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Downregulation of adiponectin expression by IL-6
&
Functional analyses of novel genetic variants of DGAT1 and DGAT2 in humans

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by

Natalie Cadotte

A thesis submitted to the School of Graduate Studies in
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Abstract

Downregulation of adiponectin expression by IL-6
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Functional analyses of novel genetic variants of DGAT1 and DGAT2 in humans

by Natalie Cadotte

Obesity is characterized by two major events: systemic low-grade inflammation and energy metabolism imbalance. Adiponectin, an adipocyte-derived protein, has insulin-sensitizing properties in muscle and liver. Its expression and plasma concentrations are decreased in obesity. Our studies examined the role of interleukin-6 (IL-6), an important obesity-related cytokine, on adiponectin gene expression. IL-6 decreased adiponectin expression in a time- and dose-dependent manner in both human primary adipocytes and 3T3-L1 cells through a p44/42 MAP kinase-dependent pathway. *In vivo* studies demonstrated that IL-6 KO mice have increased plasma adiponectin levels on both a chow and a high fat/high glucose diet. Acyl-CoA: diacylglycerol acyltransferase (DGAT) is a key enzyme in triglyceride biosynthesis. Although many studies have demonstrated the role of DGAT in rodents, very little is known about its role in humans. Our studies identified a novel stop codon in the DGAT2 gene, which results in decreased DGAT2 mRNA expression in adipocytes and is associated with decreased triglyceride levels.

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List of Abbreviations

ACAT:	acyl CoA: cholesterol acyl transferase
AMPK:	AMP-activated protein kinase
ANOVA:	analysis of variance
BMI:	body mass index
bp:	base pairs
BSA:	bovine serum albumin
CoA:	coenzyme A
CREB:	cAMP response element binding protein
DAG:	diacylglycerol
DGAT:	acyl CoA: diacylglycerol acyl transferase
DMEM:	Dulbecco's modified Eagle's medium
DNA:	deoxyribonucleic acid
DTT:	dithiothreitol
EDTA:	ethylenediamine tetraacetate
ELISA:	enzyme-linked immunosorbent assay
ER:	endoplasmic reticulum
FA:	fatty acid
FBS:	fetal bovine serum
GAPDH:	glyceraldehyde-3-phosphate dehydrogenase
GLUT4:	glucose transporter 4
HDL:	high-density lipoprotein
HDL-C:	HDL-cholesterol
HES-1:	Hairy enhancer of split –1
HF/HG:	high fat/high glucose
IBMX:	isobutylmethylxanthine
IL-6:	Interleukin-6
IRS:	insulin receptor substrate
kb:	kilo base pairs
KO:	knock out
LDL:	low-density lipoprotein
LDL-C:	LDL-cholesterol
MGAT:	acyl CoA: monoacylglycerol acyl transferase
MMLV-RT:	Moloney Murine Leukemia Virus – reverse transcriptase
NMD:	nonsense-mediated decay
PBS:	phosphate buffer saline
PCR:	polymerase chain reaction
PKA:	protein kinase A
PPAR:	peroxisome proliferator-activated receptor
PPRE:	PPAR response element
RT:	reverse transcription
SIFT:	Sorting Intolerant From Tolerant
SNP:	single nucleotide polymorphism
T2DM:	type 2 diabetes mellitus
TET:	tetra-ethylene glycol

TG:	triglycerides
TNFα:	tumor necrosis factor α
TZD:	thiazolidinediones
UCP:	uncoupling protein
WAT:	white adipose tissue
WT:	wild type

Chapter 1 – Introduction

1.1 – The obesity epidemic

Obesity has become a major health problem globally, since over 300 million people in the world are obese. Furthermore, it is anticipated that this figure will double in the next twenty years [1]. This disease is predominant but not isolated to Western countries where over thirteen percent of adults in Canada and twenty-six percent of adults in the United-States are obese [2]. This disease not only affects adults but has become quite prominent among children as well. Over the course of 15 years, there has been an increase in obesity of 50% in both boys and girls in Canada [3]; at present over thirteen percent of boys and twelve percent of girls aged 7 to 13 are obese [4]. The biggest concern related to obesity is that it is associated with numerous co-morbidities such as insulin resistance [5], type 2 diabetes mellitus (T2DM) [6;7], hypertension [8;9], dyslipidemia [10], and coronary artery disease [11;12]. Therefore, it is not surprising that the economic and social burden associated with this disease is enormous. It is estimated that, in Canada for the year 1997, the total direct cost of obesity was over $1.8 billion, which represents 2.4% of the total direct medical costs for the year [2]. In addition, obesity does not only affect the direct medical cost but other economic factors. For example, between 1988 and 1994 in the United States, there was an increase in the number of physician office visits resulting from obesity [13]. Also, obese and overweight individuals incur annual medical expenditures that are 36% higher than normal-weight individuals [14]. Apart from the direct and indirect medical costs, there are also non-medical expenditures, such as work absenteeism, the cost of which is estimated at $2.95 billion dollars for the year 2003 in the United States [15]. Therefore, research pertaining to this disease is critical from a medical and socio-economic point of view.

1.2 – The definition of obesity

Obesity is defined as increased mass of adipose tissue, and is associated with an increase risk of cardiovascular and metabolic disorders such as T2DM, hyperlipidemia, insulin resistance and coronary heart disease [16]. The clinical definition of obesity is determined with the body mass index (BMI), which considers the ratio of height and weight of individuals. A BMI higher than 25kg/m^2 is considered overweight and a BMI over 30kg/m^2 is considered obese. This measurement is widely accepted although recently debated and applies to the majority of the population. It does not take into account muscle mass and therefore, gives skewed results for any individual who has a higher muscle mass than the average population. To date, it is still the best way to diagnose obesity [17].

1.3 – The two sides of obesity

1.3.1 – Obesity as an energy imbalance

The heightened prevalence of obesity reflects mainly changes in our society and in our behavior. Although genetics are known to play a determining role in susceptibility to weight gain, a large influence is the overabundance of high energy/nutrient poor foods with high levels of sugar and saturated fat present in our diet. This leads to the common view of obesity: an energy imbalance where energy input exceeds energy output. The excess calories resulting from this imbalance are converted to triglycerides (TG) and stored as lipid droplets in adipose tissue or further stored in other tissues such as liver or muscle. The normal function of adipose tissue is to store free fatty acids (FA) as TG and to release them as needed [18] as most cells are unable to store lipids safely. However, in obesity, adipocytes become enlarged due to an overload of lipids leading to subsequent excess lipid accumulation in other cell types and eventually to impaired body homeostasis.

1.3.2 – Obesity as a proinflammatory disease

Obesity has always been seen as an energy imbalance where energy input exceeds energy output, which results in excessive body fat. This excess body fat disrupts whole body homeostasis and increases the individual's risk of developing numerous related diseases [17]. The other side of obesity that has recently come to light is that it is characterized by chronic inflammation localized specifically in adipose tissue [19]. As a result, obesity is now considered a proinflammatory condition associated with chronic low-grade inflammation [20] as well as an energy imbalance. Studies have shown that following weight gain, there is an increase in inflammatory cytokine production in adipose tissue leading to the activation of inflammatory signalling pathways [19]. This increase in inflammation leads to the recruitment of macrophages, which infiltrate the adipose tissue [19;21;22]. The critical aspect of this increased inflammation is its association with numerous co-morbidities. There is much evidence to suggest that inflammation plays a critical role in the development of insulin resistance, T2DM and therefore, the metabolic syndrome [23;24 19;25-27].

The link between obesity and inflammation was discovered over a decade ago. Tumor necrosis factor α (TNFα) was the first identified inflammatory cytokine, which was overexpressed in adipose tissue of rodent models of obesity [28;29]. Similarly, TNFα is overproduced in adipose tissue and muscle of obese individuals [30-32]. Furthermore, administration of recombinant TNFα to cultured cells or to animals impairs insulin action and conversely, mice lacking functional TNFα or TNF receptors have improved insulin sensitivity compared to wild type mice (WT) [28;33]. Thus, it is clear that overproduction of TNFα in adipose tissue is an important feature of obesity and contributes to insulin resistance.

Later studies demonstrated that obesity was characterized by a broad inflammation response and that many inflammatory cytokines, particularly interleukin-6 (IL-6), mimicked the

expression and effect of TNFα in obesity [34]. The increase in inflammation is predominantly in adipose tissue where gene expression analysis of adipose tissue has shown an increase in inflammatory markers in obese individuals [19;22;35]. The source of inflammation was determined to be the stromal vascular fraction and not mature adipocytes [19]. Macrophages have been shown to progressively infiltrate the adipose tissue as obesity develops [19;22] and some studies have localized these macrophages to necrotic adipocytes [36]. Taken together, it is clear that inflammation plays an important role in obesity and in the etiology of obesity-related diseases.

Although obesity has two distinct characteristics, increased adiposity and inflammation cannot be seen as independent events linked to obesity, but as integral partners in the development of this disease. Therefore, both effects must be studied to fully understand the impact of obesity on metabolism.

1.4 – Adipose tissue at the crossroads of obesity and inflammation

Adipose tissue has emerged as an important endocrine organ implicated in energy homeostasis and inflammation. Adipocytes are the energy storage cells of the body where excess energy is stored in the form of lipids. They are very dynamic and have an unlimited capacity for growth [37]. Furthermore, adipose tissue is responsive to both central and peripheral metabolic signals and is responsible for secreting a number of proteins named adipocytokines. These adipocytokines have been implicated in local, peripheral and central effects on energy homeostasis and inflammation [37]. Thus, adipose tissue can integrate signals from other organs and respond by regulating the secretion of multiple proteins. However, when adipose tissue is deregulated, it can negatively affect other systems. This is particularly evident when adipocytes become enlarged such that the cellular homeostasis and the secretory profile of these enlarged adipocytes become altered [38]. The other effect of enlarged adipocytes due to

increased fat intake is the subsequent lipid accumulation in non-adipocyte cells such as hepatocytes and muscle cells. This phenomenon usually referred to as lipotoxicity leads to altered cellular function and impaired insulin signalling [39]. The systemic effects of decreased insulin sensitivity associated with obesity may reflect the lipotoxic effects of fatty acids as well as the altered adipocytokine secretion profile. Interestingly, these two characteristics due to enlarged adipocytes are highly interconnected such that adipocytokines have the ability to reduce fatty acid accumulation in non-adipose tissue, which leads to increased insulin sensitivity. Independent of this effect, adipocytokines also modulate insulin sensitivity through direct signalling [5;40]. Therefore, lipotoxicity and altered adipocytokine secretion should not be viewed as independent but rather as an interconnected process where each is capable of mutually influencing or causing the other [37].

1.4.1 – Immune features of adipocytes

Since the survival of multicellular organisms depends on both the ability to store energy for low nutrient availability and the ability to fight infection, it is not surprising that metabolic and immune systems have evolved to be interdependent and to an extent, interchangeable. Many cytokines, hormones, signalling molecules and transcription factors can function in both metabolic and immune roles [34]. Normal inflammatory responses require energy redistribution and mobilization of stored lipids to adequately fight off infections [41]. The integration of metabolism and immunity, which is beneficial for maintaining good health under normal conditions, has become deleterious under situations of metabolic imbalance such as obesity [34]. A good example of the interchangeability of these two pathways is adipocytes and macrophages. Studies have highlighted the overlapping biology and function of macrophages and adipocytes in obesity. They have similar gene expression where macrophages express many "adipocyte" gene products such as fatty acid-binding protein (FABP) aP2 and peroxisome

proliferator-activated receptor (PPAR) γ, while adipocytes can express many "macrophage" proteins such as TNFα, IL-6 and matrix metalloproteinase (MMPs) [28;42-45]. Further evidence of interdependence of these two cell types is that macrophages can also take up and store lipids to become atherosclerotic foam cells. Also, preadipocytes under some conditions can exhibit phagocytic and antimicrobial properties and can even differentiate into macrophages, which suggests a potential immune role for preadipocytes [46;47]. Furthermore, the fact that macrophages infiltrate adipose tissue in obesity indicates a further dependence and interconnectivity between these cell types [19;22]. The functional and molecular overlap between fat cells and macrophages may, in part, explain the pronounced inflammation in adipose tissue of obese individuals where both macrophages and adipocytes propagate the inflammation response.

1.5 – The role of adipocytokines in metabolism

As mentioned above, adipose tissue is an important endocrine organ, which secretes numerous proteins involved in energy metabolism and insulin signalling. Although many have been identified, there is still very little known about many of them. However, some progress has been made in recent years. These bioactive molecules have been implicated in a variety of biological and physiological processes, including food intake, regulation of energy balance, insulin action, lipid and glucose metabolism, angiogenesis and vascular remodeling, regulation of blood pressure and coagulation.

1.5.1 – Adipocytokines and vascular homeostasis

Angiotensinogen (AGE) has been implicated in the development of hypertension. Although the liver is the main source, AGE, the precursor of the vasoactive peptide angiotensin II, is also secreted by adipose tissue. This observation explains the

increased circulating levels in obese individuals [48]. Overexpression of AGE in adipose tissue is associated with hypertension in mice [49]. In addition, increased AGE production can also contribute to enhance adipose tissue mass, an effect attributed to angiotensin II, which promotes new adipose cell formation [50]. Therefore, increased levels of AGE by adipose tissue of obese individuals may explain the increased risk of hypertension and associated cardiovascular diseases.

Plasma plasminogen activator inhibitor 1 (PAI 1) is the primary physiological inhibitor of fibrinolysis. The impairment of this system has been linked to the development of cardiovascular complications associated with obesity [48]. Adipose tissue, mainly visceral depots, is the main source of PAI 1 in obesity [51;52] and is responsible for the impaired fibrinolysis. In contrast, PAI 1 can also reduce preadipocyte migration [53] and can attenuate adipose tissue hypertrophy in diet-induced obesity [54], although there is some evidence to the contrary [55;56]. Taken together, this suggests that obesity-induced increased secretion of PAI 1 by adipose tissue is related to impaired fibrinolysis and cardiovascular complications but is also related to protection against diet-induced adipose tissue growth.

1.5.2 – Adipocytokines and insulin resistance

There are many adipocytokines that can negatively affect insulin sensitivity. TNFα is the most commonly known and has been discussed previously. Other adipocytokines include acylation-stimulating protein (ASP) and IL-6; the latter will be discussed in another chapter. ASP appears to be inactive as an immune modulator, unlike the other ones listed, but mostly is involved in stimulating TG storage in adipocytes. It mediates this effect by stimulating glucose uptake, enhancing fatty acid reesterification and inhibiting lipolysis [57;58]. It is still unknown if obesity leads to increased levels of ASP and whether increased levels represent increased activity or resistance to ASP [48]. The latter could promote the redirection of the fatty acid flux away

from adipose tissue, towards the liver [59]. Interestingly, ASP-deficient mice show decreased adipose tissue mass and increased insulin sensitivity, which could be a direct effect of ASP-deficiency or due to the relative leanness of these mice [59;60]. A full understanding of ASP in obesity is still unclear.

1.5.3 – Adipocytokines and insulin sensitivity

Two major adipocytokines have emerged as important factors for insulin sensitivity. They include adiponectin, which will be discussed in the next chapter, and leptin. Leptin was discovered over a decade ago due to the naturally occurring mutation in mice (ob mutation), which eventually led to the discovery of the leptin gene [61]. Leptin was later determined to be involved in long-term regulation of food intake, body weight, energy expenditure and neuroendocrine functions [62]. The ob mutation, which is associated with severe obesity, demonstrated the importance of this protein for body weight homeostasis and regulation of food intake [61]. Nevertheless, obese individuals display high levels of leptin without the beneficial effects [63], which lead to the proposed model of leptin resistance in obese individuals [64]. This was further supported by the fact that only a subset of individuals displayed weight loss following administration of very high doses of leptin [65]. Apart from its role on body weight homeostasis, leptin is also important for insulin sensitivity through different mechanisms such as increased fatty acid oxidation and reduction of ectopic fat accumulation in non-adipose tissues [66;67]. Overall, leptin serves as a satiety signal known to reduce food intake, increase energy expenditure and improve insulin sensitivity through peripheral effects.

Therefore, adipose tissue plays a central role in the development of obesity and associated co-morbidities. Adipocytes are involved in the regulation of lipid metabolism, which becomes deregulated in the obese state leading to over accumulation of TG in both adipose and non-adipose tissue. This promotes the development of insulin resistance, which is further

affected by altered adipocytokine production including numerous inflammatory cytokines. The overall effect is to create a vicious cycle of inflammation, lipids and adipocytokines all converging to promote insulin resistance.

Chapter 2 – Downregulation of adiponectin expression by IL-6

2.1 – Background

2.1.1 – Adiponectin gene structure

Adiponectin is a secreted protein that has been recently identified in adipose tissue. It was independently discovered by four groups using different methods. Adiponectin cDNA was isolated from the human adipose tissue library and named apM1 (Adipose Most Abundant gene transcript) [68]. Another group cloned the mouse adiponectin cDNA and called it Acrp30 (adipocyte complement-related protein of 30kDa). It was discovered because of its upregulation during differentiation of preadipocytes to adipocytes [69]. Another group discovered the same protein in mice and rats by using mRNA differential display techniques and named it adipoQ [70]. The last group identified the human adiponectin protein in plasma by screening for proteins with affinity for gelatin-cellulofine and therefore termed it gelatin-binding protein of 28kDa (GDP28) [71]. The human apM1 gene is located on chromosome 3 at position 3q27 [72;73]. Interestingly, genome wide scans have recently mapped a susceptibility locus for adiposity and type 2 diabetes to this same region [74;75]. The gene spans 16kb and has 3 exons (size ranging from 18bp to 4.3kb) and 2 introns (one of 0.8kb and the other of 12kb). The exon-intron junctions have classical donor and acceptor splice sites [72]. In humans there is a single 4.5kb transcript with a large 3'-untranslated region. Several transcripts are obtained from the mouse gene due to alternative polyadenylation sequences in the 3'-untranslated region, although all still retain the 5'-untranslated region and coding sequence [76].

2.1.2 – Adiponectin protein

The human adiponectin protein contains 244 amino acids. It is divided into 4 regions: a signal peptide of 20 amino acids, a N-terminal region without any homology to any known

proteins, a collagen-like region, and a C-terminal globular domain (**Figure 2.1**) [70;71]. The collagen-like region contains 22 collagen repeats (Gly-X-Y or Gly-X-Pro repeats), which are thought to mediate the formation of homotrimers [70;77]. The collagen-like region also contains conserved cysteine residues important for the formation of disulfide bonds between monomers [70;77]. These homotrimers can further combine to form hexamers and high molecular weight (HMW) forms of twelve to eighteen subunits (**Figure 2.2**) [70;71;78]. There is some evidence suggesting that different oligomers mediate different biological activities where HMW forms and hexamers can activate NF-KB but trimers cannot. Conversely, trimers can activate and increase phosphorylation of AMP-activated protein kinase a (AMPKa) while higher molecular weight forms do not mediate this effect [79].

The adiponectin protein shares sequence homology with type VIII and type X collagens, complement component Cq1, precerebellin and the hibernation-regulated proteins hib 20, 25 and 27 [70]. Interestingly, the 3D-structure of the C-terminal domain of adiponectin closely resembles TNFα cytokine even though there is no primary sequence homology [77]. The adiponectin protein undergoes several post-translational modifications and can be modified into 8 different isoforms [80]. Six of these isoforms are O-linked glycosylated at four lysine residues in the collagen-like domain [80;81]. Of note, the efficacy of adiponectin is greatly reduced if any of these lysine residues are substituted with arginine, which indicates that glycosylation is important for the bioactivity of adiponectin [80;82].

Adiponectin is mostly found in two different forms: full-length adiponectin (30kDa) and globular adiponectin (27kDa), which represents 70% of the full-length protein without the non-homologous region and part of the collagen-like domain [83]. Globular adiponectin is most likely generated by proteolytic cleavage by a yet unidentified protease. The globular form has been

Figure 2.1. The domain structure and structural features of adiponectin protein.

Adiponectin, also known as Acrp30, AdipoQ, apM1 and GBP28, was originally identified independently by four groups using different approaches. Adiponectin is composed of an N-terminal collagen-like sequence and a C-terminal globular region. The collagen-like region contains the putative sites for post-translational modifications, which include disulfide bonds and glycosylation, and is responsible for trimer formation. The globular domain is structurally similar to TNFα. A small amount of globular adiponectin is found in human plasma.

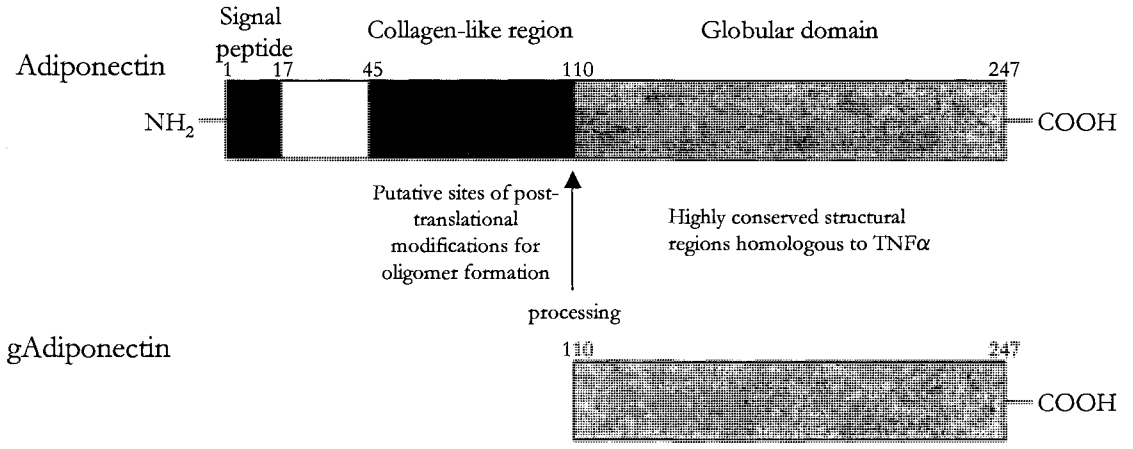
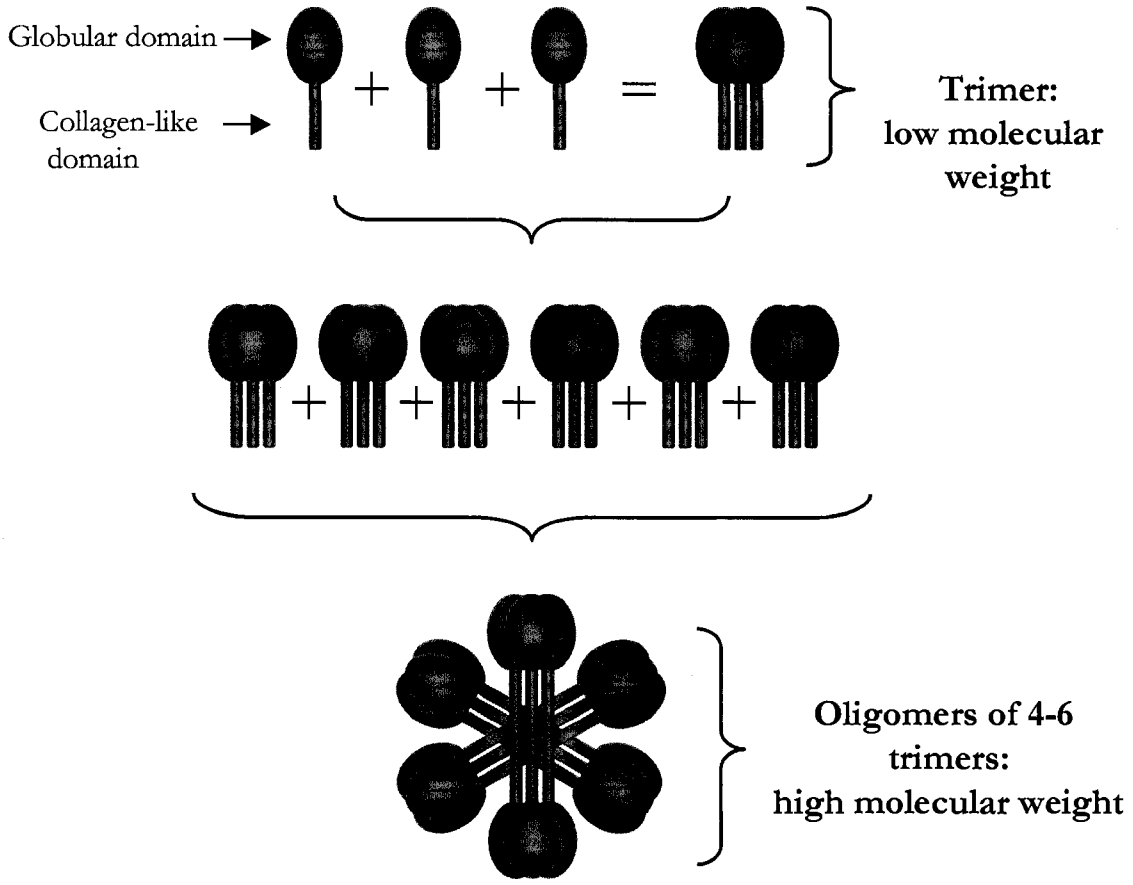

Signal peptide
Collagen-like region
Globular domain
Adiponectin
1
17
45
110
247
NH2
COOH
Putative sites of post-translational modifications for oligomer formation
Highly conserved structural regions homologous to TNFα
processing
gAdiponectin
110
247
COOH

Figure 2.2. Model for the assembly of adiponectin complexes.

Three monomers form a trimer (low molecular weight form) through associations between their globular domains. Four to six trimers associate noncovalently through interactions within the collagen-like domain to form high molecular weight entities. Both forms are thought to mediate different biological activities.


Globular domain
Collagen-like domain
Trimer: low molecular weight
Oligomers of 4-6 trimers: high molecular weight

shown to have more biological activity than the native form in some studies [83]. The difference in activity probably stems from the fact that they mediate different responses in different tissues.

2.1.3 – The function of adiponectin

Adiponectin is one of many adipocytokines, which are active molecules produced and secreted by adipose tissue [84]; they include leptin, TNFα, IL-6 and many others. Adiponectin is considered an important hormone in the regulation of insulin action and peripheral tissue lipid metabolism [16;85]. It is expressed mostly in adipose tissue where subcutaneous adipose tissue has higher expression than visceral adipose tissue [86]. It is also expressed, albeit at much lower levels, in cardiomyocytes [87], locally in the coronary circulation [88] and in osteoblasts [89-91]. Although adiponectin is expressed in other cell types, adipose tissue remains the most important physiological source. In addition, adiponectin is abundant in plasma, with concentrations ranging from 5 to 30μg/mL, thus accounting for 0.01% of total plasma proteins [92].

The major function of adiponectin is to increase insulin sensitivity and regulate energy metabolism. Firstly, it promotes fatty acid oxidation in skeletal muscle [5;83;93] and secondly, decreases hepatic glucose production [94;95] without any effect on peripheral glucose uptake [95]. Interestingly, in skeletal muscle, adiponectin increases the expression of molecules involved in fatty acid metabolism and in energy dissipation. The overall effect is to decrease tissue TG content in skeletal muscle and liver and thus coordinately increase insulin sensitivity *in vivo* [5]. Increased tissue TG content has been reported to interfere with insulin-stimulated glucose transporter 4 (GLUT4) translocation and glucose uptake resulting in insulin resistance [96]. Adiponectin modulates these insulin-sensitizing effects by activating AMP kinase (AMPK) in skeletal muscle and liver [93] and by activating peroxisome proliferator-activated receptors (PPAR) α [5] leading to fatty acid combustion and energy consumption. Interestingly, it was later

demonstrated that globular adiponectin preferentially activates AMPK in skeletal muscles [97] whereas full-length adiponectin was shown to preferentially activate AMPK in the liver [94].

Further evidence of the role of adiponectin as an insulin sensitizer is demonstrated in animal studies. Both C57Bl6 and ob/ob mice show reduced hepatic glucose uptake when treated with recombinant full-length adiponectin [94;95]. In addition, administration of globular adiponectin reduced plasma glucose and increased fatty acid oxidation in muscle [5;83;93]. Furthermore, adiponectin knock out (KO) mice showed impaired insulin sensitivity [98;99], although one study did not show any differences between KO and wild-type mice [100]. Overall adiponectin is known to increase insulin sensitivity by decreasing tissue TG content, increasing β-oxidation and decreasing gluconeogenesis in both skeletal muscle and liver [101].

2.1.4 – Adiponectin, obesity and the metabolic syndrome

In contrast to other adipocytokines such as TNFα and IL-6, which increase with obesity, adiponectin expression and secretion is reduced in diabetic and obese patients and animal models [70;102;103]. Plasma adiponectin levels negatively correlate with various indices of adiposity and metabolic syndrome such as BMI [104-108], waist circumference [109], waist-to-hip ratio [105;110;111], intra-abdominal fat [112], percentage of body fat [111;113;114], fasting plasma glucose [104;105], triglycerides [104;105], uric acid [104;105], serum insulin [104;105] and hypertension [104;115]. Conversely, weight reduction in obese patients is associated with an increase in adiponectin concentrations [116;117]. A prospective study in rhesus monkeys showed that the decrease in plasma adiponectin levels paralleled the development of insulin resistance and diabetes [118]. These observations suggest that low plasma adiponectin may contribute to diabetes mellitus in animals. In humans, it has been observed that the greater the hypoadiponectinemia, the more severe the insulin resistance and hyperinsulinemia [113]. However, there seems to be no correlation between adiponectin levels and the development of obesity [119]. The decrease observed follows the onset of obesity and

precedes the development of insulin resistance and T2DM [120] and patients with higher adiponectin concentrations are less likely to develop T2DM than those with low concentrations [121]. Obesity-induced decreases in adiponectin expression and plasma concentrations can be reversed by weight loss, either through exercise [122-126], caloric restriction or gastric partition surgery [117]. This strongly suggests that decreased adiponectin is a consequence of obesity rather than the cause of it, and that obesity leads to reduced adiponectin, which results in the progression of hyperinsulinemia, impaired glucose tolerance and eventually T2DM. Interestingly, progression of the metabolic syndrome, which includes abdominal obesity, insulin resistance, dyslipidemia and hypertension, has been linked to polymorphisms at the genomic locus of adiponectin [74]. This suggests that an alteration in adiponectin expression or secretion is causative to the development of metabolic syndrome.

2.1.5 – The role of adiponectin in cardiovascular disease

Adiponectin is also considered an anti-inflammatory adipocytokine and is thought to be protective against atherosclerosis and coronary artery disease [127;128]. Circulating levels of adiponectin are reduced in T2DM and obese individuals, and are further decreased in patients with cardiovascular disease [92;116]. Interestingly, an adiponectin haplotype (I164T) has been associated with decreased serum adiponectin levels and increased risk of developing the metabolic syndrome and cardiovascular disease [129]. Other adiponectin haplotypes have been associated with coronary heart disease and diabetes [130]. A longitudinal study demonstrated that higher levels of adiponectin were associated with decreased risk of myocardial infraction suggesting a protective effect of elevated adiponectin levels [131]. Adiponectin is thought to affect, either directly or indirectly, the vascular endothelium, which is implicated in the development of cardiovascular disease [132]. Furthermore, low levels of adiponectin are associated with endothelial dysfunction [133]. Studies suggest that adiponectin modulates the endothelial cell inflammatory

response via cAMP-protein kinase A (PKA) and NF-KB pathways [134]. Adiponectin also stimulates nitric oxide production in aortic endothelial cells, which leads to vasodilatation, increased blood flow and increased glucose disposal [135]. There is also evidence that adiponectin plays an important role in maintaining the integrity of the vessel wall [127]. Animal studies reinforce a role for adiponectin in atherosclerosis. Adiponectin KO mice display increased neointimal thickening and increased proliferation of vascular smooth muscle cells in mechanically injured arteries [98;136]. Adenovirus-mediated expression of adiponectin in these mice attenuated neointimal proliferation. Also, overexpression of globular adiponectin in the apoE-null background (atherosclerosis model) resulted in decreased atherosclerotic plaque formation [16;128;137]. Adiponectin has been localized to the injured areas of the vessel wall [128;138] where it acts to directly suppress macrophage-derived foam cell formation [139]. In short, adiponectin is involved in the regulation of the cardiovascular endothelium, which in turn affects the progression of cardiovascular disease [132]. It is now recognized as a biomarker for cardiovascular disease and a potential therapeutic target.

2.1.6 – Modulators of adiponectin gene expression and secretion

In addition to metabolic conditions, several specific drugs, proteins and interventions have been reported to modulate adiponectin gene expression and secretion (**Table 2.1**). Very little information is available on the molecular mechanisms of any of the effects listed in the table. Of particular interest on the list are PPAR agonists and β-adernergic agonists.

PPARs, nuclear hormone receptors, are involved in the regulation of adiponectin synthesis. Thiazolidinediones (TZD), which are PPAR agonists, enhance adiponectin expression in obese mice and insulin resistant humans [140]; the same effect was also observed *in vitro* in cultured 3T3-L1 adipocytes [5]. It has been hypothesized that the effects of TZD, used

Treatment	*in vivo/ in vitro*	mRNA	protein	Reference
Angiotensin II	*in vitro*	N.D.	↑	141
Atorvastatin	*in vivo*	N.D.	no effect	142
Berbedine	*in vitro*	↑	N.D.	143
Bezafibrate	*in vivo*	↑	↑	144
CB(1) receptor antagonist	both	↑	↑	145
Dexamethasone	*in vitro*	↓	N.D.	146
Fenofibrate	*in vivo*	N.D.	↑	147
Free fatty acids	*in vivo*	N.D.	↓	148
Ghrelin	*in vitro*	↓	N.D.	149
Glimeripide	*in vivo*	N.D.	↑	150
Glucocorticoids	both	↓	↓	146
Growth hormone	both	↑	↑/ no effect	151, 152
HIV protease inhibitor	both	↓	↓	153
Insulin	both	↓	↓	154
Interleukin-15	*in vitro*	N.D.	↑	155
Ionomycin	*in vivo*	↑	↑	156
Leptin	both	no effect/ ↑	no effect/ ↑	157,158
Linoleic acid	*in vivo*	↑	↑	159
Olanzapine	*in vivo*	N.D.	↑	160
Retinoic acid	*in vitro*	↓	N.D.	161
Rilmenidine	*in vivo*	N.D.	↑	162
Risperidone	*in vivo*	N.D.	↑	160
Sarpogrelate	*in vivo*	N.D.	↑	163
Sibutramine	*in vivo*	N.D.	↑/ no effect	164, 165
Testosterone	both	↓	↓	166
Topiramate	*in vitro*	↓	N.D.	167
Valproic acid	both	↓	↓	168

Table 2.1 Pharmacologic and physiologic interventions shown to modulate adiponectin gene expression and secretion. The effect of the treatments on adiponectin expression was determined by mRNA quantification and plasma/serum adiponectin levels were determined by ELISA or RIA. For *in vitro* experiments, the effect was determined in either human or mouse cell lines and the *in vivo* experiments were carried out either in rodents or in human subjects. N.D. – not determined.

therapeutically to treat insulin resistance in patients with T2DM, are mediated in two ways: first by directly activating the adiponectin promoter and second by antagonizing the downregulation mediated by TNFα [140]. Since adiponectin naturally leads to improved insulin sensitivity, the effects of TZD might be explained, in part, by the increase in adiponectin secretion [169]. TNFα, produced by white adipose tissue, is upregulated in obesity and contributes to insulin resistance by interfering with insulin receptor signalling [170]. This cytokine also suppresses adiponectin expression in white adipose tissue [156;170]. Therefore, TZD could also increase adiponectin levels by blocking the inhibitory effect of TNFα [140].

Other modulators of adiponectin expression are β_3-adrenergic agonists. Adipose tissue specifically expresses β_3-adrenergic receptors, which are involved in lipolysis through the activation of cAMP-PKA pathway. β-adrenergic agonists decrease adiponectin expression in 3T3-L1 adipocytes and the addition of an antagonist of the same receptor blocks this effect [156;171]. Interestingly, administration of the specific β_3-adrenergic agonist BRL35153 did not effect adiponectin expression in db/db mice [172]. Conversely, Delporte *et al* demonstrated that treatment with another agonist, BRL37344, decreased adiponectin expression and secretion *in vivo* and *in vitro* [173]. The difference in the agonists used may explain the discordance between these studies. Also, it is possible that the direct suppression of adiponectin by BRL35135 could be counteracted by the indirect stimulation of adiponectin production by an improved insulin sensitivity resulting in body weight reduction observed in BRL35135-treated animals [169]. Of note, weight loss has been shown to increase adiponectin levels in both mice and humans [117].

2.1.7 – Analysis of adiponectin gene expression

There have been a few published reports on the molecular architecture of the adiponectin promoter. The upstream 1.13kb fragment confers basic transcriptional activity of the human promoter [174]. It does not contain a classical TATA box but does have a CCAAT box

at –136 from the transcriptional start site [174]. It also contains numerous putative response elements of which a few have been investigated. The adiponectin promoter contains a PPAR-response element (PPRE) where PPARγ and retinoid X receptor (RXR) heterodimerize to bind this PPRE and increase promoter activity. Point mutations in the PPRE return transcription to basal levels and block TZD (PPARγ agonist) effect on adiponectin upregulation [175;176]. In conjunction, a liver receptor homologue-1 (LRH-1) response element (LRH-RE) was also identified and its activation leads to an increase in PPARγ-induced upregulation of adiponectin expression [175]. The CCAAT element and a nuclear factor-Y (NF-Y) element were also identified. CCAAT/enhancer binding protein (C/EBP) was established to be responsible for dietary responses of adiponectin expression [177;178] and important for adipocyte differentiation [179]. Another element identified in the promoter is an SP1 binding site where SP1 binding leads to increased adiponectin expression [180]. TNFα mediates the downregulation of adiponectin expression by SP1 through a decrease in SP1 binding to the adiponectin promoter [180]. To date, most studies have focused on the first 1kb of the promoter; more studies are needed to identify other response elements and their roles in the regulation of adiponectin expression.

2.1.8 – Variations in the adiponectin gene

Many genetic studies have examined single nucleotide polymorphisms (SNPs) in the adiponectin gene. Over twenty-five SNPs have been identified in various populations including European, Japanese and North Americans. Of these, only two SNPs were identified to have different frequencies in cases versus controls: +45G>T (risk allele +45G) and +276G>T (risk allele +276G) [169]. Both SNPs were associated with decreased circulating adiponectin levels, insulin sensitivity and T2DM in a Japanese population [181]. The impact of SNP276 on serum adiponectin was further studied and carriers of the TT genotype were found to have higher adiponectin levels [182-185] whereas the wild type G allele was associated with lower serum

adiponectin levels, insulin resistance, obesity and T2DM in various populations [110;181;186-190]. However, in other studies the T allele has been associated with lower serum adiponectin levels, decreased insulin sensitivity [191], adverse lipid profiles [192] and T2DM [193]. By combining both SNPs, a risk haplotype was created, which yielded much stronger associations. The haplotype was associated with higher body weight, waist circumference, blood pressure, high-density lipoprotein (HDL)/total cholesterol ratio, lower serum adiponectin levels and higher risk of T2DM [189]. The basis for this association remains unclear. The SNP276 is a synonymous mutation whereas the SNP45 is in intron 2 where no regulatory elements have been identified. It has been postulated that the most likely explanation would be that these two SNPs are in linkage disequilibrium with yet another functional SNP [169]. Although many other SNPs have been identified in the adiponectin promoter, more studies are needed to fully verify the association of various SNPs with metabolic conditions.

2.1.9 – The adiponectin receptors

To date, two receptors for adiponectin have been identified: AdipoR1 and AdipoR2. Both receptors appear to be integral membrane proteins where the N-terminal is cytosolic and the C-terminal is external [194]. In humans, both receptors are expressed in liver and in skeletal muscle [195;196], but AdipoR1 is more highly expressed in skeletal muscles and AdipoR2 in the liver. The receptors are also expressed in bone forming cells [89], pancreatic β cells [197], atherosclerotic lesions [198], macrophages [198] and the brain [199;200]. Interestingly, adiponectin does not cross the blood brain barrier [199] therefore the function of the receptors in the brain remains a mystery. AdipoR1 serves as a receptor for globular adiponectin and AdipoR2 serves as a receptor for full-length adiponectin [194]. Both receptors mediate the effect of adiponectin by increasing AMPK and PPARα ligand activity, leading to FA oxidation and glucose uptake [201]. AdipoR1 and AdipoR2 are regulated by various physiological and pathophysiological states [202]. For both

receptors, the mRNA expression in liver and skeletal muscle increases after fasting and returns to baseline after refeeding [101]. AdipoR1 and AdipoR2 expression are decreased in obesity, diabetes and non-alcoholic hepatosteatitis [203-205]. Variants in both receptors have been associated with body composition, insulin sensitivity, metabolic parameters [206;207], adiponectin levels, fasting TG levels [208] and T2DM [209]. Interestingly, sustained exercise increases AdipoR1 and AdipoR2 expression in muscle [207;210;211], which might in part explain the benefits of exercise on insulin sensitivity. Therefore, both receptors work in concert with adiponectin to promote fatty acid oxidation in muscle and decrease glucose production in the liver leading to overall improved insulin sensitivity.

2.1.10 – The role of interleukin-6 in metabolic diseases

The importance of IL-6 in obesity has been well established. IL-6, in conjunction with TNFα [212], is a major contributor to the low-grade systemic inflammation observed in obese individuals [213-215]. Furthermore, circulating levels of IL-6 have been correlated with insulin sensitivity [215;216] and the development of T2DM [215-217]. Chronic increase in systemic low-grade inflammation, characterized by increased levels of IL-6 and TNFα, can trigger insulin resistance in obese adults [24] and obese children [214]. Interestingly, weight loss, through life style modification or very low calorie diet, leads to decreased serum IL-6 levels [215;218;219]. The improved inflammation profile after weight loss is due to a decrease in macrophages within the adipose tissue [220]. IL-6 is most strongly linked to insulin resistance and glucose metabolism. Adipose tissue from insulin resistant patients secretes more IL-6 than that from normal subjects [221]. Also, subcutaneous administration of IL-6 leads to a dose-dependent increase in fasting blood glucose [222]. Interestingly, IL-6 mRNA is upregulated by insulin [223] suggesting a viscious cycle for systemic inflammation and insulin resistance. Furthermore, injection of IL-6 neutralizing antibodies in ob/ob mice leads to improved insulin sensitivity [224]. However, the

effect of IL-6 on insulin sensitivity seems to require a chronic upregulation of inflammation since acute administration of IL-6 does not lead to whole body insulin resistance [217]. Genetic studies of the IL-6 gene have shown that a SNP in the promoter is associated with obesity [225;226], insulin resistance [227] and T2DM [228;229].

Some studies in IL-$6^{-/-}$ mice have shed some light on the metabolic role of IL-6. One group found that IL-$6^{-/-}$ mice developed maturity onset obesity, with disturbed carbohydrate and lipid metabolism, and increased leptin levels [230]. This is surprising since, in humans, increased levels of IL-6 are associated with obesity and the development of insulin resistance. Therefore, one would have expected IL-$6^{-/-}$ mice to be more insulin sensitive than WT mice. Conversely, another study found that IL-$6^{-/-}$ mice did not gain weight with age and did not have altered glucose, insulin or triglyceride levels compared to WT mice [231]. When put on a high fat diet, these mice gained weight and developed insulin resistance comparably to WT mice on the same diet. The only difference observed in IL-$6^{-/-}$ mice was an increase in adiponectin levels when fed a high fat diet [231]. The discordance between these two studies has not been elucidated suggesting further research is needed to fully characterize these IL-$6^{-/-}$ mice.

2.2 – Rationale

There is much evidence suggesting that obesity is linked to inflammation and a marked decrease in adiponectin mRNA and protein levels. The primary objective of these studies was the elucidation of the role of inflammatory cytokines, namely IL-6, on adiponectin gene expression. It was hypothesized that IL-6, produced by adipose tissue, leads to the downregulation of adiponectin gene expression. Therefore, we aimed our studies at determining the link between obesity, insulin resistance and decreased adiponectin levels. Since most studies on adiponectin gene expression were carried out in animal models, it was important that we

determine if rodent and human adipose tissue behaved in a similar fashion. A further goal was to identify the active elements within the human adiponectin promoter that mediated the effect of IL-6 on adiponectin gene expression and to determine the signal transduction pathway responsible for this downregulation. In addition, the effect of IL-6 on plasma adiponectin levels was further studied to determine the physiological importance of IL-6 on metabolic changes such as decreased adiponectin levels.

2.3 – Experimental procedures

2.3.1 – Materials

Cell culture medium and reagents were purchased from Life Technologies and cell culture plasticware was purchased from Falcon. Chemical reagents were acquired from Fisher and Sigma-Aldrich chemicals. Primers were purchased from Sigma-Genosys. Restriction enzymes were purchased from New England Biolabs. Taq DNA polymerase, DNase I, and MMLV-RT were purchased from Invitrogen. Maxiprep and miniprep kits were purchased from Qiagen. Random decamer primers were purchased from Ambion. Dual-Luciferase Reporter Assay system was purchased from Promega. Nuclei isolation kit was purchased from Sigma. FastStart Master SYBR Green was purchased from Roche Molecular Biochemicals.

2.3.2 – Methods

Human preadipocyte isolation and culture

Subcutaneous adipose tissue was collected from healthy normolipemic subjects undergoing reduction mammoplasty procedures. Adipose tissue was collected in PBS and processed within an hour. Samples were finely diced and digested in type II collagenase (170U/mL) (Sigma) for 1.5 hours in 37°C shaking water bath. The stromal-vascular cells were then separated from mature adipocytes by sequential filtration down to 25μm. The stromal-

vascular cell pellet, containing the preadipocytes, was treated with red blood cell lysis buffer (150mM ammonium chloride, 5.7mM potassium dibasic sulphate, 0.1mM EDTA (pH 7.3)) for 5 minutes at room temperature and subsequently plated in 6-well plates (a starting amount of 100mL of adipose tissue yields 4 6-well plates) [232-235]. Cells were cultured in basic medium (Dulbecco's modified Eagle's medium (DMEM): Ham's F-12 medium (F-12) (1:1 v/v), 30mL/L FBS, 33μM biotin, 17μM pantothenate, 1% antibiotic/antimycotic, 1% L-glutamine) for 48 hours and then differentiated. For the first three days of differentiation, the cells were cultured in basic medium supplemented with 0.25mM IBMX, 1μM dexamethasone, 1μM rosiglitazone, and 100nM human insulin. The cells are further differentiated for another 7 days in basic medium supplemented with 1μM dexamethasone and 100nM human insulin. Cells were then treated with various concentrations of human IL-6 for different periods of time in the presence or absence of kinase inhibitors (Uo126 or LY294002).

Cell culture and differentiation

3T3-L1 preadipocytes (American Type Culture Collection) were grown in DMEM containing 25mM glucose, 10% FBS, penicillin (50U/mL) and streptomycin (50U/mL) (basic medium) at 37°C with 5% CO_2. Cells were grown two days past confluence then treated for two days in basic medium supplemented with 1μM human insulin, 0.5mM IBMX and 1μM dexamethasone. Cells were cultured for another 6 days in basic medium supplemented with 1μM human insulin where 90% of cells had accumulated fat droplets. Cells were then treated with various concentrations of human IL-6 for different periods of time in the presence or absence of kinase inhibitors (PD98059, U0126 or LY294002).

The human liposarcoma cell line SW872 (American Type Culture Collection) were cultured in DMEM: F-12 (3:1) supplemented with 5% FBS, 1% l-glutamine and 50μg/mL gentamicin in the presence of 5% CO_2 at 37°C.

RNA isolation and cDNA synthesis

Total RNA was isolated from human primary adipocytes, 3T3-L1 and SW872 cells using Trizol Reagent (Invitrogen). Cells were rinsed with PBS then scraped in 1mL of Trizol Reagent and stored at -80°C overnight. RNA was isolated following the manufacturer's protocol (Invitrogen). RNA samples were treated with amplification grade DNase I to deplete samples of any DNA contamination. RNA concentration and purity was determined spectrophotometrically using A_{260}/A_{280}. RT-PCR was performed using a two-step approach. First strand cDNA (RT reaction) was synthesized using 2.5μg of total RNA (3T3-L1 cells and SW872 cells) or 0.5μg (human primary adipocytes), 10μM random decamer primers (Invitrogen) and 200U of MMLV reverse transcriptase (Invitrogen) and incubated at 42°C for 1 hour.

Detection of adiponectin cDNA

First strand cDNA served as a template for a 25μL PCR reaction containing 0.4mM of dNTPs, 0.8μM of each primer (see below) and 1U of Vent DNA polymerase. After initial denaturation at 95°C for 3 minutes, reactions were cycled 30 times using the following parameters: 95°C for 30 seconds, 49°C for 30 seconds and 72°C for 30 seconds followed by a final 5 minutes incubation at 72°C. The following primers were used: human adiponectin forward 5'-CTTATTGGTCCTAAGGGAG-3' and reverse 5'-GGGGATAGTAACGTAAGTCT-3'; mouse adiponectin forward 5'-AAGGACAAGGCCGTTCTCT-3' and reverse 5'- TATGGGTAGTTGCAGTCAGTTGG-3'; GAPDH forward 5'- GTCGGAGTCCAACGGAT-3' and reverse 5'-CCACGACGTACTCAGC-3'. PCR reactions were separated on 1% agarose gel by electrophoresis.

Quantification of adiponectin gene expression

For the RT-PCR experiments, the following primers were designed using the LightCycler Probe Design software version 1 (Roche Molecular Biochemicals): human adiponectin forward 5'-CTTATTGGTCCTAAGGGAG-3' and reverse 5'-GGGGATAGTAACGTAAGTCT-3'; mouse adiponectin forward 5'-AAGGACAAGGCCGTTCTCT-3' and reverse 5'- TATGGGTAGTTGCAGTCAGTTGG-3'; GAPDH forward 5'- GTCGGAGTCCAACGGAT-3' and reverse 5'-CCACGACGTACTCAGC-3'. These primers were used with Taq DNA polymerase to amplify their respective sequences from a RT reaction on RNA isolated from human primary adipocytes or mouse 3T3-L1 cells. The resulting amplicons were cloned into the pCR 2.1-TOPO TA cloning kit (Invitrogen). Plasmids containing each of these genes were purified from bacterial culture using the Maxiprep DNA isolation kit (Qiagen). Serial dilutions of the plasmids were used to generate a standard curve that could be used for future runs on the LightCycler to measure mRNA abundance in experimental samples. Adiponectin mRNA expression was measured by quantitative real-time PCR in a LightCycler fluorescent temperature cycler (Roche Molecular Biochemicals, Laval, QC). One twentieth (1μL) of each RT reaction served as a template in a 20μL PCR reaction containing 3mM $MgCl_2$, 0.3μM (for adiponectin) or 0.5μM (for GAPDH) of each primer and 1X LightCycler DNA Master SYBR Green I mix. After initial denaturation at 95°C for 10 minutes, reactions were cycled 45 times using the following parameters: 95°C for 15 seconds, 49°C for 15 seconds and 72°C for 15 seconds. SYBR Green I fluorescence emission readings were monitored after each cycle. Adiponectin and GAPDH mRNA levels were quantified using the second derivative maximum method of the LightCycler Software Version 3.5 (Roche Molecular Biochemicals). The protocol determines the crossing points of individual samples by an algorithm, which identifies the first turning point of the

fluorescence curve. This turning point corresponds to the first maximum of the second derivative curve and correlates inversely with the log of the initial template concentration. Relative adiponectin mRNA levels were normalized to those of GAPDH. At the end of each PCR, melting curve profiles (cooling the sample to 72°C and heating slowly to 95°C with continuous measurement of fluorescence) were produced to confirm amplification of a single product. The specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis.

Adiponectin promoter construct

A 3.5kb fragment extending 348bp past the transcription start site of the human adiponectin promoter was cloned in the pGL3-basic vector (Promega) using the following primers: forward primer with flanking SacI restriction site 5'-GCTCGAGCTCTGTTCTCCTTTCTTGCCATT-3' and reverse primer with flanking NheI restriction site 5'-GCATGCTAGCACCCTTCCACCTTACTGACC-3'. The new vector contains the adiponectin promoter, which will be used to drive the expression of the luciferase gene. The new vector was sequenced to confirm that the promoter sequence was correct.

Electroporation of differentiated 3T3-L1 and SW872 cells

3T3-L1 cells were plated in 10cm dishes and differentiated for 10 days. Following differentiation, cells were rinsed twice with PBS followed by trypsin digest for 10 minutes. Cells were collected in 5mL of PBS by centrifugation (1000rpm for 5 minutes). Cells were washed again in PBS and resuspended in 495mL of PBS. Cells were co-electroporated with 25μg of the Firefly luciferase reporter vector pGL3-adiponectin promoter and 0.5μg of the Renilla luciferase reporter vector pRL (1:50 ratio) at 0.16kV, 950μF capacitance and 129ohms resistance for 21 milliseconds. Cells were resuspended in basic medium, plated in 24-well plate and left to adhere overnight. SW872 cells were plated in 6-well plates with 70% confluence on the day of

transfection. Cells were then co-transfected with 2μg of pGL3-adiponectin promoter and 0.5μg of the Renilla luciferase reporter vector pRL (1:50 ratio) with lipofectamine (Invitrogen) for 4.5 hours.

Both transfected cells were incubated in the presence or absence of kinase inhibitors for 1 hour (PD98059 - 50μM or LY294002 - 10μM) followed by IL-6 (30ng/mL) treatment for 24 hours. Cells were scraped and lysed in passive lysis buffer for Dual-Luciferase Reporter assay (Promega) and kept on ice until assayed. Luciferase activities derived from both Luciferase (adiponectin promoter construct) and Renilla (pRL) proteins were measured according to the manufacturer's protocol in a luminometer (Lumat LB9507, EG&G Berthold). Renilla luciferase activity was then used to standardize for electroporation efficiency.

Isolation of human primary adipocyte nuclei

Human primary adipocytes were cultured in 4 6-well plates and differentiated for 10 days. Cells were washed with PBS on ice and lysed with ice-cold lysis solution containing 1mM of DTT and 1% of Triton X-100. The cell lysates were pooled together and the nuclei were purified by centrifugation through 2M sucrose cushion solution. The nuclei were isolated according to the manufacturer's protocol (Nuclei pure prep nuclei isolation kit from Sigma).

Animal studies

IL-6 knock out mice (B6.129S2-Il6^{tm1Kopf}/J) were purchased from Jackson Laboratory. The IL-6 KO mice were bred with the wild-type mice (C57Bl6J) to generate heterozygote mice. The mice were maintained on a 12h light/12h dark schedule on a normal chow diet for 3 months. After 3 months, blood was collected in microvettes with EDTA (Sarstedt) from the saphenous vein from all 3 genotypes (n=10 females, n=10 males for each group) and plasma aliquots were frozen at -20°C until assayed. The mice were then put on a high fat/high glucose diet (protein – 26,2%, carbohydrates – 26,3%, fat – 34,9%, with 60kcal % from fat) (D12492 –

Harlan Teklad) for 6 weeks. Again blood was collected from the saphenous vein as described above. Plasma aliquots were frozen at -20°C until assayed.

Plasma adiponectin ELISA assay

Plasma adiponectin was quantified in all three genotypes after 3 months of chow diet and after 6 weeks of high fat/high glucose diet using a mouse adiponectin ELISA kit (Linco). The assay was done according to the manufacturer's protocol and all quality controls fell within the appropriate range.

Statistical analysis

Results are expressed as the mean ± SEM or SD. Where indicated, the statistical significance of the differences between groups was determined using Student's *t* test or ANOVA using GraphPad InStat v.3.06 statistical analysis software (GraphPad Software Inc.).

2.4 – Results

2.4.1 – Adiponectin mRNA detection in different cell lines

Initially, the detection of adiponectin mRNA was carried out in different adipocyte cell lines. There exist very few human adipocyte-like immortal cell lines; therefore, it was important to first find a good cellular model for adiponectin expression. The majority of previous studies determining adiponectin gene expression have been carried out in 3T3-L1 adipocytes, a mouse adipocyte cell line. Unfortunately, promoter sequences for many adipocytokines are not highly conserved across species. Therefore, we first attempted to identify a human adipocyte cell line suitable for these studies. It was also imperative to determine if previous mouse results could be applied to human cell lines. First, a human liposarcoma cell line, SW872, was tested to determine the presence of adiponectin gene expression. This cell line has been described as a good cell model for adipocyte gene expression [232-235]. Following RT-PCR, adiponectin cDNA

was amplified and run on an agarose gel. The relative intensity was determined by the ratio of the adiponectin band compared to the GAPDH (a housekeeping gene) band intensity and these values were normalized to adiponectin expression in 3T3-L1 to give values of fold increase (**Figure 2.3**). Adiponectin was detected in 3T3-L1 adipocytes and human primary adipocytes. Adiponectin expression level in human primary adipocytes was lower than the expression level in 3T3-L1 cells. It represented approximately 70% of that of 3T3-L1. Adiponectin mRNA was not detected in SW872 human liposarcoma cell line.

2.4.2 – IL-6 inhibits adiponectin mRNA expression in a dose-dependent manner

The effect of IL-6 treatment on adiponectin mRNA was determined. Human primary adipocytes and 3T3-L1 adipocytes were treated with increasing concentrations of IL-6 (15-100ng/mL) for 24 hours. Adiponectin mRNA was quantified using real-time RT-PCR. In both cases, adiponectin expression was decreased in a dose-dependent manner. The human primary adipocytes showed a significant 38% reduction of adiponectin mRNA at IL-6 concentration of 30ng/mL ($p<0.01$) and a maximal 45% decrease found at 100ng/mL ($p<0.001$) (**Figure 2.4A**). A similar pattern of inhibition was observed in 3T3-L1 adipocytes. 3T3-L1 adipocytes showed a significant inhibition of adiponectin mRNA of 23% at 15ng/mL of IL-6 ($p<0.01$) with a maximal decrease of 35% inhibition found at 30ng/mL ($p<0.001$) (**Figure 2.4B**). In both human primary adipocytes and 3T3-L1 adipocytes, IL-6 treatment at 100ng/mL did not further decrease adiponectin mRNA. This indicates that mouse adipocyte cell lines and human primary adipocytes seem to respond similarly to IL-6 treatment.

2.4.3 – IL-6 inhibits adiponectin mRNA expression in a time-dependent manner

The effect of IL-6 treatment over time on adiponectin mRNA was then determined. Human primary adipocytes and 3T3-L1 adipocytes were treated with IL-6 (30ng/mL) for various time points (6-32 hours). Adiponectin mRNA was quantified using real-time RT-PCR.

Figure 2.3. Detection and relative quantification of adiponectin cDNA in different cell types.

Human primary adipocytes and 3T3-L1 adipocytes were cultured for 7 to 10 days in differentiation medium. Total RNA was extracted and RT-PCR was performed to detect adiponectin and GAPDH cDNA. The amplicons were separated by agarose gel electrophoresis and the bands were quantified using Quantity One software. Data represent fold increase in adiponectin expression normalized to GAPDH expression compared to 3T3-L1 adipocytes. Each value represents the average of two independent RT-PCR reactions from different individual samples and the error bars represent the standard error of the mean. The two-tailed p-value from student's *t* test for both human primary adipocytes and SW872 cells compared to 3T3-L1 adipocytes is <0.0001.

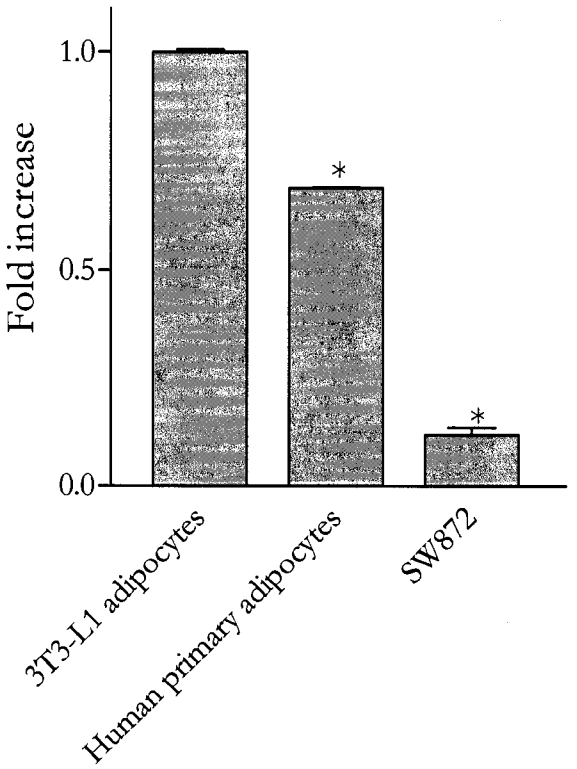
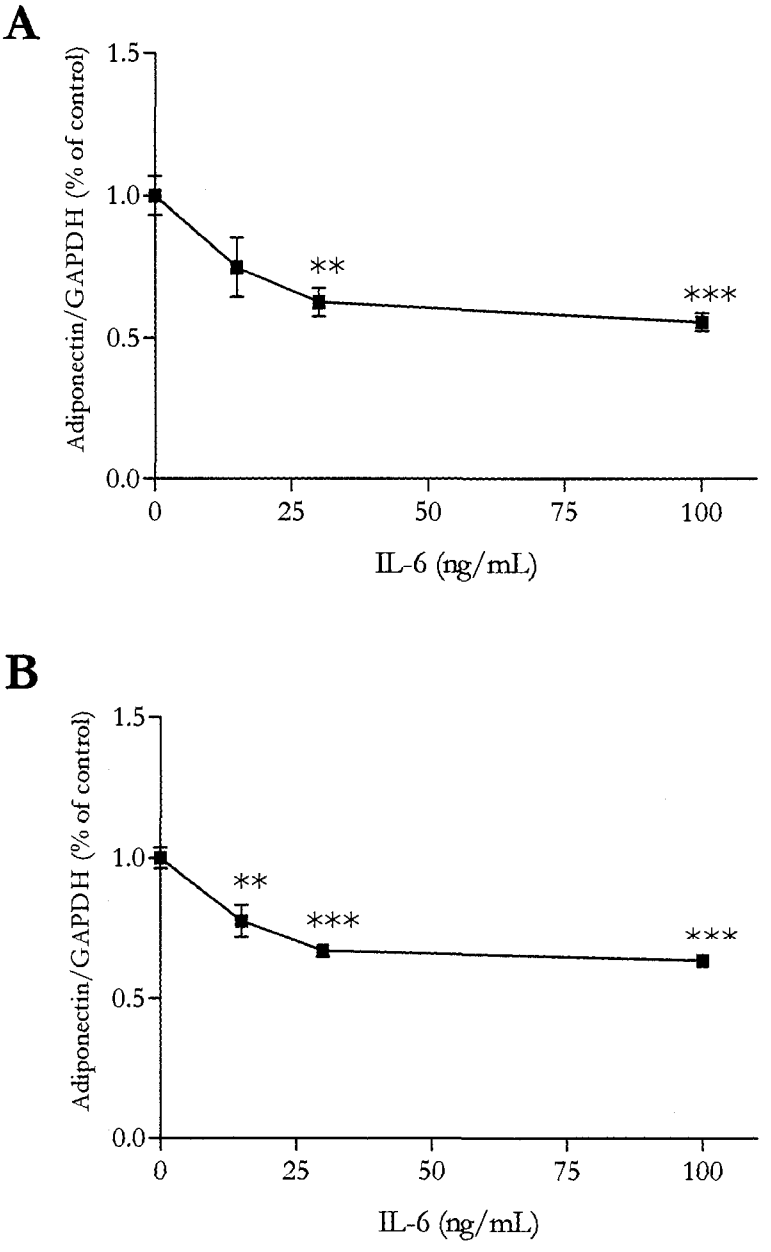

Fold increase
1.0
0.5
0.0
*
*
3T3-L1 adipocytes
Human primary adipocytes
SW872

Figure 2.4. Dose-dependent inhibition of adiponectin mRNA expression by IL-6.

Human primary adipocytes (A) and differentiated 3T3-L1 adipocytes (B) were treated with various concentrations of IL-6 for 24 hours. Reverse transcription was performed on total RNA isolated from these cells and cDNA abundance was quantified by real-time RT-PCR and normalized to GAPDH expression. Data are expressed relative to untreated control cells. Data points represent means from 12 samples and the error bars represent the standard error of the mean. The one-way ANOVA p value is <0.0001 for both cells types and differences between samples are determined using the Tukey post-test where ** indicates a $p<0.01$ and *** indicates a $p<0.001$ compared to control.


A
Adiponectin/GAPDH (% of control)
1.5
1.0
0.5
0.0
**
***
0
25
50
75
100
IL-6 (ng/mL)
B
Adiponectin/GAPDH (% of control)
1.5
1.0
0.5
0.0
**
***
***
0
25
50
75
100
IL-6 (ng/mL)

In both cell types, adiponectin expression was decreased in a time-dependent manner. The human primary adipocytes showed a significant 22% ($p<0.001$) decrease in adiponectin expression after 8 hours of IL-6 (30ng/mL) with a maximal decrease of 35% ($P<0.001$) at 24 hours (**Figure 2.5A**). The mouse 3T3-L1 adipocytes showed a slightly different inhibition profile. Adiponectin mRNA was significantly decreased by 15% ($p<0.05$) after 12 hours with a maximal decrease of 35% ($p<0.001$) after 24 hours (**Figure 2.5B**). This might indicate a difference in gene regulation in both species; although, overall the responses are quite similar.

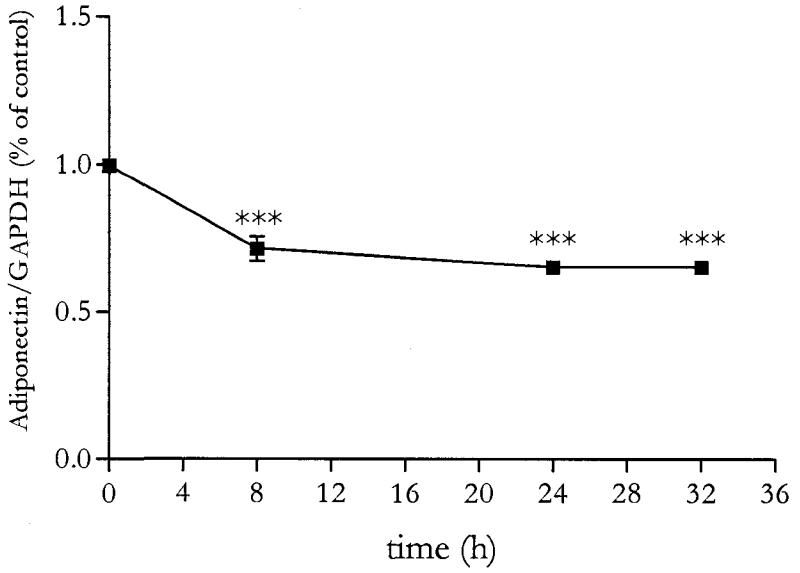
2.4.4 – Inhibition of adiponectin mRNA expression by IL-6 is mediated via p44/42 MAP kinase

We subsequently tested whether signalling proteins such as p44/42 MAP kinase or PI 3-kinase, which have been implicated in IL-6 signalling, might play a role in the downregulation of adiponectin gene expression. Human primary adipocytes and 3T3-L1 adipocytes were pre-treated with specific pharmacological inhibitors for 1 hour before IL-6 (30ng/mL) was added for 24 hours. Adiponectin expression was reduced 25% ($p<0.0001$) in human primary adipocytes and 27% ($p<0.0001$) in 3T3-L1 adipocytes after 24 hours of IL-6 treatment (**Figure 2.6**). Interestingly, inhibition of p44/42 MAP kinase by U0126 (U, 50μM) in human primary adipocytes completely reversed the IL-6 induced inhibition back to untreated levels. In contrast, inhibition of PI 3-kinase by LY294002 (LY, 10μM) did not influence the inhibition of adiponectin expression of IL-6 (**Figure 2.6A**). In mouse 3T3-L1 adipocytes, the inhibition of p44/42 MAP kinase by PD98059 (PD, 50μM) or U0126 (U, 50μM) completely reversed the IL-6 induced downregulation of adiponectin expression while treatment with LY294002 (LY, 10μM) had no effect (**Figure 2.6B**). This indicates important similarities between human primary adipocytes and mouse 3T3-L1 adipocytes in regards to the signalling pathway by which IL-6 induces its effects.

Figure 2.5. Time-dependent inhibition of adiponectin mRNA expression by IL-6.

Human primary adipocytes (A) and differentiated 3T3-L1 adipocytes (B) were treated with IL-6 (30ng/mL) for indicated periods of time. Reverse transcription was performed on total RNA isolated from these cells and cDNA abundance was quantified by real-time RT-PCR and normalized to GAPDH expression. Data are expressed relative to untreated control cells. Data points represent means from 12 samples and the error bars represent the standard error of the mean. The one-way ANOVA p value is <0.0001 for both cell types and differences between samples are determined using the Tukey post-test where * indicates a $p<0.05$ and *** indicates a $p<0.001$ compared to control.

A


Adiponectin/GAPDH (% of control)
1.5
1.0
0.5
0.0
***
***
***
0
4
8
12
16
20
24
28
32
36
time (h)

B

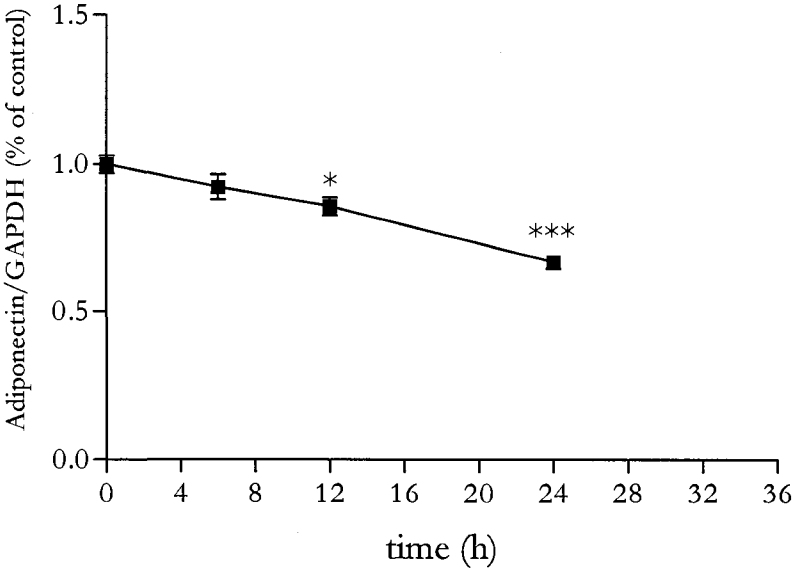
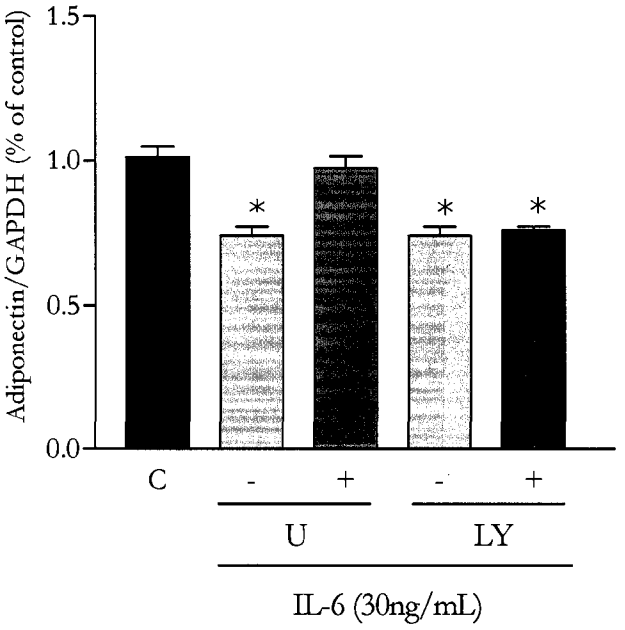

Adiponectin/GAPDH (% of control)
1.5
1.0
0.5
0.0
*
***
0
4
8
12
16
20
24
28
32
36
time (h)

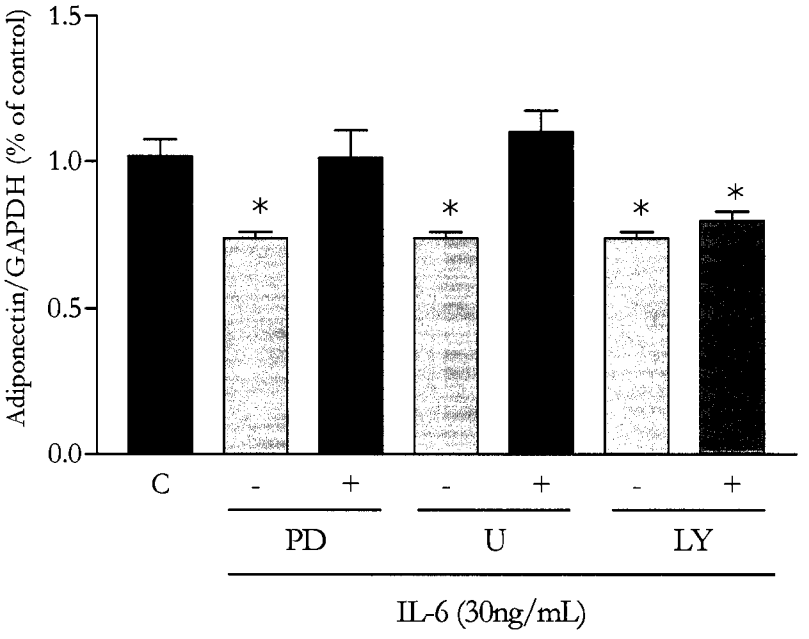
Figure 2.6. Inhibition of adiponectin mRNA by IL-6 is mediated via the p44/42 MAP kinase pathway.

Human primary adipocytes (A) and 3T3-L1 adipocytes (B) were cultured in the presence or absence of various inhibitors: p44/42 MAP kinase inhibitors, PD98059 (PD, 50μM) or U0126 (U, 50μM) or PI 3-kinase inhibitor, LY294002 (LY, 10μM) for 1 hour before IL-6 (30ng/mL) was added for 24 hours. Following the incubation, total RNA was extracted using Trizol reagent and quantitative real-time RT-PCR was performed. Adiponectin gene expression normalized to GAPDH mRNA levels is expressed relative to untreated control (C). Data represent the means of three independent experiments (each n=3) and the error bars represent the standard error of the mean. The two-tailed p-value from student's *t* test for each treatment compared to control is <0.001.

A


Adiponectin/GAPDH (% of control)
1.5
1.0
0.5
0.0
*
*
*
C
-
+
-
+
U
LY
IL-6 (30ng/mL)

B


Adiponectin/GAPDH (% of control)
1.5
1.0
0.5
0.0
*
*
*
*
C
-
+
-
+
-
+
PD
U
LY
IL-6 (30ng/mL)

2.4.5 – IL-6 effect on adiponectin promoter activity

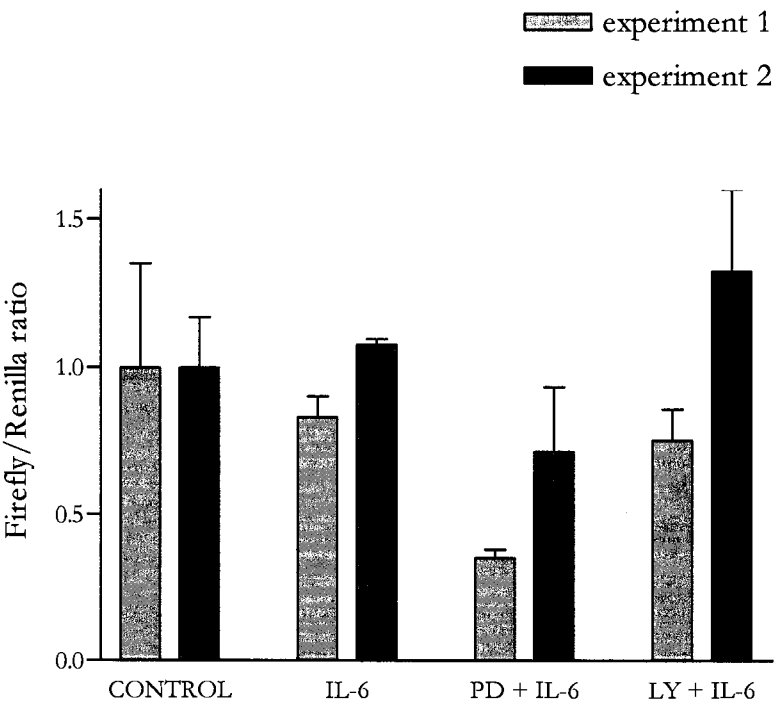
The adiponectin promoter was cloned in pGL3-basic, which is a luciferase construct. The promoter construct contained a 3.5kb fragment of the promoter including 348bp past the transcription start site. Differentiated 3T3-L1 adipocytes were electroporated and SW872 cells were transfected with pGL3-adiponectin promoter and pRL, a Renilla reporter construct used to determine electroporation efficiency. This experiment was prepared to determine the effect of IL-6 on adiponectin promoter activity. As shown previously, IL-6 can induce the downregulation of adiponectin expression. These experiments will determine if this effect is carried out through the adiponectin promoter directly. Following electroporation, cells were treated with IL-6 (30ng/mL) for 24 hours after an hour pre-treatment with p44/42 MAP kinase or PI 3-kinase inhibitors. Results obtained from reporter assays in 3T3-L1 adipocytes were inconclusive. Repeated experiments gave varying results as seen by representative results for 2 attempts (**Figure 2.7**). Even when normalized to controls, the duplicate values could not be combined to produce a trend. Although the experiment was repeated ten times, correcting for cell passage number and percentage of differentiation, the values were never reproducible. When the experiments were carried out in SW872 cells, the results obtained were unusable since firefly luciferase activity was not detected, indicating that these cells are unable to drive the expression of the firefly luciferase gene through the adiponectin promoter. Therefore, this indicates that SW872 cells lack a critical factor necessary for adiponectin gene expression.

2.4.6 – Nuclei isolation from differentiated human primary adipocytes

Human primary adipocytes were cultured and the nuclei were isolated. The nuclei were to serve as substrate to determine the active regions of the adiponectin promoter, known as the DNase sensitivity sites. Probes were designed to span six regions of the promoter. Unfortunately, the method used never yielded enough nuclei to continue the experiment.

Figure 2.7. The effect of IL-6 on adiponectin promoter activity determined by dual luciferase assay.

3T3-L1 adipocytes were cultured in differentiation medium for 10 days and subsequently co-electroporated with pGL3-adiponectin promoter (firefly luciferase construct, 25μg) and pRL (renilla luciferase reporter construct, 0.5μg). Cells were replated and allowed to adhere overnight. Cells were then treated with the p44/42 MAP kinase inhibitor PD98059 (PD, 50μM) or the PI 3-kinase inhibitor LY294002 (LY, 10μM) for 1 hour followed by IL-6 (30ng/mL) treatment for 24 hours. Following the incubation, cells were scraped in passive lysis buffer and dual luciferase assay was measured. Data normalized to renilla luciferase activity is expressed relative to untreated control. Data points represent each the average of one experiment (n=3) done in parallel and the error bars represent the standard error of the mean. No statistical analysis was performed due to inconsistent results.


experiment 1
experiment 2
Firefly/Renilla ratio
1.5
1.0
0.5
0.0
CONTROL
IL-6
PD + IL-6
LY + IL-6

Although the nuclei isolation protocol was optimized using differentiated 3T3-L1 cells, which have a similar fat content as human primary adipocytes, the amount of starting material in each case differs considerably. Based on the manufacturer's product insert, a nuclei pellet should be visible following the sucrose gradient spin. The amount of human primary adipocyte starting material was insufficient to produce a visible pellet and OD quantification further confirmed the inadequate amount. Unfortunately, human adipose tissue is in limited supply, and it yields very few human primary adipocytes. The amount obtained each time (once a week) was insufficient to allow proper and necessary nuclei isolation. Therefore, with the amount of tissue available, the DNase sensitivity sites could not be determined. However, a working protocol for determining DNase sensitivity sites was first established in differentiated 3T3-L1 cells but could not be applied to human primary adipocytes.

2.4.7 – Effects of genotype and diet on plasma adiponectin levels in IL-6 mice

The effect of genotype of IL-6 mice on plasma adiponectin levels was determined. Mice were fed a chow diet for three months and adiponectin levels were measured (**Table 2.2**). IL-$6^{+/+}$ mice have lower adiponectin levels than IL-$6^{+/-}$ mice ($p<0.01$) and IL-$6^{-/-}$ ($p<0.001$), and IL-$6^{+/-}$ have lower adiponectin levels than IL-$6^{-/-}$ mice ($p<0.01$). The trend observed is quite significant where the removal of one or two alleles of the IL-6 gene leads to progressively increased plasma adiponectin levels in mice on a chow diet ($p<0.0001$). IL-$6^{+/-}$ mice have 25% more plasma adiponectin compared to IL-$6^{+/+}$ mice and IL-$6^{-/-}$ mice have a 50% increase in plasma adiponectin levels compared to IL-$6^{+/+}$ mice. Furthermore, there is no difference between adiponectin levels in males and females when fed a chow diet. Following a three-month chow diet, the mice were put on a high fat/high glucose (HF/HG) diet for 6 weeks and adiponectin plasma levels were measured (**Table 2.3**). Again, the IL-$6^{+/+}$ mice have lower adiponectin levels than the IL-$6^{+/-}$, although this did not reach statistical significance, and have

	Adiponectin (µg/mL)
IL-6$^{+/+}$	8,6 ± 1,7
IL-6 $^{+/-}$	10,6 ± 1,5 §
IL-6$^{-/-}$	12,7 ± 1,9 ‡

Table 2.2 Plasma adiponectin levels in IL-6 mice. Mice were fed a standard chow diet for 3 months. Blood was collected from the saphenous vein, and plasma was separated then frozen at -20°C until assayed. Plasma adiponectin levels were measured using an ELISA kit from Linco. Data represent means ± standard deviation of 20 animals for each genotype. The one-way ANOVA p-value is <0.0001 and differences between genotypes are determined using the Tukey post-test where § indicates a $p<0.01$ and ‡ indicates a $p<0.001$ compared to wild-type mice.

	Adiponectin (ug/mL)			
	Males		Females	
	chow diet	high fat/high glucose diet	chow diet	high fat/high glucose diet
IL-6$^{+/+}$	7,4 ± 1,6	10,1 ± 1,2 †	9,5 ± 1,2	14,6 ± 2,3 †
IL-6 $^{+/-}$	10,7 ± 1,5 §	12,3 ± 1,4 †	10,5 ± 1,5	16,3 ± 2,7 †
IL-6$^{-/-}$	11,9 ± 2,0 ‡	16,5 ± 2,6 ‡†	13,3 ± 1,5 ‡	19,7 ± 3,6 ‡†

Table 2.3 Plasma adiponectin levels in IL-6 mice both on chow diet and high fat/high glucose diet. Mice were fed a standard chow diet for 3 months followed by a high fat/high glucose diet for 6 weeks. Blood was collected from the saphenous vein after each diet, and plasma was separated and frozen at -20°C until assayed. Plasma adiponectin levels were measured using an ELISA kit from Linco. Data represents means ± standard deviation of 10 animals for each category. The one-way ANOVA p-value for both diets in males and females is <0.001 and differences between genotypes are determined using the Tukey post test where § indicates a $p<0.01$ and ‡ indicates a $p<0.001$ compared to wild-type mice. The two-tailed p-value from a student's *t* test comparing the effect of diet on adiponectin levels for each genotype is indicated by † $p<0.001$.

lower values compared to IL-$6^{-/-}$ ($p<0.001$ for males and females). Also, IL-$6^{+/-}$ mice have lower adiponectin levels compared to IL-$6^{-/-}$ mice ($p<0.001$ for males and $p<0.05$ for females). Therefore the removal of one and two alleles of the IL-6 gene leads to a significant increase of adiponectin levels on both a chow and a HF/HG diet ($p<0.0001$ for males and females). Furthermore, the HF/HG diet induced an increase in adiponectin levels in both males ($p<0.001$) and females ($p<0.0001$) compared to the chow diet. Interestingly, the increase in females was more pronounced than in the males. The females exhibited a 55% increase due to the HF/HG diet whereas the males only increased an average of 30% on the same diet. Conversely, the males tended to have a higher increase due to the removal of the IL-6 allele compared to the females. In males on the chow and HF/HG diet, the removal of one allele led to an increase of 45% and 22%, respectively, in adiponectin levels and the removal of two alleles led to an increase of 61% and 63%, respectively. On the other hand, the females showed only a 10% and 12% increase for one allele and a 40% and 35% increase for the removal of the two IL-6 alleles on the chow diet and HF/HG diets respectively.

2.5 – Discussion

Adiponectin has emerged as an important adipocytokine, which improves insulin sensitivity and represents a novel therapeutic target for the treatment of obesity-related diseases. Adiponectin expression is downregulated in the states of obesity, insulin resistance and T2DM [70;113;116]. Various hormones and drugs influencing insulin sensitivity also regulate its expression. TZD, PPARγ agonists used clinically to improve insulin sensitivity, have been shown to increase adiponectin expression [5;140]. The upregulation by TZD is mediated through a PPRE in the adiponectin promoter [161;167;236-238] and by antagonizing the downregulation of adiponectin expression mediated by TNFα [140]. TNFα is one of many agents, including glucocorticoids [146;239]

and β-adrenergic receptor-coupled pathways [171], that are known to downregulate adiponectin expression [140;146;156].

The majority of previous studies on adiponectin have been carried out in mouse adipocyte cell lines. Since adiponectin promoter sequences are not highly conserved across species, it was essential that we determine which human adipocyte cells expressed adiponectin and determine if the regulation of expression of adiponectin in mouse 3T3-L1 adipocytes was similar to the regulation in human adipocytes either in primary cultured adipocytes or in the cultured liposarcoma cells SW872. Interestingly, adiponectin expression was not detected in SW872 cells although it is described as a good adipocyte cell model [232-235]. SW872 cells are thought to be trapped between preadipocyte stage and mature adipocyte stage. Since adiponectin is only expressed in mature adipocytes, SW872 cells must lack a critical regulator of adipocyte differentiation required for adiponectin expression. It would be interesting to do a gene chip analysis comparing the expression of differentiation genes in human primary adipocytes and SW872 cells to determine the specific genes that are not expressed in SW872 cells but are required for adiponectin expression. Both mouse 3T3-L1 and human primary adipocytes express adiponectin although human primary adipocytes seem to express less adiponectin than 3T3-L1 adipocytes.

Despite the role of adiponectin in such an important process as insulin sensitivity, these studies are the first to demonstrate that adiponectin expression is regulated by IL-6 at the mRNA level in human primary adipocytes and that this regulation parallels the effect of IL-6 in a mouse adipocyte cell line. Although the mechanism by which IL-6 induces the downregulation of adiponectin is not yet established, we have shown that mouse and human adiponectin respond in a similar pattern *in vitro* when treated with IL-6.

IL-6 induces a downregulation of adiponectin mRNA levels in a time- and dose-dependent manner in both human primary adipocytes and mouse 3T3-L1 adipocytes. The effect of IL-6 on adiponectin is not surprising since IL-6 is closely associated with metabolic diseases. IL-6 has been implicated as a pro-inflammatory cytokine and has been extensively studied with regards to numerous physiological processes and disease states. Plasma IL-6 levels correlate with obesity, insulin insensitivity and the development of T2DM [25;213;215;222;240;241]. It is considered an adipocytokine since 25% of *in vivo* systemic IL-6 originates from subcutaneous adipose tissue [242]. It is also thought to be one of a number of obesity-related inflammatory cytokines that cause insulin resistance [231]. The injection of IL-6 in healthy human volunteers increased blood glucagon and increased fasting glucose [222]. Similarly, administration of recombinant IL-6 in rodent models induced hepatic gluconeogenesis, which led to hyperglycemia and compensatory hyperinsulinemia [243]. Conversely, adiponectin increases the ability of insulin to suppress glucose production in the liver [93;94]. Therefore, downregulation of adiponectin might represent one mechanism by which hepatic glucose production is increased and glucose homeostasis is impaired in situations where IL-6 expression is increased such as insulin resistance and obesity. Moreover, the direct effect of IL-6 on insulin signalling may contribute to this effect. IL-6 impairs insulin signalling by affecting insulin receptor substrate-1 (IRS-1), GLUT4 and PPARγ mRNA and protein expression and IRS-1 tyrosine phosphorylation [221]. IL-6 treatment also leads to impaired insulin signalling and insulin-induced glycogen synthesis in hepatocytes [244]. Furthermore, our *in vitro* results suggest that IL-6 is probably an important factor contributing to hypoadiponectinemia associated with obesity and insulin resistance.

Recently, the major steps of IL-6 signalling have been elucidated (reviewed in [245]). IL-6 binds and activates a receptor complex formed of a specific α receptor and the signal

transducing unit gp130. Ligand binding leads to the homodimerization of gp130 at the plasma membrane and the activation of constitutively associated kinases such as Jak1, Jak2 and Tyk2 [246]. Thus, gp130 becomes tyrosine phosphorylated leading to the recruitment and subsequent phosphorylation of SHP-2 [247]. Activated SHP-2 can then activate downstream signalling proteins such as p38 MAP kinase, p44/42 MAP kinase and PI 3-kinase [247]. Pharmacological inhibition of p44/42 MAP kinase by both PD98056 and U0126 reversed the inhibition of adiponectin gene expression by IL-6. Therefore, p44/42 MAP kinase appears to mediate some of the negative effects of IL-6. In contrast, PI 3-kinase is probably not involved in adiponectin mRNA regulation by IL-6. This is further supported by a previous study done by Fasshauer *et al* [248] where in mouse 3T3-L1 adipocytes, IL-6 induced downregulation of adiponectin was partially reversed with pharmacological inhibitors of p44/42 MAP kinase but not by PI 3-kinase inhibitor. The difference between complete recovery in our studies and only partial recovery in their studies may stem from the fact that the initial downregulation of adiponectin expression was higher in their study (53%) compared to the downregulation we obtained of only 35%. Although the IL-6 used in both studies came from the same company, differences in potency, in cell number or passage number might explain the difference observed. However in both cases, the downregulation of adiponectin by IL-6 is probably due to a direct effect on the promoter. A potential toxic effect of IL-6 on the cells is unlikely to be the cause of reduced adiponectin levels since the reduction is reversible upon removal of IL-6 [248].

From these studies, we have developed a working model for IL-6 induced downregulation of adiponectin gene expression. Following the activation of p44/42 MAP kinase, the large subunit dissociates from its cytoplasmic anchor and translocates to the nucleus where it can phosphorylate numerous transcription factors including c-jun, c-myc and cAMP response element binding protein (CREB) [249]. There is no identified response element for any

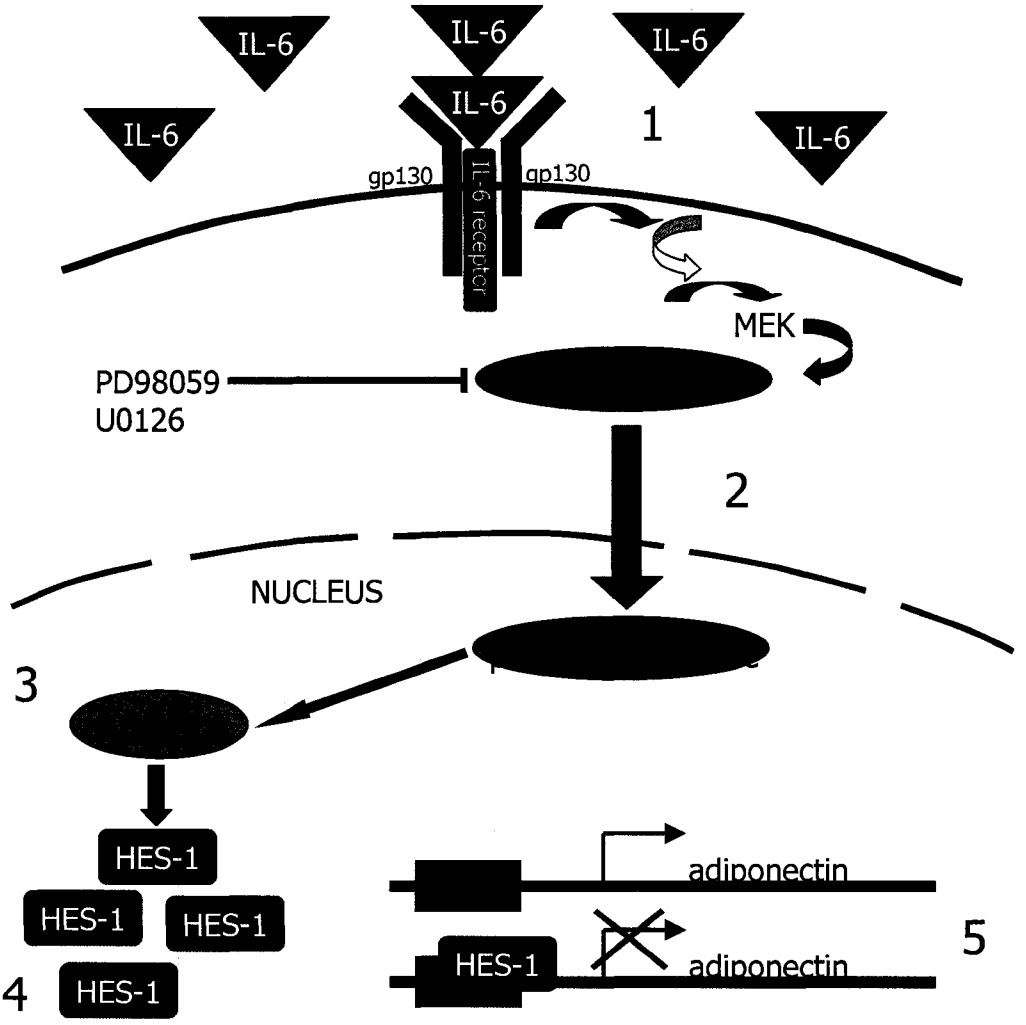
of these transcription factors in the adiponectin promoter. Nonetheless, we have identified numerous putative E-box motifs in the human adiponectin promoter, including nine B-class motifs (CANGTG) and three N-class motifs (CACNAG). Hairy enhancer of Split (HES-1) is a transcriptional repressor that recognizes both classes of E-box motifs, although it binds B-class motifs with higher affinity. Furthermore, CREB can induce the expression of HES-1 by binding cAMP response elements (CRE) within the HES-1 promoter [250]. Therefore, following the activation of CREB by IL-6 through p44/42 MAP kinase pathway, there could be an increase in HES-1 transcription and subsequent protein expression, which could lead to the downregulation of adiponectin expression through the putative E-box motifs within the adiponectin promoter. A general overview is shown in **Figure 2.8**.

Further studies are required to validate our proposed model. The characterization of the putative E-box motifs within the adiponectin promoter will permit the establishment of elements that are active for the IL-6 induced downregulation of adiponectin expression. These studies will include electrophoretic mobility shift assays (EMSA), which will determine the specific E-box motifs that bind the HES-1 transcriptional repressor. Chromatin immunoprecipitation (ChIP) assays will be used to determine the transcription factors that bind *in vivo* to the transcriptionally active promoter elements.

No one to date has looked at the effect of IL-6 on adiponectin promoter activity. Dual luciferase assays presented a clear method to determine if IL-6 had a direct effect on the adiponectin promoter and if pre-treatment with p44/42 MAP kinase inhibitor blocked the IL-6 effect on the promoter activity. This method is widely used to determine the contribution of specific elements within promoters responsible for gene regulation. Furthermore, electroporation of 3T3-L1 cells is the preferred method for transfecting these cells as they are resistant to more common forms of transfection: lipofectamine, calcium phosphate precipitation

Figure 2.8. Proposed model on IL-6 induced downregulation of adiponectin gene expression.

1. IL-6 binds and activates a receptor complex composed of a specific α-receptor and a signal transducing subunit named gp130. Ligand binding leads to the dimerization of gp130 and subsequent activation of associated kinases. 2. The resulting signalling cascade terminates by the activation of the p44/42 MAP kinase. Once activated, the large subunit of the p44/42 MAP kinase dissociates from its cytoplasmic anchor and translocates to the nucleus. 3. Once in the nucleus, it can phosphorylate and activate numerous transcription factors including cAMP response element binding protein (CREB). 4. CREB can then bind to the Hairy enhancer of Split-1 (HES-1) promoter and activate transcription. The overall effect would be an increase in HES-1 protein. 5. We propose that transcriptional repressor HES-1 binds putative E-box motifs within the adiponectin promoter to downregulate adiponectin expression. [adapted from 245].


IL-6
IL-6
IL-6
IL-6
IL-6
IL-6
1
gp130
IL-6 receptor
gp130
MEK
PD98059
U0126
2
NUCLEUS
3
HES-1
HES-1
HES-1
4
HES-1
adiponectin
HES-1
adiponectin
5

methods, etc. [251;252]. For our experiments, the main concern was that repeated electroporation did not give the same efficiency of transformation, which led to varying firefly/renilla ratio. Therefore, the method was deemed inadequate. An alternative method to electroporation would be to use a Lenti-Virus to transfect the cells. The adiponectin promoter linked to the firefly luciferase gene and the renilla luciferase gene would need to be subcloned into the two virus constructs. Differentiated 3T3-L1 adipocytes could then be infected with both viruses and dual luciferase measured.

The impact of IL-6 on adiponectin expression is well established *in vitro* but the effect of IL-6 *in vivo* on adiponectin secretion is still not fully understood. In numerous human studies, there is a significant association between plasma IL-6 and insulin sensitivity [215;241]. This is not surprising since IL-6 also downregulates adiponectin, which is known as an insulin sensitizer. Therefore, the impact of IL-6 on insulin resistance might be mediated, in part, by the downregulation of adiponectin. Interestingly, common polymorphisms in the IL-6 gene are associated with altered insulin sensitivity [229;253;254]. IL-$6^{-/-}$ mice were used to study the impact of IL-6 on adiponectin *in vivo*. We established that the removal of the IL-6 allele or alleles leads to increased plasma adiponectin levels. This is strongly suggestive of increased insulin sensitivity. Furthermore, this effect is evident whether mice are fed a chow diet or a HF/HG diet suggesting that IL-6 impacts adiponectin levels even when a healthy weight is achieved. Our data are in disagreement with a previous study [231], which showed that IL-$6^{-/-}$ mice do not have higher adiponectin levels than IL-$6^{+/+}$ mice when fed a chow diet, although the authors did observe an increase in adiponectin levels in IL-$6^{-/-}$ mice fed a high fat diet for 12 weeks. Of note, different quantification methods were used to measure plasma adiponectin. This might explain the differences in absolute values obtained in each study (30μg/mL versus 10μg/mL in our studies). It is unclear why we obtained different results, unless there are subtle differences

in strains related to either breeding strategy, or important differences in environment or diet. Our studies show that the removal of endogenous IL-6 in mice influences adipose tissue secretion of adiponectin. Furthermore, HF/HG diet produced differences between males and females in regards to adiponectin levels. Females have higher adiponectin levels when fed a HF/HG diet and the increase in adiponectin levels produced by this diet is more significant in females than in males. This is not entirely surprising since it is known that women have higher levels of adiponectin compared to men [92], and that this may be related to differing androgen [255]. This might indicate that females are more protected against obesity-related diseases through a higher increase in adiponectin expression. Further studies will be needed to characterize the sex-related differences in IL-$6^{-/-}$ mice in regards to plasma adiponectin levels.

In summary, IL-6 induces a downregulation of adiponectin mRNA expression in a time- and dose-dependent manner in both human primary adipocytes and in mouse adipocytes. This downregulation is mediated through the p44/42 MAP kinase pathway and is independent of the PI 3-kinase pathway. It is possible that the effect of IL-6 is mediated through a signalling cascade leading to HES-1 repression of adiponectin gene expression through binding of putative E-box motifs within the adiponectin promoter. From these studies, we can also establish that human primary adipocytes respond to IL-6 in a similar fashion as mouse adipocytes suggesting that species differences are not significant in adiponectin gene regulation. Furthermore, the effect of IL-6 on adiponectin was further established in an *in vivo* model. IL-$6^{-/-}$ mice have higher adiponectin levels than wild-type mice regardless of diet. Female mice have higher adiponectin levels than males when fed a HF/HG diet, which might indicate a protective effect in females. The *in vivo* studies further demonstrated that IL-6 regulates the level of adiponectin and therefore, plays a critical role in insulin sensitivity either in healthy individuals or obese and diabetic individuals.

Chapter 3 – Functional analysis of novel genetic variants of DGAT1 and DGAT2 in humans

3.1 – Background

3.1.1 – The DGAT enzymes

DGATs are enzymes responsible for catalyzing the last step of TG synthesis. As their name implies, acyl Coenzyme A (CoA): diacylglycerol acyl transferase (DGAT), convert diacylglycerol (DAG) and fatty acyl-Coenzyme A into TG [256]. Two different genes have been identified, which code for DGAT1 [257;258] and DGAT2 [259;260]. They are encoded by distinct genes with similar enzymatic functions. DGAT1 was identified due to its similarity to acyl CoA: cholesterol acyl transferase (ACAT) genes [257] whereas DGAT2 belongs to an independent gene family, which also contains genes for acyl CoA: monoacylglycerol acyl transferase (MGAT) [261-264]. Although both enzymes are expressed in a variety of tissues, the highest levels of expression are in tissues normally implicated in TG metabolism [265]. DGAT1 is primarily expressed in small intestine, adipose tissue and liver [257] and DGAT2 is highly expressed in adipose tissue and liver [259]. Interestingly, DGAT is also expressed in both skeletal muscle, heart, brain and prancreas, where TG serve as stores of FA for oxidative metabolism [265,266;267]. The role of DGAT in the various tissues will be discussed later in more detail.

3.1.2 – TG synthesis and DGAT biochemistry

There are two major pathways for mammalian TG synthesis involving fatty acyl CoA substrates. The glycerol-phosphate pathway is probably present in all tissues [268]. In this pathway, glycerol-3-phosphate is combined with two fatty acyl CoA to form a phosphatidate. Through the removal of a phosphate by phosphatidate dephosphorylase, the phosphatidate is converted to diacylglycerol, which can be covalently linked to a fatty acyl CoA

via DGAT to form a TG. Another pathway of TG synthesis, prominent in enterocytes of the small intestine, is the monoacylglycerol pathway. Dietary fats yield monoacylglycerol, which undergoes step-wise acylation to form TG. DGAT is also believed to catalyze the final step of this pathway [265]. The monoacylglycerol pathway is also active in suckling rat liver [269] and rat adipocytes [270]. Although DGATs can use a variety of fatty acyl CoA substrates, DGAT1 appears to prefer monounsaturated oleoyl CoA to saturated palmitoyl CoA [259]. In addition, DGATs are more active with sn-1,2-stereoisomers of DAG than with sn-2,3-diacylglycerol [268]. In all tissues, DGAT activity has been predominantly localized to the endoplasmic reticulum (ER) membrane [268;271;272]. Interestingly, there is evidence suggesting that DGAT enzymes may have different activities whether present on the cytosolic side or the lumenal side of the ER [273;274].

3.1.3 – DGAT knock out studies

DGAT1-deficient mice

The importance of DGATs is well presented in animal models. DGAT1-deficient mice are viable and are protected against diet-induced obesity and hepatic steatosis [275]. Adult DGAT1-deficient mice have less tissue TG, ~50% less adipose tissue mass and smaller adipocytes than WT mice on a chow diet [275;276]. Although tissue TG content is reduced by 50% in DGAT1-deficient mice, levels of both DAG and fatty acyl CoA, substrates for the DGAT reaction, are not significantly elevated and actually, are lower in skeletal muscle and liver of these mice [276]. Despite the absence of DGAT1, these mice have normal serum TG levels [275]. This suggests that DGAT1 is not rate limiting for the secretion of TG from the liver. Furthermore, DGAT1-deficient mice have a lower body weight compared to WT mice when fed a high fat diet and DGAT1$^{+/-}$ have an intermediate phenotype [275]. The difference is explained by lower fat mass and not lean body mass as DGAT1-deficiency does not affect the latter. DGAT1-

deficient mice also show an increase in insulin sensitivity [276]. Insulin-stimulated glucose uptake is increased in skeletal muscle and white adipose tissue of DGAT1-deficient mice and $DGAT^{+/-}$ also shown improved insulin sensitivity [277]. Interestingly, they also have increased food intake compared to WT mice [278] but increased energy expenditure through at least two mechanisms: increased physical activity and increased expression of uncoupling protein 1 (UCP1) – important for non-shivering thermogenesis in rodents [275-277;279]. Furthermore, the hyperphagia exhibited by these mice is more pronounced during cold exposure [278]. A proposed explanation for the increased energy expenditure in DGAT1-deficient mice is their increased leptin sensitivity [279]. Leptin is an adipocytokine that mainly functions through the central nervous system to induce satiety and increase energy expenditure. Leptin also affects peripheral tissues, enhancing insulin sensitivity and fatty acid oxidation [280-282]. DGAT1-deficient mice lose more weight than WT mice in response to subcutaneous leptin infusion [276]. However, leptin seems insufficient to explain the metabolic changes in DGAT1-deficient mice since neither decreased food intake nor alteration in hormone levels (mediating the effect of leptin on energy expenditure) was observed [256]. The idea that leptin is not the only modulator affected in DGAT1-deficient mice is supported by the fact that ob/ob mice (leptin deficient) with hepatic and adipose DGAT1 inhibited by antisense inhibitors also displayed improved insulin sensitivity [256]. However, no metabolic changes were observed when ob/ob mice were crossed with DGAT1-deficient mice [276]. This might suggest that the effect of DGAT1-deficiency on energy and glucose metabolism requires an intact leptin pathway [279].

Other observed phenotypes of DGAT1-deficient mice include altered endocrine function of white adipose tissue (WAT). Change in DGAT1 expression alters adipocyte size in mice [276;283], and alterations in adipocyte size are known to correlate with changes in the endocrine function of WAT [279]. Therefore, it is not surprising that DGAT1-deficient mice have an altered

expression and secretion pattern of adipocytokines that modulate energy and glucose metabolism [284]. Although, it is not yet known which adipocytokine is responsible for the phenotype of DGAT1-deficient mice, one candidate is adiponectin. WAT of DGAT1-deficient mice have a 2-fold increase in adiponectin expression when fed a high fat diet, and in obese models, DGAT-1 deficient mice produce more adiponectin than WT mice [284]. Although the preceeding is suggestive of a role of adiponectin in the phenotype of DGAT1-deficient mice, further research is needed to fully establish this link.

DGAT1-deficient mice also show impaired function at the level of the skin and mammary gland, consistent with the broad expression pattern of DGAT1. These mice develop several skin abnormalities such as dry fur and hair loss after puberty [285]. This is associated with atrophic sebaceous glands and the absence of wax diesters, a major species of fur lipids in rodents [279]. Therefore, water repulsion and thermoregulation after water immersion are impaired in DGAT1-deficient mice [285]. Conversely, these abnormalities are absent in $DGAT^{+/-}$ mice suggesting that the effect of DGAT1-deficiency on skin requires a complete absence of the enzyme. Furthermore, these mice also have altered mammary gland physiology. Postpartum DGAT1-deficient mice are unable to nurse their offspring due to an absence of milk production [275]. Again, DGAT1+/- mice do not have an altered phenotype in terms of lactation ability.

DGAT2-deficient mice

In contrast to DGAT1-deficient mice, DGAT2-deficient mice are not viable and die within hours after birth [286]. These mice have a severe reduction in total TG content (~ 90% reduction) leading to a deficiency in oxidative metabolism substrates [286]. Also, DGAT2-deficient mice lack essential fatty acids resulting in abnormalities of skin lipids and impaired epidermal barrier function causing rapid dehydration [279]. This suggests that DGAT2 is essential for TG metabolism [279]. $DGAT2^{+/-}$ mice are not protected against diet-induced obesity, suggesting that

50% reduction in DGAT2 expression is not limiting for adipocyte TG synthesis [279]. Although both enzymes catalyze the same reaction, this is compelling evidence that they play different roles throughout the development process and that each one mediates different critical steps at different time points.

3.1.4 – The role of DGAT in energy metabolism

DGATs play an important role in energy metabolism [256]. These enzymes are mainly expressed in tissues linked to TG synthesis such as adipose tissue, which is involved in energy storage, the liver, where TG are important for lipoprotein production, and the intestines where TG synthesis is important for dietary fat absorption. However, DGATs are also expressed in other tissues such as skeletal muscle and pancreas where excess accumulation of TG is associated with insulin resistance and impaired insulin secretion. Finally, DGAT is also expressed in the heart, where again lipotoxicity can play a role in cardiomyopathy and endothelial dysfunction, and in the brain, where DGAT may play a direct role on the central nervous system to regulate energy metabolism. However, DGAT function in various tissues still remains unclear and warrants further investigation.

DGAT in adipose tissue

Adipose tissue is the most important site for TG storage. In general, the amount of TG stored in adipocytes remains constant although it is in a dynamic state with a high turnover rate. This allows adipose tissue to quickly respond to metabolic perturbations and varying energy needs of the body. Two predominant states exist: the fasting state where lipolysis is dominant and FA are quickly released to provide energy throughout the body and the fed state where TG synthesis is dominant and lipolysis is inhibited by higher levels of circulating insulin. Therefore, the expression of enzymes involved in lipid metabolism must also follow this oscillation between fed and fasting states. This is observed for acetyl-CoA carboxylase 1 (ACC1), fatty acid

synthase (FAS) and DGAT2 [287]. DGAT2 has the highest expression in adipose tissue and oscillation in expression of this gene is thought to be solely responsible for alteration in total DGAT activity between the fed and fasted states [259;287]. DGAT2 expression is elevated in WAT of ob/ob mice [287] and undergoes compensatory upregulation in DGAT1-deficient mice [276]. Conversely, DGAT1 expression does not fluctuate between fed and fasted states [256]. Its activity is tightly regulated at the posttranscriptional level such that a 20-fold increase in mRNA levels of DGAT1 is only associated with a 2-fold increase in protein expression and activity [288]. Interestingly, DGAT1 is responsible for the majority of DGAT activity in adipose tissue [259;287] whereas DGAT2 is thought to play a secondary role.

DGAT1 is also thought to play a role in adipocyte differentiation. 3T3-L1 adipocytes express DGAT1 after reaching complete differentiation [289]. Overexpression of DGAT1 with recombinant adenovirus in differentiated 3T3-L1 adipocytes resulted in a 2-fold increase in DGAT activity without any effect on DGAT2 activity [288]. Furthermore, overexpression of DGAT1 in WAT of ap2-DGAT1 mice (transgenic mice overexpressing DGAT1 specifically in adipose tissue) resulted in weight gain (~20%) when the mice were fed a high fat but not a chow diet, and this weight gain was due to increased fat mass via adipocyte hypertrophy [283]. The moderate weight gain observed may be the result of a lack of a concerted increase in other enzymes important for TG synthesis thereby limiting the effect of DGAT1 overexpression [256]. This phenomenon was observed in obese Zucker rats [290]. Interestingly, in the ap2-DGAT1 mice that gained weight, there was no loss of insulin sensitivity [283]. How these mice were protected from obesity-induced insulin resistance is not well understood. Perhaps the modest weight gain was not associated with lipid accumulation in other tissues, which would cause insulin resistance. Conversely, overexpression of DGAT1 might be beneficial such that increased TG synthesis might be associated with a decrease in DAG, which in turn would decrease the levels of DAG-

responsive protein kinase C (PKCs), which are associated with insulin resistance [291;292]. Furthermore, these ap2-DGAT1 mice did not show a reduction in adiponectin levels following their weight gain [283]. Since adiponectin is important for insulin sensitivity and is usually downregulated in obesity [92], the lack of alteration in adiponectin expression might explain the normal insulin sensitivity in these mice.

The importance of DGAT in TG synthesis is well established. However, it is not well understood if DGAT is protective or harmful in states of lipid metabolism imbalance. Although there is still much to learn, DGAT represents a potential therapeutic target for the treatment of obesity.

DGAT in the liver

The metabolic role of the liver includes the uptake and secretion of lipids, mainly the uptake of albumin bound FA and the secretion of TG in chylomicrons and very low-density lipoproteins (VLDL). Although the liver primarily deals with lipids, it does not store TG for long periods of time. However, it is capable of rapidly responding to changes in fuel availability and metabolic demands [256]. TG storage in the liver becomes problematic when excess FA are continually being taken up or if *de novo* synthesis of FA is elevated for extended periods of time, thus leading to the metabolic condition known as non-alcoholic fatty liver disease (NASH). In this case, the liver is incapable of compensating with an increase in VLDL secretion, although the reason for this remains unclear [256]. It has been proposed that two different pools of TG exist where one pool is reserved for cytoplasmic storage and therefore unavailable for secretion and a second pool would be used exclusively for secretion in lipoprotein particles [273]. This model is supported by evidence that DGATs have two different activities depending on their localization on the ER membrane. DGAT enzymes exposed to the cytosolic side of the ER membrane would be responsible for TG synthesis reserved for storage inside the cell whereas

DGAT enzymes exposed to the lumenal side of the ER would be coupled with lipoprotein secretion [273]. Interestingly DGAT on the lumenal side of the ER requires the presence of carnitine acyltransferases responsible for delivering substrates to the lumenal side of the ER [274]. The expression of DGAT1 and DGAT2 does not seem to be associated with these different activities [293]. A better explanation for the dual activity of DGAT depending on localization would be that there exists another DGAT protein not yet identified or that through differential RNA splicing or post-translational modifications, the same protein would be localized on the different faces of the ER membrane [256].

While it is clear that DGAT plays a part in TG synthesis in the liver, it is uncertain how quantitatively important DGAT is in determining the level of hepatic secretion of TG in lipoprotein particles. *In vitro*, DGAT1 overexpression can increase TG synthesis leading to increased VLDL secretion [256]. Also, drugs used to lower hepatic TG production, such as fenofibrate and gemfibrozil, have been correlated with a decrease in DGAT activity [294;295]. But not all reagents that decrease hepatic TG production also influence DGAT activity; for example, omega-3-polyunsaturated fatty acids and Gamichunggantang, an oriental herbal medicine, both lower TG in plasma but have no effect on DGAT expression or activity [294;296;297]. Therefore, further studies are needed to fully understand the role of DGAT in hepatic lipid homeostasis.

DGAT in the intestines

Dietary-derived TG are partially hydrolyzed in the lumen of the small intestine by pancreatic lipase to generate fatty acids and 2-monoacylglycerol [256]. The latter is subsequently taken up by enterocytes and reesterified to yield diacylglycerol and TG catalyzed respectively by MGAT and DGAT [268;298]. The TG are then secreted into the lymphatic system in the form of chylomicrons. The efficiency of this process is probably enhanced with the aid of multi-enzyme complexes formed of MGAT, DGAT and other relevant enzymes [299].

Although DGAT1 is most abundantly expressed in the small intestine, it is considered non-essential for TG synthesis in enterocytes [256]. DGAT1-deficient mice have normal intestinal absorption of dietary TG when fed a chow diet [300]. This is not surprising since DGAT2 and diacylglycerol transacylase, an enzyme which transfers one acyl group from one DAG to another DAG to produce a monoacylglycerol and TG, are both also expressed in enterocytes [263;300]. Furthermore, intestinal enterocytes also express two different MGATs, which display some DGAT activity *in vivo* [261-264]. Interestingly, DGAT1-deficient mice have delayed fat absorption especially when fed a high-fat diet [300]. A possible explanation is that DGAT1-deficient mice are unable to meet the high demand for reesterification when overloaded with a high-fat dietary content [256]. Furthermore, these mice display intracellular lipid droplets (containing both TG and DAG) in the enterocytes [300]. This suggests that the incorporation of TG into chylomicrons, and not TG synthesis, is the rate-limiting step for intestinal chylomicron TG export [256]. In summary, DGAT1 does not play a critical role in TG synthesis in enterocytes probably due to the redundancy of TG synthesizing enzymes present in the small intestine.

DGAT in skeletal muscle and pancreas

Although TG synthesis in muscle and pancreas is much less significant than in adipose tissue, liver and small intestine, it can have a significant impact on insulin sensitivity and insulin secretion in skeletal muscle and pancreas respectively via a phenomenon termed lipotoxicity [301-303].

Skeletal or active muscles mainly use glucose as their source of energy and are therefore, more suited to store glycogen than TG. However, prolonged exercise can lead to an increase in FA uptake and utilization. This probably explains the fact that endurance athletes have a higher TG content in muscle compared to untrained individuals [304-306]. Conversely, in non-athletes, lipid accumulation in muscle is highly predictive of insulin resistance, especially associated with

obesity or T2DM [307-309]. In general, intramyocellular TG content is a better predictor of total body insulin resistance than intra-abdominal fat [256]. Although lipid accumulation in muscle is probably not directly responsible for insulin resistance since endurance athletes still display high insulin sensitivity even when they have lipid accumulation in muscle [306], the fact that the accumulation is not associated with an increase in fatty acid oxidation in non-athletes is probably one of the causes of insulin resistance [256]. Also, other lipid intermediates resulting from this accumulation, such as fatty acid derivatives and ceramide, might also cause insulin resistance [310;311]. Taking this into consideration, it is still unclear what role DGAT plays in muscle, especially in regards to insulin resistance. It is highly probable that DGAT is protective against lipotoxicity, since storing FA as TG has been shown to be protective against FA-induced apoptosis [312].

In the pancreas, lipid metabolism affects insulin secretion such that lipotoxicity has been associated with a decreased capacity of β-cells to secrete insulin [313-315]. Again, it is not known if excess TG is the cause of lipotoxicity. More likely is that chronic oversupply of FA to the pancreatic islets may lead to lipotoxicity, decreased insulin secretory capacity and β-cell apoptosis [18]. Therefore, increased TG synthesis and accumulation may reflect the chronic oversupply rather than being the cause of lipotoxicity in these cells [256]. Therefore, just like in muscle, DGAT may be protective against harmful intermediate lipids and be beneficial for β-cells and insulin secretion [316].

Accumulation of lipids in non-TG-storing tissues, like skeletal muscle and pancreas, appears to be associated with lipotoxicity and organ dysfunction. The role of DGATs in TG synthesis in these tissues still remains unknown, and whether DGATs are protective or contributory to insulin resistance needs further investigation.

3.1.5 – Human genetics studies of DGAT

Although a fair amount of research has been carried out in mice, there is still very little known about DGATs in humans. To date, there has not been any identified DGAT mutation resulting in a non-functional protein. One polymorphism in the DGAT1 promoter has been identified and was shown to reduce promoter activity [317]. This SNP also correlated with BMI, plasma HDL-cholesterol (HDL-C) and blood pressure in Turkish women [317]. However, this same genetic variant had no apparent effect in Turkish men or in a French population [317;318]. Other SNPs in DGAT have been associated with reduced milk production in cattle [319;320]. Furthermore, DGAT1 has also been mapped within suggestive quantitative trait loci (QTL) related to growth rate, intramuscular fat and fatty acid composition in pigs [321], although later studies excluded this gene as a candidate for fatty acid composition QTL [322]. Thus, further genetic research, trying to identify common and rare variants in the DGAT genes, is necessary to establish the role of DGATs in energy metabolism in humans.

3.1.6 – DGAT as a therapeutic target for the treatment of obesity

Excess TG accumulation in adipose tissue leads to obesity and when it occurs in non-adipose tissue, is associated with insulin resistance. Therefore, inhibiting TG synthesis represents a potential therapeutic strategy for the treatment of obesity and T2DM [323]. Evidence from DGAT1 knock out studies has provided insight into the metabolic effects of inhibiting TG synthesis [275]. Importantly, the tissue distribution pattern of DGAT expression is similar in humans and in mice [324]. In mice, DGAT1-deficiency was associated with obesity resistance, increased energy expenditure and improved glucose metabolism [275]. Furthermore, $DGAT^{+/-}$ mice display an intermediate phenotype [275] suggesting that partial inhibition or loss of DGAT1 is still beneficial in regards to obesity. As an enzyme, DGAT1 represents an excellent target for small molecule inhibitors [279]. Although there are currently no known synthetic inhibitors of

DGAT, several natural compounds have been reported to inhibit DGAT activity *in vitro* [325-330]. The effects of these compounds on energy and glucose metabolism in animal models have not yet been studied. Also, it is not known if these compounds inhibit DGAT1 specifically or inhibit all DGAT enzymes. Of note, synthetic inhibitors have been generated for ACAT enzymes, which are DGAT1 homologues [331].

Although initial results suggest that DGAT1 may be a potential therapeutic target for the treatment of obesity, many problems may still arise. It is not known if pharmacological inhibition of DGAT1 will produce the same results as its genetic inactivation. Also, the effects in rodents and in humans might be considerably different. This is especially true for the increased energy expenditure observed in DGAT1-deficient mice, which is associated with the upregulation of UCP-1, which mediates non-shivering thermogenesis in rodents in brown adipose tissue. Since non-shivering thermogenesis through UPC does not play an important role in energy metabolism in human adults, DGAT inhibition in humans might not produce an effect as robust as the one observed in mice. Nonetheless, developing DGAT inhibitors and studying their effects on energy metabolism represents an interesting new field of research, which might yield exciting results for the treatment of obesity in humans.

3.2 – Rationale

DGATs are important enzymes in lipid metabolism and represent a potential therapeutic target for the treatment of obesity as seen in DGAT1-deficient mice. Although a fair amount is known about these enzymes in rodents, very little research has been carried out in humans. To date, there are no identified naturally occurring SNPs in these genes, which result in non-functional protein. The main purpose of these studies was to identify new rare variants in both DGAT1 and DGAT2 in extreme populations of obese or lean individuals. Also, the

effect of these variants on BMI, lipid profile values or DGATs expression was to be examined. Because of the impact of DGAT-deficiency in mice, it was hypothesized that rare mutations in both genes predicted to affect protein function would lead to an overall decrease in plasma TG levels and potentially to an increase in HDL-C levels. Also, the loss of one DGAT might be compensated with an increased expression of another DGAT enzyme. Therefore, we examined the presence of rare variants in DGAT enzymes and their impact on lipid metabolism.

3.3 – Experimental Procedures

3.3.1 – Materials

AmpliTaq Gold was purchased from Applied Biosystems. Trizol Reagent, MMLV-RT, DNase I and pCR 2.1TOPO TA cloning kit were purchased from Invitrogen. Maxiprep and miniprep kits were purchased from Qiagen. Random decamer primers were purchased from Ambion. Primers were purchased from Sigma-Genosys. FastStart Master SYBR Green I was purchased from Roche Molecular Biochemicals.

3.3.2 – Methods

Subjects and DNA isolation

Obese subjects (n=761) were recruited from the Ottawa Weight Management Clinic and age- and sex-matched lean subjects (n=760) were recruited from the Ottawa area. Blood was drawn for lipid analysis and DNA purification, and detailed biodata were taken from each participants. Genomic DNA was extracted from white blood cells by standard methods [332]. Adipose tissue biopsies were obtained from select individuals. Informed consent was obtained from all subjects after the purpose and potential risks of the study were explained to them. The protocol was approved by The Human Research Ethics Board of the University of Ottawa Heart Institute.

Sequencing and data analysis

Exons and intron/exon boundaries of the DGAT1 and DGAT2 genes were resequenced in all 1521 subjects. Primers were designed using the Exon Locator and extractor for Resequencing program (EXLR). Ten nanograms of DNA from each sample was amplified in a 10μL PCR reaction using AmpliTaq Gold and cleaned using tetra-ethylene glycol (TET). Sequencing reactions were performed using BigDye Terminator and cleaned using TET before separation on a 3730xl DNA Analyzer (ABI). All sequence variants identified were verified by manual inspection of the chromatograms and by a second independent sequencing reaction. The effect of all variants identified was predicted using two different well-known programs, PolyPhen (http://www.bork.embl-heidelberg.de/PolyPhen/) [333;334] and Sorting Intolerant From Tolerant (SIFT) (http://blocks.fhcrc.org/sift/SIFT.html) [335].

Adipose tissue biopsies and RNA isolation

Subcutaneous abdominal adipose tissue was obtained in the fasting state using a periumbilical incision and local lidocaine anesthesia. A 3mm incision was made and 5 to 10mg of tissue was collected using a liposuction canula [336;337]. Total RNA was isolated from subcutaneous adipose tissue using Trizol Reagent (Invitrogen) following the manufacturer's protocol. RNA samples were treated with amplification grade DNase I to deplete samples of any DNA contamination according to the manufacturer's instructions (Invitrogen). RNA concentration was determined spectrophotometrically using A_{260}/A_{280}. First stand cDNA was synthesized using 1μg of total RNA, 10μM random decamer primers (Ambion) and 200U of MMLV reverse transcriptase (Invitrogen) and incubated at 42°C for 1hour.

Analysis of DGAT1 and DGAT2 expression

For the RT-PCR experiments, the following primers were designed using the LightCycler software version 1 (Roche Molecular Biochemicals): DGAT1 forward 5'-

GTGGTCTTACTGGTTGAG-3' and reverse 5'- GGTAGGTCAGATTGTCCG-3'; DGAT2 forward 5'- GGCATACGGCCTTACC-3' and reverse 5'- TGTCTTGCTGGGTTGGG-3'; GAPDH 5'- GTCGGAGTCAACGGAT-3' and reverse 5'- CCACGACGTACTCAGC-3'. These primers were used with Taq DNA polymerase to amplify their respective sequences from an RT reaction on RNA isolated from human abdominal adipose tissue. The resulting amplicons were cloned in a pCR 2.1-TOPO TA cloning kit (Invitrogen). Plasmids containing each of these genes were purified from bacterial culture using the Maxiprep DNA isolation kit (Qiagen). Serial dilutions of the plasmids were used to generate a standard curve that can be used for future runs on the LightCycler to measure the mRNA abundance in experimental samples. DGAT1 and DGAT2 mRNA expression was measured by quantitative real-time PCR in a LightCycler fluorescent temperature cycler (Roche Molecular Biochemicals). One twentieth (1μL) of each RT reaction served as a template in a 20μL PCR containing 3mM $MgCl_2$, 0.5μM of each primer and 1X LightCycler DNA Master SYBR Green I mix. After initial denaturation at 95°C for 10 minutes, reactions were cycled 45 times using the following parameters: 95°C for 15 seconds, 49°C (GAPDH), 56°C (DGAT1) or 63°C (DGAT2) for 15 seconds and 72°C for 15 seconds. SYBR Green I fluorescence emission readings were monitored after each cycle. mRNA levels were quantified using the second derivative maximum method of the LightCycler Software Version 3.5 (Roche Molecular Biochemicals), which determines the crossing points of individual samples by an algorithm, which identifies the first turning point of the fluorescence curve. This turning point corresponds to the first maximum of the second derivative curve and correlates inversely with the log of the initial template concentration. Relative mRNA levels were normalized to those of GAPDH. At the end of each PCR, melting curve profiles (cooling the sample to 72°C and heating slowly to 95°C with continuous measurement of fluorescence)

were produced to confirm amplification of a single product. The specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis.

Statistical analysis

Results are shown as mean ± SEM. Where indicated, the statistical significance of the differences between groups was determined using Student's *t* test using GraphPad InStat v.3.06 statistical analysis software (GraphPad Software Inc.) or Statistical Analysis System (SAS Institute Inc.).

3.4 – Results

3.4.1 – DGAT1 rare non-synonymous variants

The exons and intron/exon boundaries of the DGAT1 gene were sequenced in 377 very obese (BMI 47.5 kg/m^2), 380 obese (BMI 37.3 kg/m^2), 374 very lean (BMI 19.5 kg/m^2) and 386 lean individuals (BMI 20.9 kg/m^2). Seven rare (<1% allele frequency) non-synonymous variants unique to either the obese or lean population were discovered (see **Table 3.1**). Five variants, G58S, R70H, L172V, S210del and A485V, were each identified in one very obese individual whereas, one variant, R219C, was identified in the obese group. Only one variant, V301A, was identified in the ultra lean group whereas none were found in the lean group. For each variant identified, the subjects affected were all heterozygous for the mutation.

Effect of the R70H variant on BMI and lipids

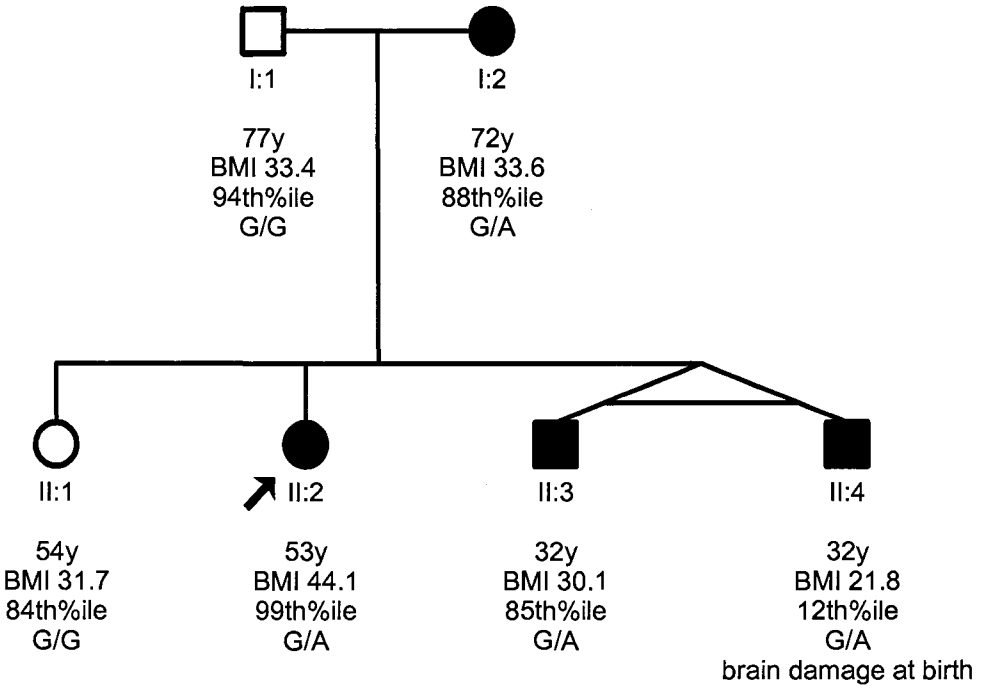
Based on the severe nature of the R70H variant as predicted by PolyPhen and SIFT, we assessed this variant for segregation in the proband's available pedigree (**Figure 3.1**). However, we found no relationship between this variant and BMI (GG: 32.6 kg/m^2 vs. GA: 32.4 kg/m^2). Nevertheless, we further examined the subjects bearing the R70H mutation to determine if this non-synonymous mutation affected plasma lipid profiles. Fasting plasma total cholesterol, TG,

Cohort	Rare non-synonymous mutations	Predicted effect	
		PolyPhen	SIFT
Very obese BMI 47.5 ± 0.4	G58S (1/306)	no prediction	affected
	R70H (1/373)	possibly damaging	affected
	L172V (1/376)	benign	tolerated
	S210del (1/367)	in-frame 3bp deletion	
	A485V (1/372)	benign	affected
Obese BMI 37.3 ± 0.3	R219C (1/380)	probably damaging	affected
Ultra lean BMI 19.5 ± 0.1	V301A (1/374)	possibly damaging	tolerated
Lean BMI 20.9 ± 0.1	none		

Table 3.1 Rare non-synonymous mutations identified by sequencing in the DGAT1 gene. Extreme populations of obese (n=761) and lean (n=760) individuals (each divided into two subpopulations) were recruited and DNA sequencing was performed. Exons and intron/exon boundaries of the DGAT1 gene were resequenced and non-synonymous variants were identified; the number of subjects with each mutation and the total number of subjects sequenced are identified in parentheses. For each variant, the effect of the mutation was analyzed using two programs, PolyPhen and SIFT and the predicted effect is shown for each single nucleotide polymorphism. BMI, body mass index given in kg/m^2.

Figure 3.1. Familial pedigree for the DGAT1 R70H mutation.

Genotypes of individuals from whom sequence data were obtained are presented. Black-shaded individuals in the pedigree are carriers of the R70H mutation and the proband is marked with an arrow. Square symbols denote males and circles denote females. Roman numerals and Arabian numerals indicate generation and sequential numbering, respectively. Current age, BMI and percentile of BMI of each member are given bellow each symbol. BMI, body mass index given in kg/m^2; %tile, percentile.


I:1
77y
BMI 33.4
94th%ile
G/G
I:2
72y
BMI 33.6
88th%ile
G/A
II:1
54y
BMI 31.7
84th%ile
G/G
II:2
53y
BMI 44.1
99th%ile
G/A
II:3
32y
BMI 30.1
85th%ile
G/A
II:4
32y
BMI 21.8
12th%ile
G/A
brain damage at birth

LDL-C, HDL-C and glucose were measured in carriers (n = 4) and non-carriers (n = 2) of the mutation in this pedigree. No difference was observed between carriers and non-carriers in regards to lipid profiles (**Table 3.2**). These studies demonstrated that the R70H variant does not affect weight or lipid levels in affected individuals. Unfortunately, the subject was unavailable for an adipose tissue biopsy and therefore, the effect of this mutation on DGAT1 and DGAT2 expression was not verified.

Effect of S210del variant on DGAT1 and DGAT2 gene expression

Another interesting variant identified in the obese group was the S210del, which results in a 3bp in-frame deletion. No prediction for the effect of this mutation was available since the programs used (PolyPhen and SIFT) only predict effects for substitutions. For this variant, only the proband was available for the study, therefore segregation of this variant with BMI or lipids was not possible. However, the effect of the variant on DGAT1 and DGAT2 mRNA expression was examined. An adipose tissue biopsy was performed on the one subject with the mutation and total RNA was extracted. DGAT1 and DGAT2 mRNA expression was quantified using semi-quantitative real-time RT-PCR. Interestingly, mRNA levels for both DGAT1 and DGAT2 were comparable to those of an obese control subject (BMI 35.5 kg/m^2) **(Figure 3.2)**.

Effect of the R219C variant on BMI and lipids

The third variant from the obese cohort that was examined is the R219C variant. This mutation was predicted to be probably damaging according to the PolyPhen program and predicted to have an effect on protein structure according to the SIFT program. Although this mutation was not found in the very obese group but only in the obese group, it still presented interesting potential effects on protein function. We therefore assessed this variant for segregation in the proband's available extended pedigree (**Figure 3.3**). Again, we found no

Sex	Current age	Relationship to proband	Genotype	BMI	Total cholesterol	TG	LDL-C	HDL-C	Glucose
F	53	proband	G/A	44,1	4,0	1,01	2,3	1,28	5,1
F	72	mother	G/A	33,6	5,1	0,94	2,8	1,87	6,0
M	32	brother twin	G/A	21,8	3,8	1,26	2,3	0,88	4,9
M	32	brother twin	G/A	30,1	5,7	2,92	3,3	1,09	5,1
			mean	**32,4**	**4,7**	**1,53**	**2,7**	**1,28**	**5,3**
M	77	father	G/G	33,4	5,4	2,92	3,2	0,83	5,6
F	54	sister	G/G	31,7	5,7	0,56	3,2	2,26	5,4
			mean	**32,6**	**5,6**	**1,74**	**3,2**	**1,55**	**5,5**
			p value	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

Table 3.2 Lipid profile values of individuals from the R70H variant family. Members of the family of the proband were recruited and fasting blood lipid levels were determined for each available member. BMI is measured in kg/m^2 and all other values are given in mmol/L. The average of each value is given for each genotype and the p-value from a student's *t* test comparing each genotype is given. BMI, body mass index; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; N.S., not significant.

Figure 3.2. Effect of DGAT1 S210del variant on DGAT expression.

Adipose tissue biopsy was performed on the proband with the S210del variant and tissue was stored at -80°C. Reverse transcription was performed on total RNA isolated from the adipose tissue and DGAT1 and DGAT2 cDNA abundance was quantified by real-time RT-PCR, normalized to GAPDH expression. Data are expressed relative to control (obese subject). Data points represent means from 6 reactions and the error bars represent the standard error of the mean.

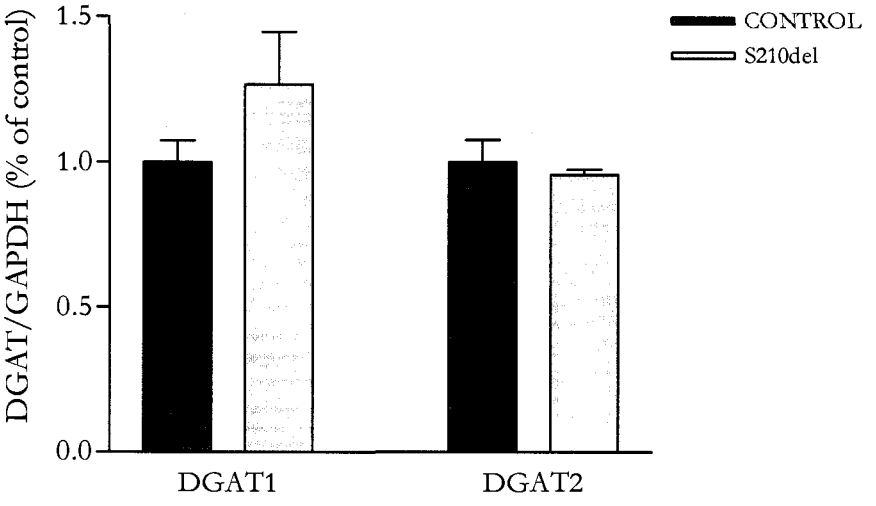
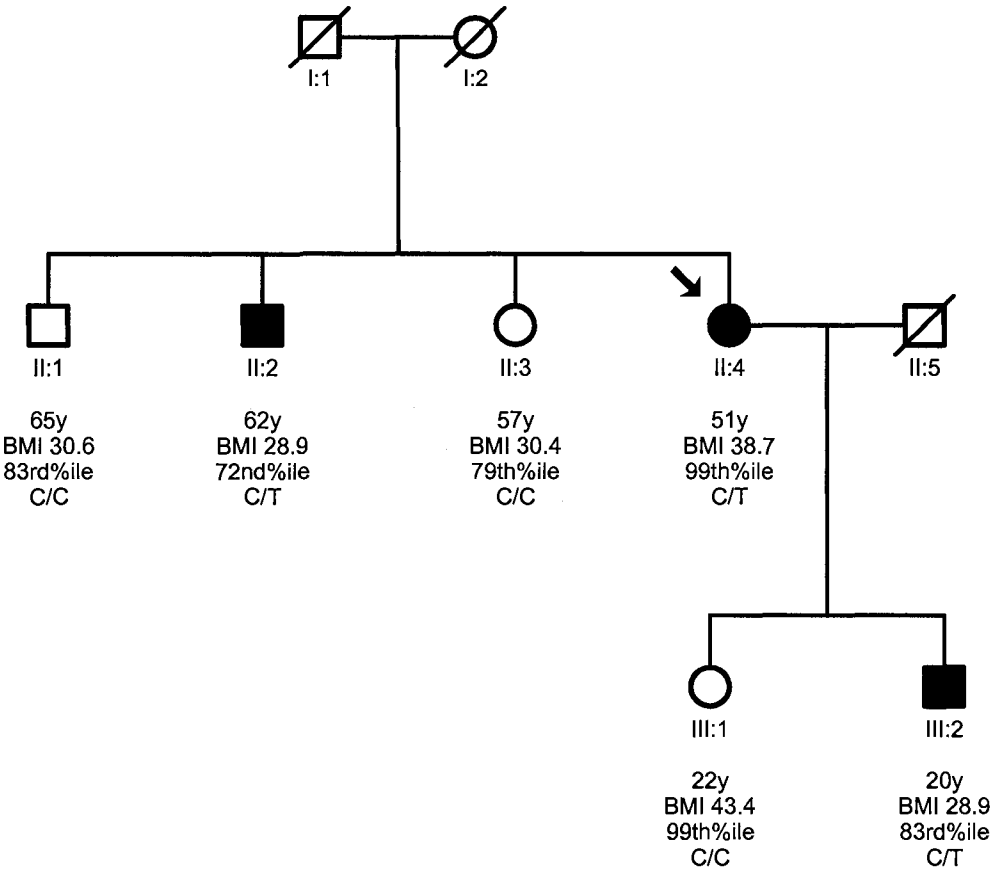

DGAT1/GAPDH (% of control)
1.5
1.0
0.5
0.0
DGAT1
DGAT2
CONTROL
S210del

Figure 3.3. Familial pedigree for the DGAT1 R219C mutation.

Genotypes of individuals from whom sequence data were obtained are presented. Black-shaded individuals in the pedigree are carriers of the R219C mutation and the proband is marked with an arrow. Square symbols denote males and circles denote females. Roman numerals and Arabian numerals indicate generation and sequential numbering, respectively. Current age, BMI and percentile of BMI of each member are given bellow each symbol. A slash across symbol indicates a deceased family member. BMI, body mass index given in kg/m^2, %tile, percentile.


I:1
I:2
II:1
65y
BMI 30.6
83rd%ile
C/C
II:2
62y
BMI 28.9
72nd%ile
C/T
II:3
57y
BMI 30.4
79th%ile
C/C
II:4
51y
BMI 38.7
99th%ile
C/T
II:5
III:1
22y
BMI 43.4
99th%ile
C/C
III:2
20y
BMI 28.9
83rd%ile
C/T

correlation between this variant and BMI (CT: 34.6 kg/m^2 vs. CC: 32.2 kg/m^2). We subsequently examined wether this variant was associated with any lipid profile values. Carriers (n = 3) and non-carriers (n = 3) had similar total cholesterol levels, triglycerides, LDL-C, HDL-C, and glucose levels (**Table 3.3**). This suggests that the R219C variant in the DGAT1 gene does not affect lipid profile values in this pedigree.

Effect of the R219C variant on DGAT1 and DGAT2 expression in adipose tissue

We also determined if the R219C mutation had any effect on the expression of DGAT1 and DGAT2 in adipose tissue. An adipose tissue biopsy was performed on the proband and adipose tissue RNA was extracted. DGAT1 and DGAT2 mRNA expression was quantified using semi-quantitative real-time RT-PCR. Quantification revealed no difference in expression level of DGAT1 or DGAT2 in the subject with the R219C mutation compared to an obese control subject (BMI 35.5 kg/m^2) (**Figure 3.4**). This further established that the R219C mutation had no discernible systemic effect.

3.4.2 – DGAT2 rare non-synonymous variants

For DGAT2, nine rare (<1% allele frequency) non-synonymous variants unique to either the obese or lean population were discovered (**Table 3.4**). Four variants, V82A, L105F, D222A and G250D, were each identified in one very obese individual. The Y285STOP variant was identified in one very obese individual and one obese individual. Three variants, A7S, C99R and R134H, were identified in the ultra lean group whereas none were identified in the lean group. For each variant identified, all subjects were heterozygous for the mutation.

Effect of the Y285STOP variant on BMI and lipids

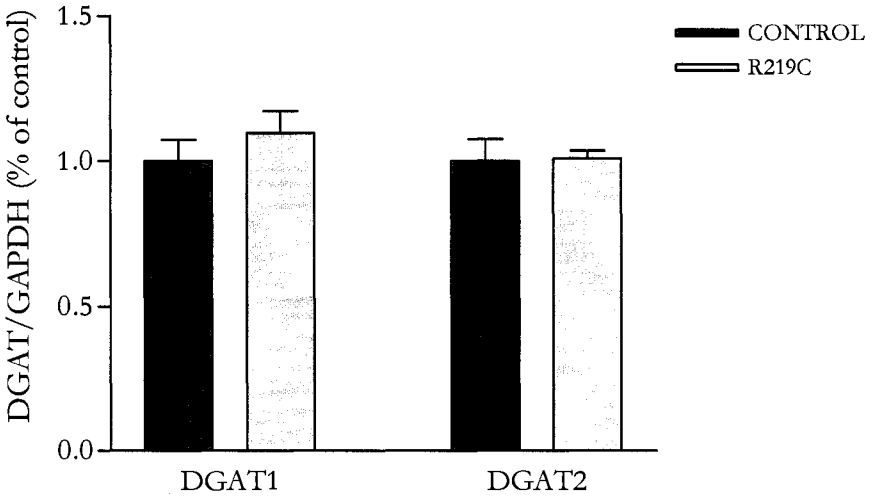
The nonsense Y285STOP mutation was examined further since it was found in 2 subjects of the obese group. It was established that the mutation resulted in a premature STOP codon, which truncated the protein at $2/3_{rds}$ of its normal length. Only one proband was

Sex	Current age	Relationship to proband	Genotype	BMI	Total cholesterol	TG	LDL-C	HDL-C	Glucose
F	51	proband	C/T	38,7	4,10	1,75	2,3	0,99	5,0
M	62	brother	C/T	28,9	6,50	1,60	4,4	1,33	4,3
M	20	son	C/T	28,9	4,30	0,91	3,0	0,93	5,5
			mean	**34,6**	**5,1**	**1,37**	**3,2**	**1,26**	**5,1**
M	65	brother	C/C	30,0	5,03	1,01	3,3	1,24	5,4
F	57	sister	C/C	30,4	5,11	1,33	2,8	1,70	5,0
F	22	daughter	C/C	43,4	5,30	1,77	3,6	0,85	5,0
			mean	**32,2**	**5,0**	**1,42**	**3,2**	**1,08**	**4,9**
			p value	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

Table 3.3 Lipid profile values of individuals from the R219C variant family. Members of the family of the proband were recruited and fasting blood lipid levels were determined for each available member. BMI is measured in kg/m^2 and all other values are given in mmol/L. The average of each value is given for each genotype and the p-value from a student's *t* test comparing each genotype is given. BMI, body mass index; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; N.S., not significant.

Figure 3.4. Effect of DGAT1 R219C variant on DGAT expression.

Adipose tissue biopsy was performed on the proband with the R219C mutation and tissue was stored at -80°C. Reverse transcription was performed on total RNA isolated from the adipose tissue and DGAT1 and DGAT2 cDNA abundance was quantified by real-time RT-PCR, normalized to GAPDH expression. Data are expressed relative to control (obese subject). Data points represent means from 6 reactions and the error bars represent the standard error of the mean.


DGAT/GAPDH (% of control)
1.5
1.0
0.5
0.0
DGAT1
DGAT2
CONTROL
R219C

Cohort	Rare non-synonymous mutations	Predicted effect	
		PolyPhen	SIFT
Very obese (BMI 47.5 ± 0.4)	V82A (1/369)	benign	tolerated
	L105F (1/376)	benign	tolerated
	D222A (1/377)	benign	tolerated
	G250D (1/369)	probably damaging	affected
	Y285STOP (1/360)	nonsense	
Obese (BMI 37.3 ± 0.3)	Y285STOP (1/373)	nonsense	
Ultra lean (BMI 19.5 ± 0.1)	A7S (1/370)	benign	affected
	C99R (1/374)	probably damaging	tolerated
	R134H (1/365)	possibly damaging	affected
Lean (BMI 20.9 ± 0.1)	none		

Table 3.4 Rare non-synonymous mutations identified by sequencing in the DGAT2 gene. Extreme populations of obese (n=761) and lean (n=760) individuals (each divided into two subpopulations) were recruited and DNA sequencing was performed. Exons and intron/exon boundaries of the DGAT2 gene were resequenced and non-synonymous variants were identified; the number of subjects with each mutation and the total number of subjects sequenced are identified in parentheses. For each variant, the effect of the mutation was analyzed using two programs, PolyPhen and SIFT and the predicted effect is shown for each single nucleotide polymorphism. BMI, body mass index given in kg/m^2.

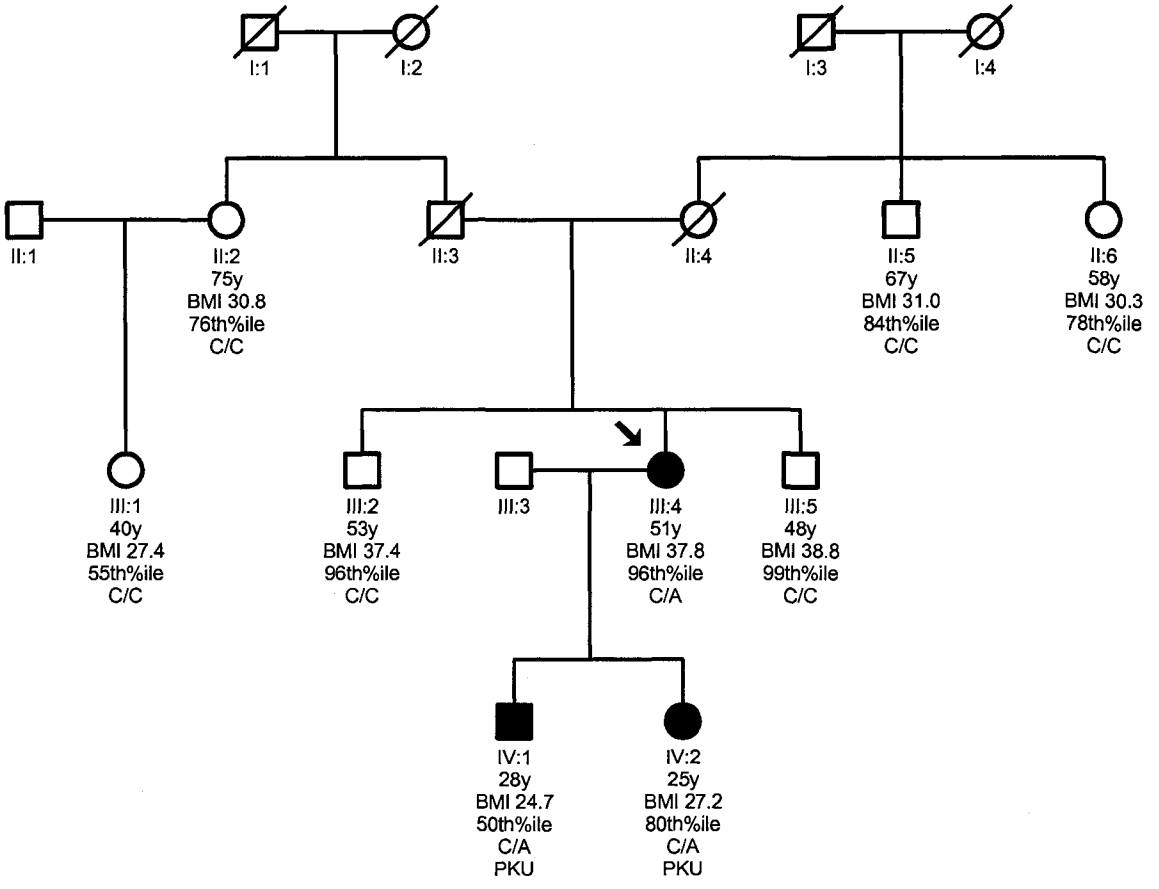
available for further studies. Therefore, the association of this mutation with BMI was examined in the available proband's extended pedigree (**Figure 3.5**). Although not reaching statistical significance, there is a trend towards lower BMI in carriers (n = 3) of the mutation compared to non-carriers (n = 7). Carriers of the mutation with the genotype CA had an average BMI of 29.9 kg/m^2 compared to 32.6 kg/m^2 in non-carriers with the genotype CC (N.S.). In addition, all plasma lipid values tended to be lower in carriers of the mutation compared to non-carriers (**Table 3.5**). Furthermore, TG levels were significantly lower in carriers (0.85 mmol/L) than in non-carriers (1.51 mmol/L) ($p<0.04$). These TG values were then evaluated according to the Canadian Heart Health Survey [338] and the percentile range for each value was determined for each subject according to age and sex. Percentile ranges represent reference values for lipids in adults in Canada and are affected by age and by sex [338]. Overall, carriers of the Y285STOP mutation seem to be in a lower TG percentile range than non-carriers. This is suggestive of lower TG synthesis and secretion in the plasma of carriers of the Y285STOP mutation regardless of age and sex.

Effect of the Y285STOP variant on DGAT1 and DGAT2 expression

We subsequently examined the effect of the Y285STOP mutation on DGAT1 and DGAT2 mRNA expression. An adipose tissue biopsy was performed on the proband and adipose tissue total RNA was extracted. DGAT1 and DGAT2 expression was quantified using real-time RT-PCR. In adipose tissue, DGAT2 expression showed a decrease compared to obese controls (n=2, average BMI 35.8 kg/m^2) (**Figure 3.6A**). Conversely, DGAT1 mRNA expression was upregulated in the proband (**Figure 3.6B**). Adipose tissue was then collected from the other two carriers) of the mutation and DGAT1 and DGAT2 expression was quantified. Both other carriers of the mutation showed a reduction in DGAT2 mRNA expression (**Figure 3.6A**).

Figure 3.5. Familial pedigree for the DGAT2 Y285STOP mutation.

Genotypes of individuals from whom sequence data were obtained are presented. Black-shaded individuals in the pedigree are carriers of the Y285STOP mutation and the proband is marked with an arrow. Square symbols denote males and circles denote females. Roman numerals and Arabian numerals indicate generation and sequential numbering, respectively. Current age, BMI and percentile of BMI of each member are given bellow each symbol. A slash across symbol indicates a deceased family member. PKU, phenylketonuria; %tile, percentile; BMI, body mass index given in kg/m^2.


I:1
I:2
I:3
I:4
II:1
II:2
75y
BMI 30.8
76th%ile
C/C
II:3
II:4
II:5
67y
BMI 31.0
84th%ile
C/C
II:6
58y
BMI 30.3
78th%ile
C/C
III:1
40y
BMI 27.4
55th%ile
C/C
III:2
53y
BMI 37.4
96th%ile
C/C
III:3
III:4
51y
BMI 37.8
96th%ile
C/A
III:5
48y
BMI 38.8
99th%ile
C/C
IV:1
28y
BMI 24.7
50th%ile
C/A
PKU
IV:2
25y
BMI 27.2
80th%ile
C/A
PKU

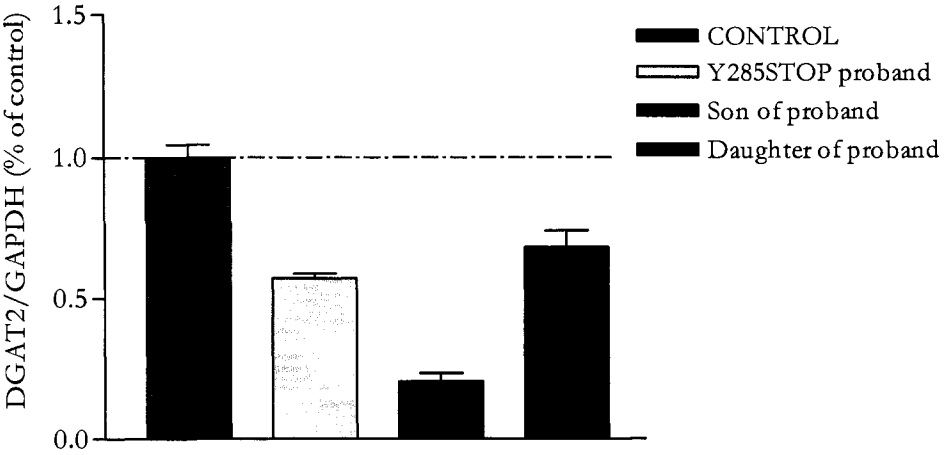
Sex	Current age	Relationship to proband	Genotype	BMI	Total-C	TG	TG %ile	LDL-C	HDL-C	Glucose
F	51	proband	C/A	37,8	6,2	1,23	25th - 50th	4,3	1,32	5,6
M	28	son	C/A	23,0	4,4	0,88	10th - 25th	3,0	1,04	4,3
F	25	daughter	C/A	22,4	3,4	0,44	< 5th	2,0	1,16	4,5
			mean	**27,7**	**4,7**	**0,85**		**3,1**	**1,17**	**4,8**
F	58	maternal aunt	C/C	30,3	6,3	1,99	50th - 75th	4,2	1,21	4,5
M	67	maternal uncle	C/C	31,0	4,8	1,56	25th - 50th	3,3	0,76	6,9
M	52	brother	C/C	37,4	6,9	1,46	25th - 50th	4,7	1,57	6,0
M	48	brother	C/C	38,8	6,0	0,92	10th - 25th	4,5	1,13	6,6
F	40	paternal 1rst cousin	C/C	27,4	5,5	1,81	75th - 90th	3,2	1,50	4,7
F	75	paternal aunt	C/C	30,8	6,4	1,33	25th - 50th	3,9	1,94	6,0
			mean	**32,6**	**6,0**	**1,51**		**4,0**	**1,35**	**5,8**
			p value	N.S.	N.S.	<0.04		N.S.	N.S.	N.S.

Table 3.5 Lipid profile values of individuals from the Y285STOP variant family. Members of the family of the proband were recruited and fasting blood lipid levels were determined for each available member. BMI is measured in kg/m^2 and all other values are given in mmol/L. The percentile range is given for TG levels according to the Canadian Heart Health Survey (). The average of each value is given for each genotype and the p-value from a two-tailed student's *t* test comparing each genotype is given. BMI, body mass index; total-C, total cholesterol; TG, triglycerides; TG %ile, TG percentile; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; N.S., not significant.

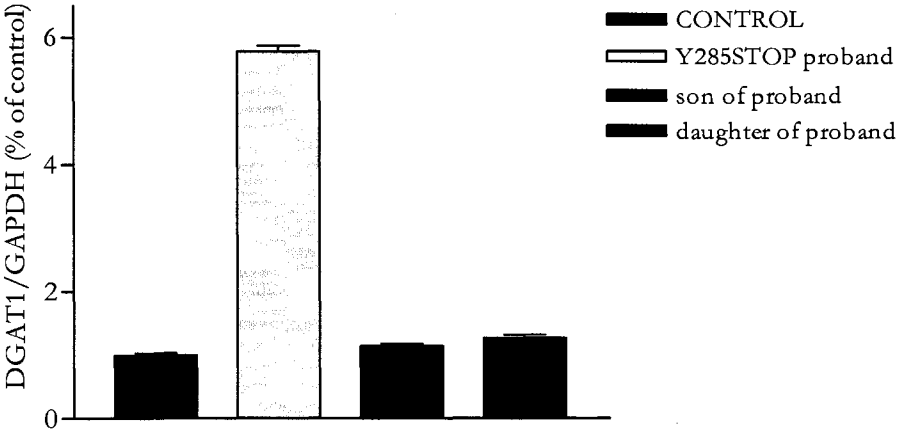
Figure 3.6. Effect of DGAT2 Y285STOP variant on DGAT expression.

Adipose tissue biopsies were performed on all the carriers (proband, son of proband and daughter of proband) of the Y285STOP mutation within the available kindred, and tissue was stored at -80°C. Reverse transcription was performed on total RNA isolated from the adipose tissue and DGAT2 (A) and DGAT1 (B) cDNA abundance was quantified by real-time RT-PCR and normalized to GAPDH expression. Data are expressed relative to controls (2 obese subject). Data points represent means from 12 reactions and the error bars represent the standard error of the mean.

A


DGAT2/GAPDH (% of control)
1.5
1.0
0.5
0.0
CONTROL
Y285STOP proband
Son of proband
Daughter of proband

B


DGAT1/GAPDH (% of control)
6
4
2
0
CONTROL
Y285STOP proband
son of proband
daughter of proband

However, DGAT1 mRNA expression did not seem to be different to that of the controls (**Figure 3.6B**).

3.5 – Discussion

DGATs have been implicated in the development of diet-induced obesity primarily through rodent-based studies. Here, we explored the potential genetic contribution of these genes to plasma lipid profiles through DNA sequencing in two extreme populations (each divided into 2 sub-populations), followed by familial and gene expression studies. Based on the relatively large sample size, it appears that rare non-synonymous changes in these genes are infrequent and their contribution to overall lipid levels in humans is likely to be minor. Specifically, DGAT1 rare variants seem unlikely to strongly contribute to lipid metabolism. Nevertheless, the findings regarding the Y285STOP mutation in DGAT2 suggest that variants in DGAT2 may affect lipid profiles and may potentially be important in the etiology of lipid disorders.

Of all the variants identified in the DGAT1 gene, which were predicted to have an effect on protein structure or function, none resulted in altered lipid profiles or difference in DGAT1 and DGAT2 gene expression. Thus, mutations in the coding region of DGAT1 seem unlikely to contribute to lipid disorders. Nevertheless, other SNPs have been identified in the DGAT1 promoter, which were correlated with BMI, high-density lipoprotein levels and blood pressure in Turkish women [317]. However, this association was not observed in Turkish men [317] or in a French obese population [318]. Therefore, more genetic studies are needed to determine the impact of common or rare variants in DGAT1 on body weight and plasma lipid values. Also, the overall contribution of DGAT1 mutations in human diseases and disorders is still under

investigation and remains to be determined. Furthermore, new variants in the promoter may also yield interesting results, which still need to be investigated.

Conversely, the Y285STOP DGAT2 nonsense mutation resulting in a premature stop codon, which truncates the protein at 2/3 of its normal length, yielded interesting results. Family genetic studies provided insights on the effect of this variant on plasma lipid levels. It was hypothesized that this DGAT2 mutation might lead to an overall decrease in plasma TG levels and potentially an increase in HDL-C levels. Interestingly, carriers of the mutation had lower TG compared to non-carriers within the same family. In addition, when looking at the percentile range for each subject, carriers seem to be in lower percentiles for TG compared to non-carriers. However, the mutation did not affect other lipid values or glucose levels. Nevertheless, carriers of this mutation did display a trend towards lower levels of all lipids, including HDL-C. Therefore, lower TG levels in this case were not associated with a concomitant increase in HDL-C levels. Further studies demonstrated that DGAT2 gene expression showed a decrease in all 3 carriers of the DGAT2 mutation. This suggests that the mutant allele of the DGAT2 gene is either not expressed or expressed at very low levels. Another possibility is that the mutant allele is expressed at normal levels but is degraded very rapidly. Nonsense-mediated decay (NMD) is a complex process, which eliminates mRNAs containing premature termination codons and therefore limits the synthesis of abnormal proteins [339]. Since this DGAT2 mutation leads to a premature stop codon, the cell could recognize the aberrant transcript and rapidly degrade it leading to an overall decrease in DGAT2 mRNA. In addition, varying levels of DGAT2 mRNA in the carriers of this mutation may be explained by the variability in NMD efficiency of each individual. Previous reports relating to other genes have shown that individuals with identical genetic mutations can have varying severity of the associated phenotype due to different efficiency in the NMD process [340].

However this remains to be determined for this DGAT2 mutation. Detection of the mutant truncated protein would indicate that a different mechanism of transcription regulation is in place for this particular mutation. However, the Y285STOP mutation in the DGAT2 gene appears to be associated with lower levels of mRNA expression compared to controls.

In addition, there was a potential reciprocal upregulation of DGAT1 mRNA in some carriers of the DGAT2 mutation. This was especially evident in the proband, where there was a 6-fold increase in DGAT1 mRNA expression. Interestingly, the proband had the highest level of TG compared to other carriers of the mutation. This may partly be explained by age or exercise although diet may also play a role. With age, TG levels tend to rise and may explain the higher TG level in the proband [338]. Also, differences in level of physical activity in the proband compared to her children may explain the differences. Since adipose tissue storage of TG is influenced by physical activity [341], plasma TG may also reflect the level of exercise in the different individuals. Diet may also affect these lipid values. Dietary restriction is not practiced by the proband as indicated by a lifestyle questionnaire. Conversely, both children of the proband suffer from phenylketonuria and abide by a strict diet. A major component of their diet is protein shakes, which contain very little fat. Therefore, the difference in diet might explain, in part, the differences in TG levels. This may also explain the robust reciprocal upregulation of DGAT1 in the proband. Because of the overload of dietary TG in the case of the proband, the body would respond by upregulation DGAT1 because of the partial DGAT2 deficiency. This is not as evident in the other two carriers of the mutation probably due to the fact that their diets are relatively low in fat content. Furthermore, the 6-fold upregulation of DGAT1 mRNA in the proband may not be associated with an equivalent increase in protein expression or activity. Previous studies have shown that a 20-fold increase in DGAT1 mRNA levels results in only a 2-fold increase in protein levels due to a tight post-translational regulation

[288]. Therefore, DGAT1 overexpression may only be sufficient to bring overall DGAT activity back to normal levels. There are some studies that have shown that DGAT2 is reciprocally upregulated in DGAT1-deficient ob/ob mice [276]. However, this is the first time a naturally occurring mutation in DGAT2 has been associated with a potential reciprocal upregulation in DGAT1 expression. Further studies are needed to fully determine if DGAT1 and DGAT2 are coordinately regulated.

Overall, none of the identified DGAT1 variants affected BMI, lipid values or DGAT expression levels. Conversely, the Y285STOP DGAT2 nonsense variant was associated with lower TG levels and reduced DGAT2 mRNA expression. The overall effect of this mutation needs to be examined in a larger population since these studies were only carried out in one family. However, common variants in the DGAT genes seem unlikely to contribute to lipid disorders. Furthermore, several experiments suggest that there are several mechanisms for TG synthesis. DGAT1-deficient mice still synthesize TG and show only a 20% reduction in total carcass TG [275]. In addition, there is redundancy in DGAT activity [260] such that several other enzymes with DGAT activity have been identified [263;300]. Therefore, it may be possible that these enzymes compensate for the functional abnormalities in the DGAT enzymes resulting from SNPs. Conversely, many SNPs may not have an effect on expression or activity of the related DGAT enzymes. Also, the effect of SNPs may only present themselves if other aggravating factors are present, such as a high fat diet with TG overload or other lipid metabolism imbalances. Nevertheless, unidentified mutations in DGAT genes may still be linked to altered lipid profiles and may potentially be associated with resistance to diet-induced obesity as observed in DGAT1-deficient mice [279]. Therefore, identifying common or rare variants in DGAT genes may still prove useful in establishing a link between obesity and altered lipid metabolism.

Chapter 4 – Conclusions and future directions

Obesity is a complex disease that is associated with numerous co-morbidities, namely insulin resistance and T2DM. This work has attempted to address potential therapeutic targets for the treatment of obesity and related conditions through the study of the two distinct characteristics of this disease.

The first aspect investigated was the role of obesity-induced inflammation on adiponectin gene expression. Adiponectin is a protein secreted mainly by adipocytes, which is known to promote insulin sensitivity. Its downregulation in the obese state has been shown to lead to the development of obesity-induced insulin resistance and T2DM. These studies have demonstrated that adiponectin is downregulated by IL-6 in a dose- and time-dependent manner through the p44/42 MAP kinase pathway. Also, *in vivo* studies have shown that a high fat/high glucose diet induces the expression of adiponectin, more so in female mice than male mice, and that the removal of the IL-6 allele leads to increased adiponectin levels. Although modulating adiponectin may not represent a means of treating obesity, increasing its expression could protect against obesity-induced complications such as insulin resistance and T2DM. Conversely, IL-6, which is secreted by adipose tissue, represents an important contributor to systemic inflammation associated with obesity. Therefore, reducing inflammation associated with obesity and subsequently increasing adiponectin expression could represent a potential therapy for reducing the complications associated with obesity. The overall effect would be increased insulin sensitivity in obese patients and a more normal glycemic control.

Further experiments to be carried out in regards to adiponectin gene expression would be to characterize the active element within the adiponectin promoter responsible for the IL-6 induced downregulation of adiponectin expression. By better understanding the specific

regulation of adiponectin gene expression in situations of high inflammation, insights could be gained in determining specific inhibitors, which would selectively block the negative effects of inflammation associated with obesity.

The second aspect investigated was the role of DGAT enzymes on lipid homeostasis. DGAT enzymes are responsible for the synthesis of TG mainly in adipose tissue, liver and the small intestine. They are also thought to be involved in the development of obesity where overabundance of lipids in fat-enriched diets leads to increased TG synthesis and storage in adipocytes. The present studies were the first to demonstrate that rare non-synonymous mutations in the DGAT2 gene can be associated with an altered lipid profile; in particular, the Y285STOP mutation in DGAT2, which potentially leads to decreased TG synthesis and plasma levels. The mutation also correlated with a reduction in DGAT2 expression possibly through a mechanism known as nonsense-mediated decay. Furthermore, DGAT1 and DGAT2 may be coordinately regulated such that one enzyme can compensate for the loss of the other. This reciprocal regulation could be dependent of dietary fat content, age, level of exercise and many other factors that can affect lipid homeostasis. In our study, we found that in general, naturally occurring mutations in the DGAT1 and DGAT2 genes did not correlate with BMI and did not protect against obesity. Further studies are necessary to fully establish the role of the Y285STOP DGAT2 mutation and to determine the prevalence of this mutation in different populations. Furthermore, DGAT1 still represents a potential therapeutic target for the treatment of obesity as demonstrated by DGAT1-deficient mice, which are protected against diet-induced obesity. Since most studies have been carried out in mice, more human genetic and epidemiologic studies are necessary to further characterize the role of DGAT enzymes on lipid homeostasis, obesity and potentially cardiovascular diseases.

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Contributions of collaborators

All experiments and results presented in this thesis are the work of the thesis author, Natalie Cadotte except for the following:

- Human primary adipocytes were isolated with the help of Thet Naing
- All mouse studies were carried out with the help of Paulina Lau
- Recruitment for obese subjects was done in collaboration with Dr. Robert Dent of the Ottawa Weight Management Clinic
- Recruitment for lean subjects was done in collaboration with GlaxoSmithKline and Sybil Hebert
- Sequencing and data analysis was performed in collaboration with the Lawrence Berkeley National Laboratories and the Joint Genome Institute in California
- Plasma lipid and glucose levels were measured at the Ottawa Hospital Civic Laboratory.
- Adipose tissue biopsies were performed by Dr. Ruth McPherson and Heather Doelle

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Non Peer-Reviewed Conference Presentations:

1. Arnaout, A., Ahituv, N., **Michaud, N.**, Kavaslar, N., Pennacchio, L.A., McPherson, R. Genetic Variation in DGAT1 and DGAT2 in Obese and Lean Individuals. The Endocrine Society's 88th Annual Meeting 2006, Boston, MA, June 24-27. Poster

2. **Michaud, N.**, Gauthier, A. & McPherson, R. Interleukin-6-Induced Down-regulation of Adiponectin Gene Expression. Canadian Lipoprotein Conference (CLC) 2004, Banff, AB, October 21-24. Poster