stroke), cardiovascular (hypertension, atrial fibrillation, etc.), respiratory (obstructive sleep apnea, asthma, etc.) diseases, as well as gastrointestinal disorders (reflux, hepatobiliary disease, etc.). In addition, there are higher rates of kidney disease and osteoarthritis, as well as anesthetic and surgical complications (Malnick and Knobler 2006). Uncontrolled glycemia levels, whether hyperglycemic or hypoglycemic, can lead to sometimes severe adverse effects. Hypoglycemia may cause lethargy, impaired mental functioning, irritability, shaking, weakness, loss of consciousness, and may even lead to brain

damage (de Courten-Myers et al. 2000; NIDDK 2008a; NIDDK 2008b). Conversely, hyperglycemia can lead to microvascular complications such as neuropathy, nephropathy, retinopathy and macrovascular complications, such as myocardial infarction and stroke.

Previous *in vitro* screening studies conducted by our team, evaluated the potential of plants to protect against glucose toxicity and deprivation in PC12-AC pre-neuronal cells (neuropathy), as well as their anti-oxidant potential (since ROS leads to cellular damage and contributes to complications associated to T2D). *P. balsamifera* was among the plants that protected pre-neuronal cells (PC12-AC) against glucose toxicity. Therefore, it is of interest to evaluate in the same animal model used in this thesis, the potential of *P. balsamifera* and its active salicortin, as well as *L. laricina* at minimizing complications associated to obesity and diabetes. This could be achieved by looking more closely at their anti-oxidant potential and their ability to protect against the development of nephropathy and neuropathy. Their anti-oxidant potential can be evaluated by looking at malondialdehyde (MDA) levels (marker of oxidative stress), or anti-oxidant enzymes such as catalase, superoxide dismutase (SOD), glutathione reductase or glutathione peroxidase, or anti-oxidant molecules such as glutathione levels (GSH), uric acid, bilirubine (Yeum et al. 2004; Valko et al. 2007; Weydert and Cullen 2009). Evaluating glomerular histopathology, as well as the albumine/creatinine ratio in urine (Soler 2012), will give insight on the potential of the plant extracts or the active principle to decrease nephropathy. Measuring sorbitol pathway intermediates, conducting physiological and behavioral tests, such as tactile or thermal algesia response, as well as sensory and motor nerve conduction velocity, lipid peroxidation products and lipidomics will help determine the efficacy of the plant extracts or the active principle at improving neuropathy. It is to note, that lipidomics is the study of all lipids present in a specific cell type, for example dorsal root ganglions, and their implication in disease

development and prediction, such as peripheral neuropathy (Obrosova et al. 2004; Watson 2006; Obrosova et al. 2007). One of our collaborators has been looking at the lipidomics profile, more specifically glycerophospholipids such as platelet activating factors (PAF) and lysophosphatidylcholines (LPC), of dorsal root ganglions collected from the mice used in this thesis. PAFs and LPCs are potent inflammatory lipids associated with diabetic retinopathy and neurodegeneration (Smith et al. 2008; Cheng et al. 2009; Kennedy et al. 2011). They have been looking at the potential of balsam poplar, salicortin, and tamarack in decreasing these inflammatory mediators as compared to their HFD controls, and therefore at their likelihood in preventing or reducing peripheral neuropathy.

5.6 Limitations

Although both plant species and the active principle yielded very promising results in both the prevention and treatment protocols in terms of antiobesity and antidiabetic agents, there are certain limitations that exist in terms of data interpretation. For instance, assays usually utilized to further confirm the antidiabetic potential of any agent, such as oral-glucose tolerance test (OGTT) or intra-peritoneal insulin tolerance test (ITT) were not performed in the studies that compose this thesis. Both OGTT and ITT require fasting the animals. Since the plant extracts or active principle were incorporated into the HFD, dietary interruption would result in concomitantly removing the treatment. Moreover, the mouse model used is primarily an obesity model resulting in T2D. Thus fasting the animals can result in a disruption of the metabolic alterations of the model, creating false positives when looking at overall weight gain and food intake.

Throughout the course of these studies, we discovered that the longer the animals were being administered a HFD, i.e. those in the treatment protocol, the more variability existed amongst them. This was confirmed in the studies of Peyot and collaborators (Peyot et al. 2010), which stated that pooling animals with different characteristics, such as low weight gain, weak IR and near-normal glycemia, with animals with high weight gain, frank IR, and hyperglycemia, can yield misleading results. Therefore in order to correct for this variability, animals administered a HFD were stratified into two groups according to the effect of the high-fat diet on body weight: low responders (LR) and high responders (HR). This stratification was based on body weight after 8 weeks of administration of a HFD, and allowed for analysis and comparison of the effect of *P. balsamifera*, salicortin, and *L. laricina* on mice fed a HFD (treatment protocol), in response to varying levels of obesity and insulin resistance. LR mice exhibited a much more normal metabolic profile. Treatment with the plant extracts or the active principle essentially had little if any effect. This confirms the validity of the segregation. However this decreased by half our number of animals per group, compromising the statistical power of many of the assays. This can be corrected in the future by starting off with twice the amount of animals.

In order to simulate the traditional CEI preparations of the plant extracts, prepared mostly as infusions, the ethanolic plant extracts used in this thesis were compared to their aqueous extracts. The data pertaining to this study was not presented within the thesis. However it is important to mention that the effects produced by the aqueous extracts of *P. balsamifera* on physiological and systemic parameters associated to obesity and diabetes were similar to its ethanolic extract, but of lesser magnitude (approximately ¼ of the effect observed with its ethanolic counterpart). Solvents such as ethanol are able to extract more phenolic phytochemical compounds than water for example. It is these compounds that have been associated with beneficial effects against a wide range of diseases, including obesity, diabetes, cardiovascular

disease, and cancer (King and Young 1999; Dai and Mumper 2010). At this time, we cannot conclude that the traditional method of preparation and usage is not efficient. Firstly, the dosage used for the aqueous extracts was identical to the ethanolic one and, as mentioned, phenolic compounds are less concentrated in water extracts. Secondly, the traditional method of preparation remains confidential but boiling times and temperatures have been shown to be crucial in preparations since they can affect the amount of phenolics that are extracted with water (Dai and Mumper 2010). Further studies are required in order to properly evaluate the potential of water extracts at preventing or treating obesity, and to identify their signaling pathways, by using higher doses and by preparing water extracts that more closely resemble traditional preparations.

Although basic *in vitro* screening studies were carried out on some of the mechanisms of action of *P. balsamifera* and *L. laricina*, more detailed analysis of the effects of these plant extracts and the active salicortin on pathways involved in glucose and lipid metabolism need to be conducted. The best and most cost-efficient approach would be to screen these pathways *in vitro*, since it will help orient the experiments in an *in vivo* context. Furthermore, it would be pertinent to identify the active components of *L. laricina* through *in vitro* bioassay guided fractionation in the adipogenesis and glucose uptake assays. Thereafter, an evaluation of the active principles' potential as an antiobesity and antidiabetic agent in an *in vivo* context would be of great importance. Such studies are currently underway within our research team. Identification of an active principle is crucial, being a marker of quality control, as such allowing to correlate the temporal and geographical variations in traditional preparations with variations in phytochemical profile (and even biological activity in bioassays), thus paving the way to more uniform efficacy.

One of the limitations of natural product research is that information is scant or simply lacks as pertains to the stability, solubility, absorption, metabolism, as well as mechanism and duration of action of the plant extracts. There is a wide range of components found within each plant extract, each acting on various targets. This can lead to complications when trying to identify signaling pathways implicated in the observed effects of the plant species.

It remains that the best prevention or even treatment for obesity and diabetes is diet and exercise. However when these prove to be insufficient, bariatric surgery and medications are available to treat either one of these diseases (Figure 53).

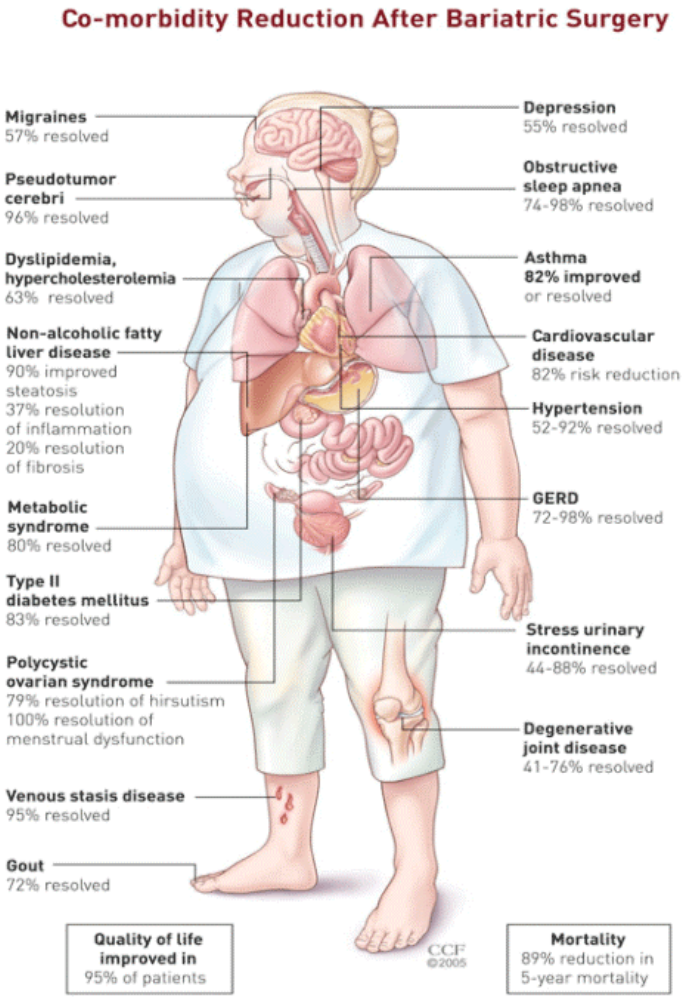


Figure 53: Examples of complications associated with adult obesity and to what degree bariatric surgery improves them (taken from: http://www.hippocrateon.com/index.php?pageid=59).

5.7 General knowledge on natural products identified as antidiabetic and antiobesity agents, their mechanisms of action, and how *P. balsamifera*, salicortin, and *L. laricina* compare to them

As reported, the use of complementary or alternative medicine to respond to one's primary health care needs has been growing in popularity on a worldwide scale (Tilburt and Kaptchuk 2008). As stated, the CEI have a low compliance to modern therapies, wherefrom the need to identify and validate alternative and more culturally acceptable treatments stemming from their own traditional pharmacopoeia. This will help them prevent (predisposition due to genetics and lifestyle) or even treat (high incidence of already existing cases) both obesity and T2D. In addition, the high costs and undesirable, sometimes hazardous side-effects, observed with obesity and diabetes treatments has prompted research in the field of natural products, in order to develop and identify treatments, to be used as substitutes or alone, for both these diseases.

These natural products, whether they are under the form of crude extracts or isolated compounds, can be arranged into different categories based on their antidiabetic or antiobesity effects.

5.7.1 Natural products as antidiabetic agents

Targets for antidiabetic medications include stimulating insulin secretion, increasing insulin sensitization (i.e. increasing glucose uptake, decreasing hepatic glucose production, and improving circulating TG by promoting adipogenesis), inhibiting intestinal carbohydrate digestion and absorption, and mimicking incretin-based therapies (incretin mimetics and DPPIV inhibitors). More specifically, there is evidence that a variety of plants possess antidiabetic properties by inhibiting hepatic glucose production through normalization of glucose-6-

phosphatase activity. Examples are *Momordica charantia* (Singh et al. 1989; Shibib et al. 1993), *Allium sativum* (garlic) and *Allium cepa* (onion)(Sheela et al. 1995). Others such as *Gymnema sylvestre*, *Momordica charantia*, *Nigella sativa* have been shown to inhibit glucose absorption in the small intestine (Matsuda et al. 1999; Persaud et al. 1999; Meddah et al. 2009). There have also been some plants that have been associated with enhancing insulin signaling or secretion, such as *Cinnamomum zeylanicum* (cinnamon) (Kahn 2003), *Nigella sativa* (Benhaddou-Andaloussi 2008), *Aloe vera* (Ajabnoor 1990), and *Trigonella foenum graecum* (also known as fenugreek) (Vats et al. 2003).

5.7.2 Natural products as antiobesity agents

Concerning plant extracts with antiobesity potential, they can be separated into 5 categories depending on their site of action: lipid absorption, energy intake, energy expenditure, pre-adipocyte proliferation and differentiation, lipogenesis and lipolysis.

5.7.2.1 Natural products as antiobesity agents acting on lipid absorption

Natural products that act on pancreatic lipase, inhibit this key enzyme. The latter is implicated in TG absorption by hydrolyzing TG into monoglycerides (MG) and fatty acids. Inhibition of pancreatic lipase leads to decreased fat absorption and possibly increased fat excretion in stool, as observed with the antiobesity medication Orlistat. Some examples of plants inhibiting pancreatic lipase include *Panax japonicus*, *Juniper communis*, *Trigonella foenum graecum* (Yun 2010).

The effects of *P. balsamifera*, salicortin, and *L. laricina* on inhibiting pancreatic lipase remain to be determined. Such an action would help explain, in part, the improvement in body weight and insulin sensitivity. An attempt was made to answer this hypothesis by collecting a sample of feces over a period of a week from mice of both the prevention and treatment protocols

and analyzing them in a bomb calorimetry assay (looking at differential energy release). However this assay was inconclusive due to unknown daily fecal matter excreted. This can be corrected by using metabolic chambers to measure total daily fecal excretion per mouse in future assays, allowing for proper analysis and conclusions.

5.7.2.2 Natural products as antiobesity agents acting on energy intake

Body weight regulation through appetite control is multifactorial, involving neurological and hormonal interrelationships. These take into account the physiological and psychological experience and behavioral expression of appetite, metabolism and peripheral physiology, and the central nervous system (CNS) neural pathway's functioning (Yun 2010). Neurotransmitters such as serotonin, histamine, and dopamine, along with their receptors, are associated with satiety regulation, and represent potential targets for drugs geared towards reducing energy intake. Appetite suppressants usually affect hunger control centers in the brain, creating a sense of fullness. However this decrease in food intake can lead to increased ghrelin, which stimulates appetite (Yun 2010). Therefore, in the quest to find antiobesity treatments, it may be of interest to look at products that blunt this response, i.e. ghrelin antagonism, as well as those acting as melanin-concentrating hormone (MCH; orexigenic hypothalamic peptide) receptor antagonists (Yun 2010). A few examples of natural products acting as appetite suppressants include, *Hoodia gordonii*, a leafless spiny succulent plant from South African countries. It increases ATP content in hypothalamic neurons, decreasing AMPK activity and increasing malonyl-CoA. This signals the anorexigenic/orexigenic neuropeptide system to suppress food intake.

Based on this information, the effect of *P. balsamifera* on satiety needs to be looked at in more detail. One hypothesis may be that it inhibits AMPK in the brain, leading to decreased food intake (proposed reason for anorexic effect of metformin). It could also act centrally in the

arcuate nucleus of the hypothalamus, ex. neuropeptide Y and agouti-related peptide (orexigenic), which regulate melanocortin system responsible for feeding behavior.

Other targets for appetite control and modulating eating behavior include increasing the release of 5-hydroxytryptamine and serotonin. This was observed with hydroxycitric acid prepared from *Garcinia cambogia*. Alternatively, treatment can amplify signaling in the basal hypothalamus' energy-sensing function, as occurs with the Korean red ginseng. Supplementing the diet with certain dietary fats, such as conjugated linoleic acid, lauric acid, and salatrim can also suppress energy intake, by slowing down gastric emptying and modulating gastrointestinal hormone secretion (Yun 2010).

5.7.2.3 Natural products as antiobesity agents acting on energy expenditure

Obesity results when there is an imbalance between energy intake and energy expenditure. Energy expenditure encompasses physical activity, obligatory energy expenditure, and adaptive thermogenesis. In that context, cellular targets for antiobesity (even for antidiabetic) medications include the mitochondria. For example, metformin and thiazolidinediones are said to inhibit complex I of the electron transport chain, activating AMPK in the process and leading to increased glucose and lipid metabolism, as well as energy expenditure.

Several of the plant species belonging to the CEI traditional pharmacopoeia have been found to interfere with the normal functioning of mitochondrial respiration. Among them is *L. laricina*, which was identified as a mitochondrial uncoupler *in vitro* and confirmed in an *in vivo* context in this thesis, by measuring mitochondrial respiratory control ratio and heat expenditure (surface skin temperature). Its ability to activate the AMPK pathway, as observed *in vitro*, remains to be determined in an *in vivo* context.

Concerning *P. balsamifera*, it also exhibits increased heat expenditure, which correlated with an increase in UCP-1 expression in brown adipose tissue (associated with non-shivering thermogenesis). However there are non-pleasant side effects that have been associated with mitochondrial uncoupling agents, such as increased sweating, cataracts, and fatal hyperthermia.

There are key players involved in energy expenditure as heat. These include UCP-1 found in brown adipose tissue. Another example is acquisition of BAT features, i.e. rich in mitochondria with increased capacity for FA oxidation, within white adipose tissue. More recent evidence suggests the implication of UCP-3, found in various tissues including skeletal muscle, in mediating thermogenesis regulated by thyroid hormone, β3-adrenergic agonists and leptin (induction of AMPK and skeletal muscle thermogenesis)(Solinas et al. 2004; Cline 2006; Yun 2010; Henry et al. 2011)(Figure 54).

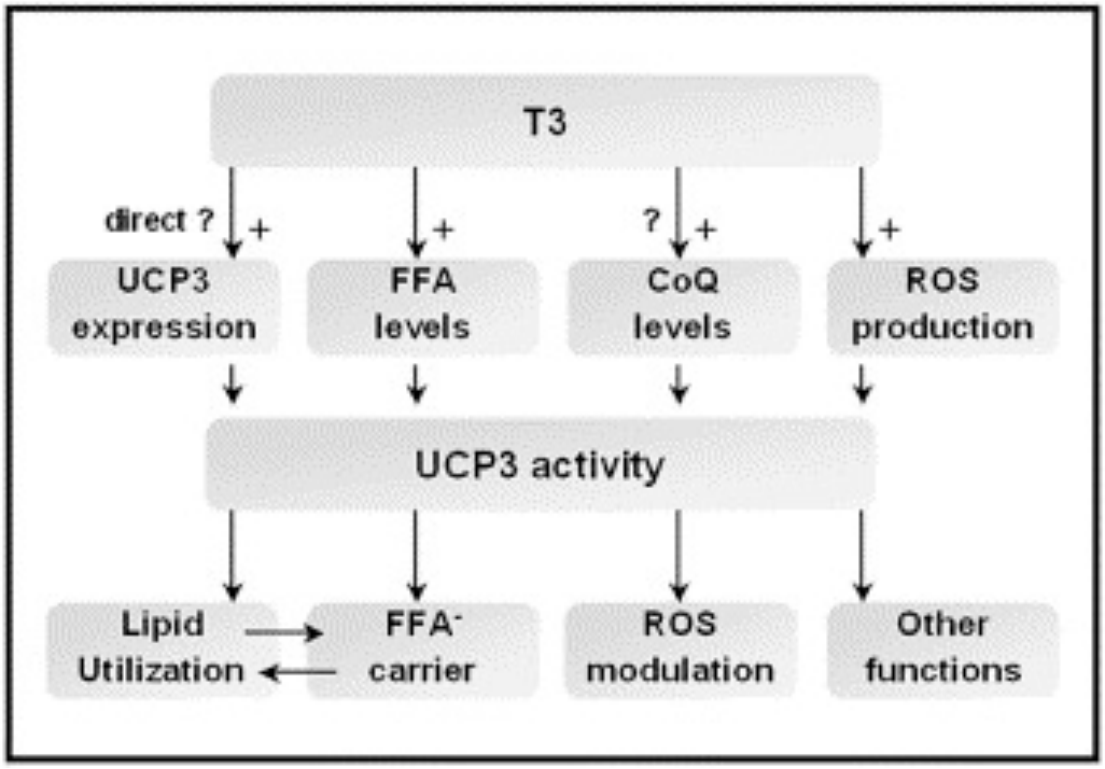


Figure 54: Thyroid hormone upregulates UCP-3 activity (Lanni et al. 2003).

In any case, the putative hyperthermia observed in animals treated with *P. balsamifera* and *L. laricina* needs to be looked at more closely. It can be done by implanting microchips and looking at daily variations in temperature. Another possibility is by determining whether hyperthermia is exercise-induced by placing animals in metabolic chambers (to measure physical activity). It can also be hormonally induced (ex. thyroid hormone), in which case looking at their plasma levels is crucial. It is also important to analyze UCP (UCP-1 to 3) proteins expression in various tissues (liver, skeletal muscle, BAT and WAT) (Figure 55).

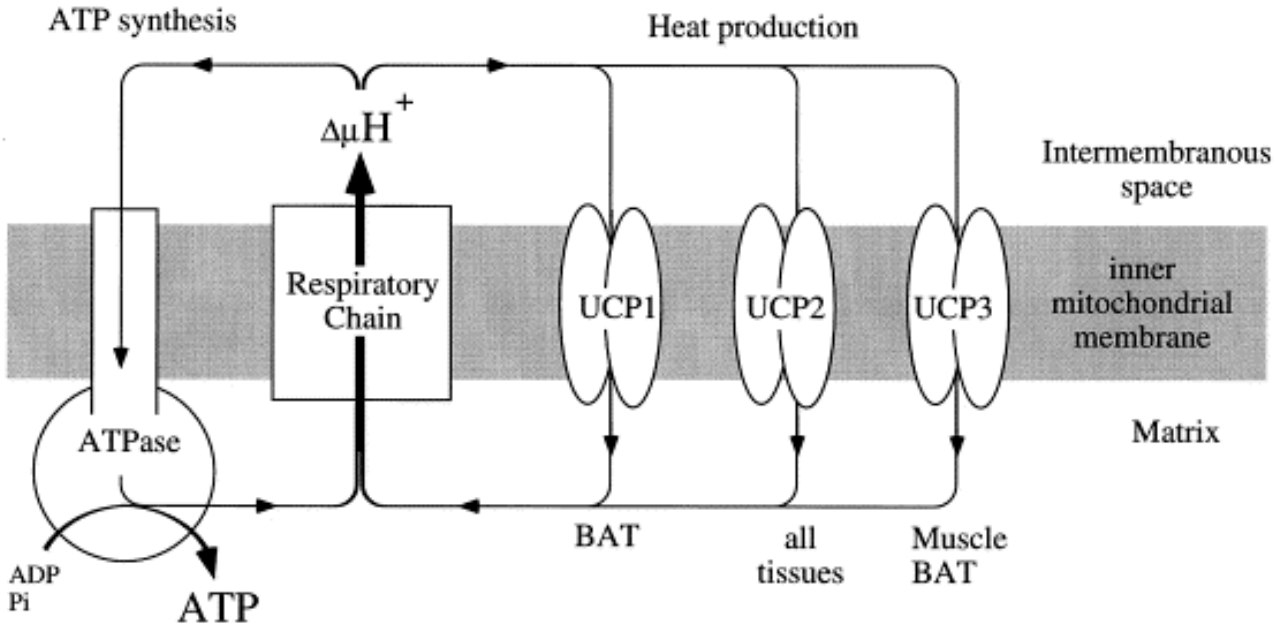


Figure 55: Summary of the distribution of UCP 1 to 3, and how a proton leak leads to decrease ATP production and increased heat production (Fleury and Sanchis 1999).

Natural products that cause energy expenditure through heat are useful in the treatment of obesity. Some examples include *Pinellia ternate* (Korean medicinal herb upregulating UCP1 expression in BAT), *Solanum tuberosum* (increases UCP3 expression in BAT and liver), and naturally occurring n-3 polyunsaturated fatty acids and fucoxanthin (marine origin) (increases

BAT features in WAT deposits) (Yun 2010). Other compounds that have exhibited weight loss properties through increased energy expenditure include extracts of *Pinellia ternate* and *Panax ginseng* berries, caffeine, capsaicin, as well as green tea and its extracts (catechins, epicatechin gallate/EGC, epigallocatechin gallate/EGCG). Caffeine is reported to inhibit phosphodiesterase degradation of cAMP thereby increasing energy expenditure. Capsaicin upregulates catecholamine secretion from adrenal medulla (thermogenic properties) (Rayalam et al. 2008) (Figure 56). Finally, EGCG is said to stimulate thermogenesis by inhibiting the o-methyltransferase involved in the degradation of norepinephrine (Yun 2010).

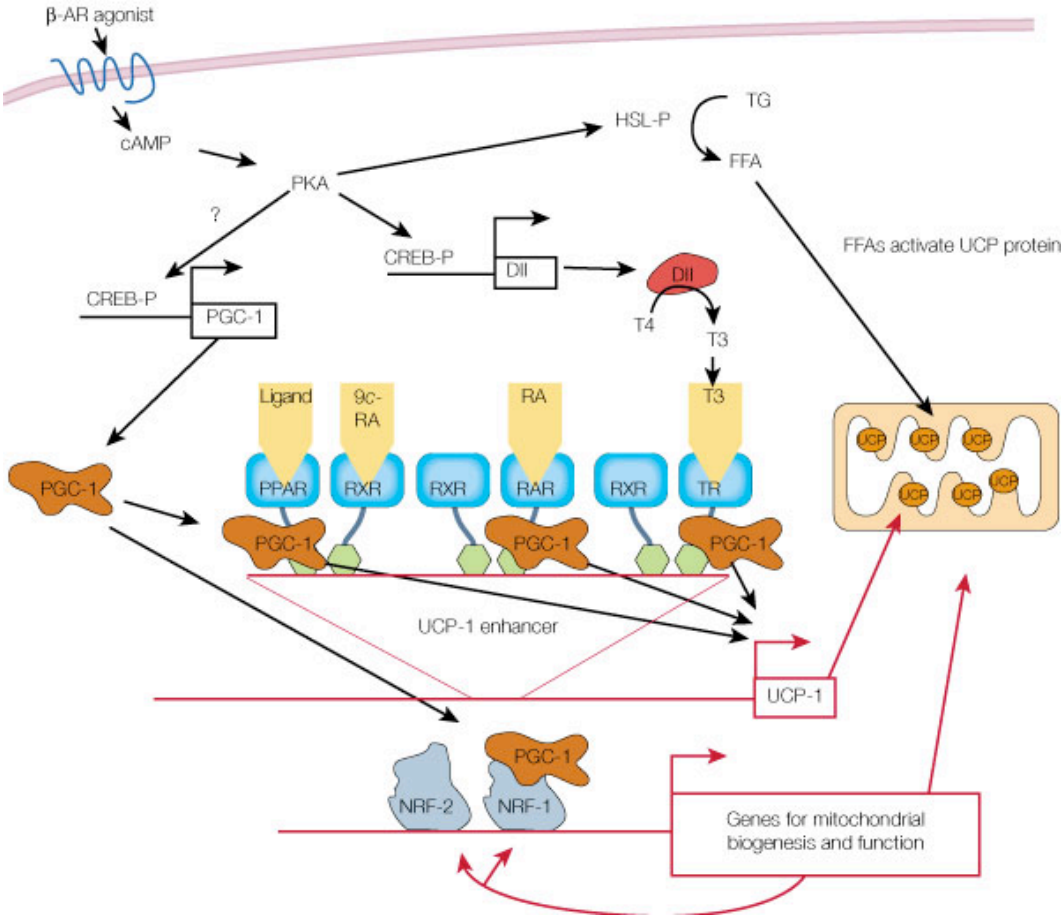


Figure 56: β3-agonists and PKA upregulate UCP-1 expression and lead to thermogenesis (Lowell and Spiegelman 2000).

5.7.2.4 Natural products as antiobesity agents acting on adipose tissue (adipocyte proliferation and differentiation)

Adipocytes play a central role in the maintenance of lipid homeostasis and energy balance. They do so by storing TG or releasing FA in response to changing energy demands. Adipose tissue growth can occur as a result of hyperplasia (proliferation) and hypertrophy (differentiation). Therefore natural products that act either to inhibit pre-adipocyte proliferation, to increase apoptosis of maturing adipocytes, or to inhibit adipocyte differentiation are being investigated with regards to potential antiobesity treatments. The latter mechanism of action is the case of *P. balsamifera* and its active principle salicortin. Some examples of phytochemicals exhibiting the abovementioned properties include resveratrol, quercetin, genistein, EGCG, and capsaicin. Pathways of interest in the adipocyte include ERK (cell proliferation, differentiation, apoptosis), AMPK (fatty acid oxidation), and sirtuin1 (Sirt1; decreases adipogenesis by promoting fat mobilization and suppressing PPARγ). The inhibition of adipogenesis or adipose tissue expansion can nonetheless have unhealthy consequences, by increasing ectopic fat storage, and as such promoting the development of T2D and other metabolic diseases.

5.7.2.5 Natural products as antiobesity agents acting on lipid metabolism

Antiobesity treatments can also target lipid metabolism by increasing TG hydrolysis (adipose tissue triglyceride lipase/ATGL, HSL, monoglyceride lipase lipoprotein lipase) and the oxidation of those newly released FA (Langin 2006). Due to the fact that excessive lipolysis can result in high circulating FA levels and the development of dyslipidemia, blocking FA release and promoting fatty acid oxidation can be of therapeutic interest (Langin 2006; Yun 2010). Fatty acid oxidation can occur through AMPK and PPARα activation, as can be seen with *Panax ginseng* and *Salacia oblonga* root (Yun 2010).

As mentioned, the AMPK pathway is also involved in improving obesity by enhancing the β-oxidation of FA (increasing energy expenditure). Some examples of plant extracts that stimulate the AMPK pathway include the majority of plant extracts from the CEI traditional pharmacopoeia (Eid et al. 2010a; Martineau et al. 2010), including *L. laricina*. Other plants originating from Southern Brazil, such as *Ilex paraguariensis* (Yun 2010), do the same.

In addition, treatments can inhibit lipogenesis by using for example PPARγ antagonists, such as resveratrol and quercetin. Alternatively, they can target fat absorption through inhibition of pancreatic lipase, as is the case for instance with peanut shell extract (*Arachis hypogaea*), green tea, and pomegranate. Active ingredients identified in this context include ellagic acid and tannic acid.

Chapter 6: Conclusion

The results of this thesis confirm in an *in vivo* context of diet-induced obesity in C57BL/6 mice that *P. balsamifera*, its active salicortin, and *L. laricina* possess antiobesity and antidiabetic activity. These plant species were evaluated in two varying degrees of the metabolic syndrome, what were termed as prevention (mild state) and treatment (advanced state) protocols.

Both balsam poplar and the active principle were effective in altering obesity and diabetes parameters in each one of the protocols. In the prevention protocol, *P. balsamifera* produced an immediate and sustained effect on the measured parameters, effectively reducing body weight gain, retroperitoneal fat pad weight, liver and muscle lipid content, as well as circulating glucose, insulin and leptin levels. It also activated (or strong tendencies) glucose and lipid oxidation pathways, as well as thermoregulation. In the treatment protocol balsam poplar increased in effectiveness throughout the study, while salicortin's activity waned with time. *P. balsamifera* and its active salicortin mostly improved obesity parameters, with a consequent enhancement of insulin sensitivity (improving glycemia, insulinemia, adipokine, and hepatic TG levels). They also activated (or strong tendencies) pathways involved in lipid and glucose metabolism.

L. laricina in both protocols effectively decreased glycemia levels, improved insulin resistance and slightly decreased abdominal fat pad and body weights. It also increased energy expenditure as demonstrated by elevated skin temperature in the prevention study, and showed strong tendencies to improve mitochondrial function and ATP synthesis in the treatment. Concerning *L. laricina*, in the prevention study its efficiency increased with time. In the treatment study, *L. laricina's* effect was maintained when looking at glycemia and insulinemia levels, but tapered off when looking at body weight. *L. laricina* was therefore primarily antidiabetic, and indirectly altered body weight parameters, similarly to the mode of action of Metformin (AMPK activation, mitochondrial uncoupling, indirect body weight modulation).

In summary, both obesity and diabetes parameters were more efficiently altered in a less advanced state of the metabolic syndrome. This confirms how crucial weight loss is in reestablishing insulin sensitivity, and improving diabetes.

Overall the results presented within this thesis demonstrate that *P. balsamifera* and its active salicortin, as well as *L. laricina* represent valuable culturally adapted alternative therapeutic agents in attenuating the development of obesity and insulin resistance (prevention), or improving the established state of both these diseases (treatment). Although salicortin's activity waned with time at the dose used for this treatment study, it nevertheless represents an important marker for quality control, ensuring safety, characterizing composition and optimizing antiobesity biological activity of traditional preparations of *P. balsamifera*. It can also serve as a template for the development of new therapeutic agents for the treatment of obesity and T2D. It is to note, that salicortin was identified as the most potent phytochemical constituent of *P. balsamifera* in inhibiting adipogenesis in 3T3-L1 cells. This however does not preclude the possibility of identifying, through bio-assay guided fractionation, other constituents responsible for additional beneficial effects. The latter include improving other aspects of obesity or T2D and their associated secondary complications, such as peripheral neuropathy, inflammation, oxidative stress, and so on. This reinforces the importance in identifying the active principle(s) of *L. laricina*, through a bioassay guided-fractionation approach in the glucose uptake and adipogenesis assays, and testing them in an *in vivo* context of diet-induced obesity. As mentioned, *L. laricina* closely resembles, in its mechanism of action, the commonly prescribed antidiabetic medication Metformin. Therefore identifying active principles and evaluating their pleiotropic effects in an *in vivo* context, can potentially lead to the development of new pharmaceutical agents with great potential at improving diabetes and body weight parameters.

In conclusion, *P. balsamifera*, salicortin, and *L. laricina* modulated body weight and insulin resistance parameters in a manner comparable to modern day pharmaceuticals used in the treatment and management of both obesity and diabetes. Therefore, notwithstanding the work still necessary (for ex. more detailed signaling pathways in insulin-sensitive tissues as reported in section 5.5 Perspectives), the present thesis represents a major contribution to the validation and understanding of the antiobesity and antidiabetic potential of *P. balsamifera*, salicortin, and *L. laricina*. Indeed, we have identified properties that had not yet been ascribed to these plants or their phytochemical constituents. These thus represent valuable alternative and culturally-adapted therapies stemming from the CEI traditional pharmacopoeia. They provide a strong impetus for their integration in culturally-adapted clinical studies among the CEI, alongside lifestyle changes such as healthy diet and exercise, including culturally relevant traditional foods and activities (fishing, hunting, trapping).

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Appendix A: Diet composition and preparation

Figure 1:

Diet composition of standard laboratory chow diet used in all experimental protocols that compose this thesis (sent to the authors from a representative at Charles River Labs.).

5075
CHARLES RIVER 18% AUTOCLAVIBLE RODENT CHOW

CHEMICAL COMPOSITION*

NUTRIENTS**

GENERAL CONSTITUENTS	
Crude Protein, %	18.1
Crude Fat, %	4.5
Crude Fibre, %	3.4
Ash, %	6.7
Nitrogen Free Extract (by difference), %	57.3
CHEMICAL CONSTITUENTS	
Neutral Detergent Fibre (NDF), %	14.1
Acid Detergent Fibre (ADF), %	4.6
ENERGY	
Total Digestible Nutrients (TDN), %	75.0
Gross Energy (GE), kcal/g	4.1
Physiological Fuel Value***(PFV), kcal/g	3.4
AMINO ACIDS	
Arginine, %	0.99
Cysteine, %	0.46
Glycine, %	0.83
Histidine, %	0.40
Isoleucine, %	0.70
Leucine, %	1.28
Lysine, %	1.06
Methionine, %	0.39
Phenylalanine, %	0.82
Tyrosine, %	0.51
Threonine, %	0.63
Tryptophan, %	0.22
Valine, %	0.83

MINERALS	
Calcium, %	0.97
Phosphorus, %	0.85
Potassium, %	0.67
Magnesium, %	0.17
Sodium, %	0.30
Chloride, %	0.54
Fluorine (max.), mg/kg	35.00
Iron, mg/kg	170.00
Zinc, mg/kg	70.00
Manganese, mg/kg	87.00
Copper, mg/kg	12.00
Cobalt, mg/kg	0.34
Iodine, mg/kg	0.50
Selenium, mg/kg	0.20
VITAMINS	
Menadione (added), mg/kg	8.8
Thiamin, mg/kg	80.0
Riboflavin, mg/kg	7.0
Niacin, mg/kg	98.0
Pantothenic Acid, mg/kg	30.0
Choline, mg/g	1.6
Folic Acid, mg/kg	8.0
Pyridoxine, mg/kg	9.0
Biotin, mcg/kg	350
Vitamin B_{12}, mcg/kg	26
Vitamin A, IU/g	41
Vitamin D_3, IU/g	2.2
Vitamin E, IU/kg	90

* The chemical composition data is based upon ingredient analysis information. Nutrient composition may vary due to the natural variation in ingredient composition.

** Nutrients have been calculated on a 90% dry matter basis.

*** PFV (kcal/g) = ((Protein, % x 4) + (Fat, % x 9) + (NFE, % x 4)) ÷ 100

Figure 2:

Diet composition of high-fat diet used in all experimental protocols that compose this thesis (taken from: http://www.bio-serv.com/pdf/F3282_S3282.pdf).

Bio-Serv
Delivering Solutions...
Nutritional • Enrichment • Medicated • Special Needs

Nutritional Profile

Product# F3282 - Mouse Diet, High Fat, Fat Calories (60%), 1/2" Soft Pellets, 5 kg/Box
Product# S3282 - Mouse Diet, High Fat, Fat Calories (60%), 1/2" Soft Pellets, 5 kg/Box - Sterile

Proximate Profile

Protein	%	20.5
Fat	%	36.0
Fiber	%	0.0
Ash	%	3.5
Moisture	%	<10
Carbohydrate	%	35.7

Caloric Profile

Protein	kcal/gm	0.82
Fat	kcal/gm	3.24
Carbohydrate	kcal/gm	1.43
Total	**kcal/gm**	5.49

Amino Acids

Alanine	gm/kg	5.3
Arginine	gm/kg	7.3
Aspartic Acid	gm/kg	12.8
Cystine	gm/kg	0.6
Glutamic Acid	gm/kg	40.6
Glycine	gm/kg	4.9
Histidine	gm/kg	5.5
Isoleucine	gm/kg	11.0
Leucine	gm/kg	16.6
Lysine	gm/kg	14.8
Methionine	gm/kg	7.1
Phenylalanine	gm/kg	8.9
Proline	gm/kg	20.5
Serine	gm/kg	11.4
Threonine	gm/kg	8.7
Tryptophan	gm/kg	2.2
Tyrosine	gm/kg	11.4
Valine	gm/kg	13.0

Carbohydrates

Monosaccharides	gm/kg	1.3
Disaccharides	gm/kg	146
Polysaccharides	gm/kg	200

Fatty Acids

C18:2 Linoleic	gm/kg	36.6
C18:3 Linolenic	gm/kg	3.6
Total Saturated	gm/kg	141
Total Monounsaturated	gm/kg	162
Total Polyunsaturated	gm/kg	40.2

Minerals

Calcium	gm/kg	5.6
Chloride	gm/kg	0.86
Copper	mg/kg	3.6
Chromium	mg/kg	0.41
Fluoride	mg/kg	11.0
Iodine	mg/kg	0.31
Iron	mg/kg	40.8
Magnesium	gm/kg	0.49
Manganese	mg/kg	46.7
Phosphorus	gm/kg	5.8
Potassium	gm/kg	5.6
Selenium	mg/kg	0.21
Sodium	mg/kg	571
Sulfur	mg/kg	668
Zinc	mg/kg	21.6

Vitamins

Choline	mg/kg	1148
Folic Acid	mg/kg	0.75
Niacin	mg/kg	15.0
Pantothenic Acid	mg/kg	5.5
Pyridoxine	mg/kg	4.1
Riboflavin	mg/kg	2.3
Thiamin	mg/kg	3.0
Vitamin A	IU/kg	3162
Vitamin B_{12}	mcg/kg	40
Vitamin D_2	IU/kg	1000
Vitamin E	IU/kg	25.7
Vitamin K_3 (Menadione)	mg/kg	0.52

Ingredients

Lard, Casein, Maltodextrin, Sucrose, Mineral Mix, Vitamin Mix, DL-Methionine, Choline Chloride

These are typical amounts of nutrients calculated from available information. Actual assay results may vary.
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Figure 3:

Example of the calculation used for plant extracts or active principle dosing. It is based on assessment of average food consumption and body weight.

If for example we are administering 125 mg/kg of body weight of either balsam poplar or tamarack to mice weighing on average 33 g:

$$125\ mg\ of\ plant\ extract \rightarrow 1000\ g\ (1kg)\ of\ body\ weight$$
$$x\ mg\ of\ plant\ extract \rightarrow 33\ g\ of\ body\ weight$$
$$x = 4.125\ mg$$

In other words, 4.125 mg of plant extract needs to be ingested by the mice in order for them to be receiving 125 mg/kg. Since the plant extracts or the active principle are incorporated into the high-fat diet consumed by the mice, this implies that 4.125 mg of plant extract needs to be incorporated into 2.6 g of average daily food consumed by each mouse. Therefore, if we prepare around 230 g of food (enough for 1 week of administration to a group of 12 mice):

$$4.125\ mg\ of\ plant\ extract \rightarrow 2.6\ g\ of\ average\ daily\ food\ consumption$$
$$x\ mg\ of\ plant\ extract \rightarrow 230\ g\ of\ weekly\ food\ consumption\ for\ a\ group\ of\ 12\ mice$$
$$x = 365\ mg\ of\ plant\ extract\ to\ incorporate\ in\ 230\ g\ of\ high\ fat\ diet$$

It is to note that the incorporation of the plant extract or the active principle into the high fat diet did not alter its texture. In addition, although the preparation was made for 1 week of administration, it was maintained in the refrigerator (4°C) in individual containers for each dose and each treatment. The control high-fat diet was also kept in the refrigerator (4°C). Each one of these diets was periodically administered to the animals throughout the week. Body weight, food and water intake, as well as glycemia were measured every second day. This close monitoring of the animals allowed for proper assessment of dose administration, based on variations in body weight, and if any on food intake as well.