GLOBAL GENE EXPRESSION PROFILES OF SUBCUTANEOUS ADIPOSE TISSUE IN OBESE AND NON-OBESE YOUNG MEN

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Global gene expression profiles of subcutaneous adipose tissue in obese and non-obese young men.

By: Curtis R. French

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Abstract

Objective: The current study was designed to compare global gene expression profiles of subcutaneous adipose tissue (SCAT) from obese and non-obese young men using whole human genome microarrays.

Research methods and procedures: Sixteen healthy young men aged 20-27 participated in the study. Total RNA was isolated from SCAT, and competitively hybridized with a universal human reference RNA to Agilent's 44K whole human genome chip. A paired study design was used; ratios for each obese individual were directly compared to those of a matched non-obese individual.

Results and Discussion: We identified 114 significantly up-regulated and 204 down-regulated genes and transcripts in obese SCAT, 232 of which have defined names. For 181 of the genes with defined names, adipose tissue expression has not been reported previously. Gene Ontology (GO) analysis revealed an over-representation of genes involved in apoptosis, cell adhesion, cellular morphogenesis, and cell growth as up regulated in obese subjects. Genes with functions in the mitochondria, including genes involved in ATP coupled electron transport, were down regulated in obese subjects. Clustering analysis identified a sub-set of 27 genes that was able to accurately distinguish obese from non-obese subjects. Moreover, numerous genes that may influence the rate of lipolysis were also found to be differentially expressed.

Key Words: adiposity, whole human genome microarrays, cluster analysis, lipolysis.

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List of Abbreviations and symbols

ADRB2:	adrenergic β-2 receptor
ADRBK2:	adrenergic β receptor kinase 2
AGPAT2:	1-acylglycerol-3-phosphate O-acyltransferase 2 protein
AGPAT4:	1-acylglycerol-3-phosphate O-acyltransferase 4 protein
AMP:	Adenosine monophosphate
AMPK:	adenosine monophosphate activated protein kinase
ATP:	Adenosine triphosphate
ATP5S:	ATP synthase, S subunit
BF:	Body fat
BIA:	Biolelectrical impedance analysis
BMI :	Body mass index
cAMP:	Cyclic Adenosine monophosphate
CART:	Cocaine and amphetamine regulated transcript
cATP:	Cyclic Adenosine triphosphate
CCK:	Cholecystokinin
cDNA :	Complementary deoxyribonucleic acid
CEPBA:	CCAAT/enhancer binding protein (C/EBP), alpha
CREB:	Cyclic AMP response element binding protein
CREB3L3:	Cyclic AMP response element binding protein 3-like-3
CY3:	Cyanine 3
CY5:	Cyanine 5

C6orf107:	Chromosome 6, open reading frame 7
DNTP:	Deoxyribonucleotide triphosphate
DNA:	Deoxyribonucleic acid
DTT:	Dithiothieitol
DXA:	Dual energy X-ray absorptiometry
EST:	Expressed sequence tag
GPR109B:	Nicotinic acid receptor
GRP10:	G-protein coupled receptor 10
HDL-C:	High density lipoprotein cholesterol
HOMA-R:	Homeostatic assessment model for insulin resistance
HOX:	Homeotic family of genes
HSL:	Hormone sensitive lipase
IGF:	Insulin-like growth factor
LDL-C:	Low density lipoprotein cholesterol
LPD:	Lipid storage droplet
MCH:	Melanin-concentrating hormone
mRNA:	Messenger ribonucleic acid
MMLV-RT:	Moloney murine leukemia virus reverse transcriptase
NADH:	Reduced nicotinamide adenine dinucleotide
NDUFB5:	NADH:ubiquinone oxidoreductase (Complex I), subunit 5
NDUFB6:	NADH:ubiquinone oxidoreductase (Complex I), subunit 6
NEFA:	Non-esterified fatty acid
NL:	Newfoundland and Labrador

NPY:	Neuropeptide Y
NPYR:	Neuropeptide Y receptor
NPR3:	Natriuetic peptide receptor 3
NTP:	Nucleotide triphosphate
PEG:	Polyethylene glycol
PKA:	Protein kinase A
PPP2A:	Protein phosphatase 2A
PPP1CA:	Protein phosphatase 1, catalytic subunit alpha
PLTP:	Phospholipid transfer protein
PRKAR2A:	Protein kinase A, regulatory subunit 2 alpha
PRKAR2B:	Protein kinase A, regulatory subunit 2 beta
RNA:	Ribonucleic acid
SIRT1:	Sirtuin (silent mating type information regulation 2 homolog) 1
SCAT:	Subcutaneous adipose tissue
SNP:	Single nucleotide polymorphism
SSC:	saline-sodium citrate Buffer
TG:	Triglyceride
TORC2:	Transducer of CREB 2
UCP:	Uncopupling protein
VAT:	Visceral adipose tissue
VLCD:	Very low caloric diet
Wnt:	Family of genes ortholagous to the Drosophila *wingless* gene

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Chapter 1: Introduction

Chapter 1.1: Introduction to obesity

Obesity is quickly becoming one of the major health concerns in the developed world. Between 1985 and 2001, the number of overweight Canadian women increased by 32%, while the incidence of obesity doubled (Health Canada report, 2003). This trend was even more evident in men, where the number of overweight individuals rose by 41% and the incidence of obesity almost tripled (Health Canada report, 2003). This has put an enormous strain on the Canadian healthcare system. It has been calculated that in 2001 alone, 5.3 billion dollars was spent treating the consequences of obesity, accounting for 2.2 % of all Canadian healthcare costs (Katzmarzyk *et al.,* 2004).

Once thought to be a condition of behavior, that is, too much food intake and not enough exercise, obesity is now recognized as a chronic disease with molecular causes. This change in point of view has spurred a wave of research into the genes that may cause or predispose an individual to obesity and associated complications such as insulin resistance and type 2 diabetes. Defining genes that contribute to obesity has provided many problems for researchers. Obesity can result from single gene mutations, such as those in the leptin gene (Montague et al., 1997) and the melanocortin-4 receptor gene (Lubrano-Berthelier et al., 2004), but they are extremely rare in the population (Snyder et al., 2004). Only 173 human obesity cases due to single gene mutations in ten different genes have been reported (Perusse *et al.,* 2005). The common form of obesity is thought to be a complex disease, and results from the combined effects of many genes and environmental factors. In this model, many genes can have a small influence on the status

of adiposity in a given individual or predispose an individual to becoming obese, and the effect of these genes can be influenced by environmental factors.

To further complicate the study of obesity, many other disorders tend to be common in individuals who are obese, and these disorders may have separate or overlapping genetic causes or predispositions. Insulin resistance is common in obese individuals, and can lead to numerous complications such as the metabolic syndrome and type 2 diabetes. The metabolic syndrome is collection of metabolic abnormalities that include glucose intolerance, insulin resistance, central obesity, dyslipidaemia, and hypertension (Eckel *et al.,* 2005). The above are all risk factors for the development of cardiovascular disease, a condition that is also common in obese individuals. Insulin resistance and obesity are also the primary risk factors for the development of type two diabetes. A strong correlation between obesity, defined by body mass index (BMI), and the relative risk for diabetes has been shown in both men (Chan *et al.,* 1994) and women (Colditz *et al.,* 1995) **(Figure 1).** The study of the genetic control of obesity and related metabolic disorders will thus help in the understanding and treatment of individuals who may be predisposed to develop these conditions.

Chapter 1.2: Adipose tissue and its active role in the etiology of obesity

Adipose tissue has long been considered a passive, inactive tissue simply present for the storage of triglycerides (Kim *et al.,* 2000). However the discovery of numerous endocrine, paracrine and autocrine functions have shed new light on adipose tissue, and the active role it may play in the development of obesity and related metabolic disorders (Mohamed-Ali *et al.,* 1998). A number of factors are known to be predominately

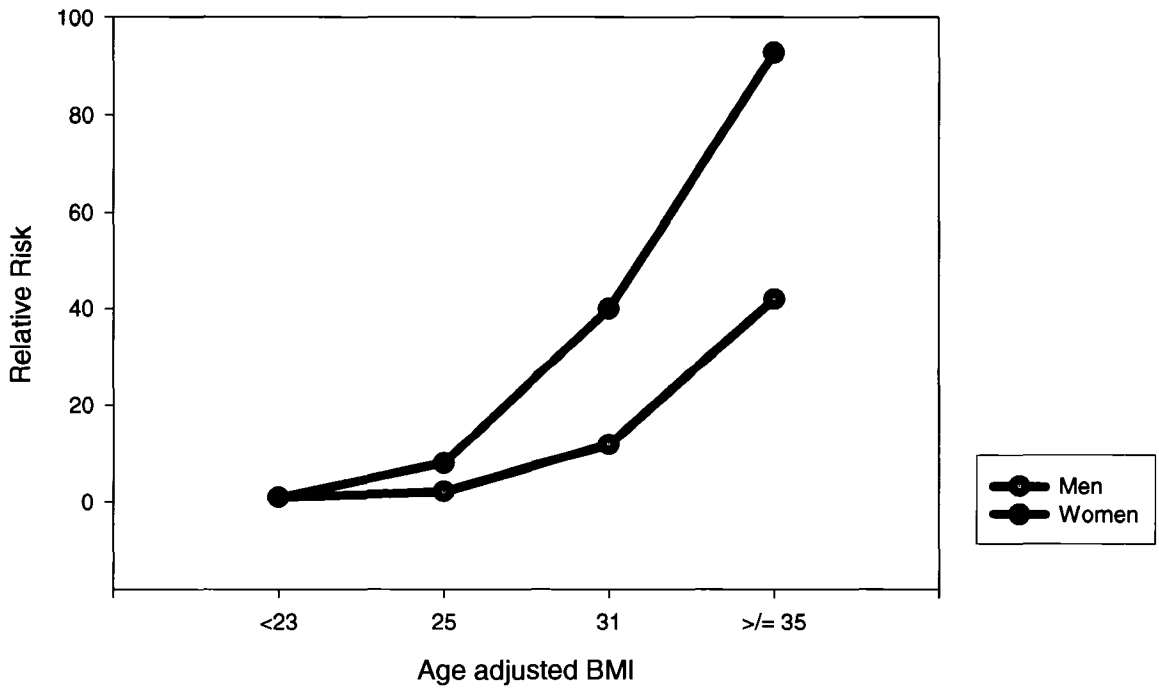

Figure 1: Age adjusted risk of developing type 2 diabetes according to body mass index. Adapted from (Sharma, 2003), original data from (Chan *et al.*, 1994 and Colditz *et al.*, 1995).

expressed and secreted by adipose tissue (**Table 1**), allowing for communication between adipose tissue and other tissues and organs. Thus the study of obesity has, to a large degree, become the study of adipose tissue. Defining how adipose tissue works on the genetic level and how it functions differently in lean and obese individuals will provide obesity candidate genes that may act as targets for the development of obesity related drugs.

The use of animal models has greatly increased our knowledge about how adipose tissue functions, and how important it is to maintain a healthy level of adipose tissue. Moitra and associates (1998) created a transgenic mouse that lacks adipose tissue. These mice express a dominant-negative protein, termed A-ZIP/F, under the control of the adipose-specific aP2 enhancer/promoter. A-ZIP/FF mice were diabetic, had elevated serum glucose, insulin, free fatty acids and triglycerides (TG). Furthermore, the mice had reduced fecundity and premature death. In humans, mutations in genes such as 1-acylglycerol-3-phosphate O-acyltransferase 2 (*AGPAT2*) cause a similar phenotype, as these individuals have reduced fat, or a complete lack of fat tissue. Similar to the mouse model, these individuals have early onset diabetes, hypertriglyceridema, and severe insulin resistance (Simha *et al.,* 2003). Thus, the profound effects of lacking adipose tissue reiterates its importance in maintaining energy homeostasis and the overall health of the body.

Table 1: Proteins that are predominately expressed and/or secreted from adipose tissue that have been implicated in the pathophysiology of obesity.

Proteins released	Omental fat	Sub-cutaneous fat
Lipoprotein lipase	+	+
Acylation stimulating protein	+	++
Cholesterol-ester transfer protein	++	+
Retinol binding protein	+	+
Plasminogen activator inhibitor-1	++	+
Estrogens	+	+
Leptin	+	++
Angiotensinogen	++	+
Adiponectin	++	+
Tumor necrosis factor α	+	+
Interleukin-6	++	+
Insulin-like growth factor-1	+	+
Insulin-like growth factor binding protein-3	+	+
Monobutyrin	+	+
Peroxisomal proliferators activated receptor-γ	+	++
Uncoupling protein-1	+	++
Uncoupling protein-2	+	++

++ indicates increase expression when compared to the other fat deposit.

Adapted from (Wajchenberg, 2000).

1.3: Overview of lipolysis

Lipolysis is arguably one of the most important processes that occurs in adipose tissue. This involves the break down of triglycerides in the lipid storage droplet, and the subsequent release of free fatty acids and glycerol. Catecholamines are powerful regulators of lipolysis in human adipocytes through stimulatory β1 and β2 adrenoreceptors and inhibitory α2 adrenoreceptors (Fain *et al.,* 1983). A third β adrenoreceptor (β3) can also stimulate lipolysis, but is only active in the omental fat deposit. On the other hand, lipolysis can be inhibited in human adipocytes through the inhibitory action of the insulin receptor (reviewed by Large *et al.,* 1998) and the adenosine receptor (Schoelch *et al.*, 2004). Lipolysis may also be inhibited by the AMP activated protein kinase (AMPK) in human adipocytes (Winder *et al.,* 1999).

The stimulatory action of catecholamines induces lipolysis through the second messenger cAMP. This molecule activates the cAMP dependant protein kinase, otherwise know as protein kinase A (PKA). The PKA holoenzyme is a tetramer consisting of two regulatory and two catalytic subunits. In mice, at least four isoforms of the regulatory subunit are transcribed (RIα, RIβ, RIIα, and RIIβ), and two isoforms of the catalytic subunit are transcribed (Cα and Cβ) (Planas *et al.,* 1999). Binding of cAMP causes the dissociation of the regulatory subunits, allowing free catalytic subunits to phosphorylate downstream targets. Different isoforms of the regulatory subunits have tissue specific patterns of expression, leading to the belief that isoform diversity confers at least some specificity by assembling different holoenzyme complexes in different tissues or different parts of the cell (Planas *et al.,* 1999). With respect to lipolysis, the RIIβ subunit may be

of importance as it is the most abundant subunit expressed in the adipose tissue and has limited expression elsewhere (Planas *et al.,* 1999).

Upon activation by cAMP, PKA can phosphorylate hormone sensitive lipase (HSL) at three distinct sites: serine 563, 659, and 660 (reviewed by Holm, 2003), which leads to activation and translocation to the surface of the lipid storage droplet. HSL plays a crucial role in the hydrolysis of triacylglycerol and cholesteryl esters in various tissues including adipose tissues (Sekiya *et al.,* 2004). PKA can also phosphorylate perilipin, a protein that acts as a barrier to protect the lipid droplet from HSL. Phosphorylation by PKA causes perilipin to translocate away from the lipid droplet (Zhang *et al.,* 2003), allowing HSL access to the stored triglycerides. In opposition to this process, AMPK can phosphorylate HSL at a fourth site (serine 565) and this is believed to inhibit PKA's ability to phosphorylate and activate HSL (Holm *et al.,* 2003). The process of lipolysis may also be inhibited through the actions of insulin and insulin-like growth factors (IGFs). Binding of insulin and other related growth factors to their receptors has an inhibitory effect on the concentration of cAMP (Holm, 2003), leading to less activation of PKA and thus less activated HSL and perilipin. An overview of the lipolytic pathway is seen in **Figure 2**.

In vitro studies have shown that alterations in the sensitivity of lipolytic induction may contribute to obesity. For example, variations in the DNA sequence of beta adrenoreceptors have been shown to affect function and their ability to induce lipolysis, and these variations also associate with obesity (reviewed by Arner, 2001). Furthermore, genes involved in lipolytic induction such as the natriuetic peptide receptor 3 (*NPR3*) and the adrenergic β2 receptor (*ADRB2*), were found to have decreased expression in human

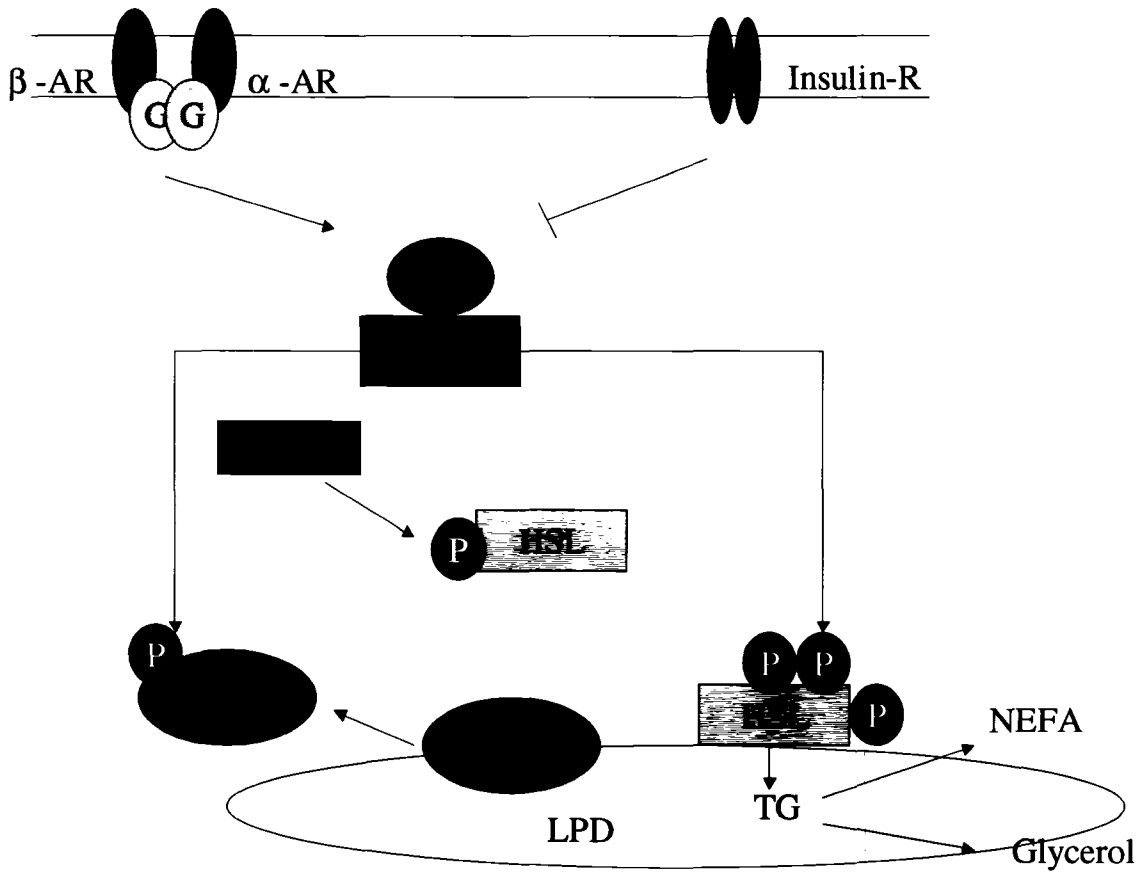

Figure 2: Overview of the lipolytic pathway. Stimulation via α or β adrenoreceptors causes accumulation of cAMP, which binds and activates protein kinase A (PKA). PKA can then phosphorylate perilipin causing it to translocate away from the lipid storage droplet (LPD). Activated PKA can also phosphorylate hormone sensitive lipase at three sites, causing activation of HSL, and subsequent hydroloysis of trigylcerides (TG), forming glycerol and non-esterified fatty acids (NEFA). Signaling via the insulin receptor inhibits this process via lowering cAMP levels. The AMP activated protein kinase (AMPK) can also phosphorylate HSL at a fourth residue, presumably blocking phosphorylation and/or activation by PKA.

visceral adipose tissue from obese males, while genes that inhibit lipolysis, such as the neuropeptide Y receptor (*NPYR*) and the adrenergic β receptor kinase 2 (*ADRBK2*) have increased expression (Gomez-Ambrosi *et al.,* 2004). The study of animal models also adds strength to this observation. RIIβ knockout mice (knock out of the principle regulatory PKA subunit) are lean, resistant to diet induced obesity, and have a blunted capacity for catecholamine induced lipolysis (Planas *et al.,* 1999). Since obesity may result from a reduced rate of lipolysis, thereby allowing for the excessive accumulation of triglyceride in the lipid storage droplet, interventions that increase the rate of lipolysis may represent a means of treatment for obesity.

Chapter 1.4: Energy charge and the mitochondrial involvement with obesity

It has long been believed that the production of ATP within the mitochondria of adipocytes can affect the accumulation or release of body fat stores (Rossmeisl *et al.,* 2004). Supporting evidence for this theory was gained by the generation of transgenic mice that ectopically express uncoupling proteins, which reduce the amount of ATP produced in the mitochondria by uncoupling ATP synthesis from electron transport. Ectopic expression of uncoupling protein 1 in the white adipose tissue of mice results in less ATP production as well as protection from dietary and genetic induced obesity (Rossmeisl *et al.,* 2004). Similarly, levels of UCP in brown adipose tissue were found to be lower in obese humans when compared to lean controls (Oberkofler *et al.,* 1997), which is consistent with reduced energy expenditure in obese individuals. However it remains to be determined if reduced expression of uncoupling proteins plays a significant

role in the pathogenesis of obesity as brown adipose tissue accounts for only a small percent of adipose tissue in adults.

In order for energy production in the mitochondria to be involved in the pathophysiology of obesity, there must be a link between cellular ATP concentration and the rate of lipolysis and/or lipogenesis. This link may be provided through the actions of AMPK. This enzyme responds to changes in the AMP/ATP ratio. A decrease in cellular ATP concentration activates this enzyme which can then proceed to stimulate processes that produce ATP, while inhibiting processes that consume ATP (reviewed by Hardie *et al.,* 2001). AMPK has been shown to inhibit both lipolysis and lipogenesis in adipose tissue (Winder *et al.,* 1999), and will do so under conditions of low cellular ATP levels. Thus, the generation of cellular energy in adipose tissue mitochondria may have implications on the status of whole body energy balance and it is therefore plausible that perturbation of this system can lead to obesity. Similarly, drugs that can target this system may be useful in the treatment of obesity.

Chapter 1.5: Microarrays as useful tools for studying obesity.

The advent of microarrays has provided a new tool for the study of complex diseases such as obesity. Microarrays facilitate the simultaneous quantification of thousands of different messenger RNA (mRNA) molecules in a single experiment. This aids in the study of complex diseases by determining what genes are expressed in a give cell, tissue, or organ, and how their expression patterns may be altered in the disease state. This is an especially powerful tool for the study of obesity as a large number of genes in a wide range of processes have been implicated in its etiology. The basic

principal of this technique relies on competitive hybridization of mRNA molecules to cDNAs or synthetic oligonucleotides on a glass slide. Detection of the mRNA molecules is accomplished by attaching dye molecules to the mRNA prior to hybridization, followed by detection of the fluorescent signal post hybridization. It is now possible to assay all known genes in the human genome in a single experiment, using mRNA from adipose tissue, or other tissue/organs that may be involved in the development of obesity. This allows for the detection of genes and the generation of genetic profiles that may be pathogenic with respect to obesity, or predispose an individual to obesity.

Chapter 1.6: Adipose tissue microarray studies

A number of microarray studies reporting gene expression profiles in adipose tissue have been reported in animal models and humans. These studies have shown that genes involved in lipolysis, as well as many other biological processes, may be involved in the pathophysiology of obesity (Gomez-Ambrosi *et al.,* 2004, Castro-Chavez *et al.,* 2003, and others). To date, the focus has mainly been on the visceral adipose tissue depot, as it has been established that the accumulation of visceral fat is associated with a higher risk for obesity related phenotypes (Mohamed-Ali *et al.,* 1998), such as hypertension, insulin resistance, impaired insulin secretion, type 2 diabetes, cardiovascular disease, and hyperlipidemia (Montague *et al.,* 1997, Lubrano-Berthelier *et al.,* 2004). Yang and associates (2003) generated gene expression profiles from visceral adipose tissue and discovered that a number of genes encoding receptors for appetite regulating peptides are produced in this depot, including *leptin, melanin concentrating hormone* (*MCH*), *cholecystokinin* (*CCK*), *neuropeptide Y* (*NPY*), and *orexin.* This

suggests a regulation of appetite by adipose tissue through autocrine/paracrine mechanisms (Yang *et al.,* 2003). While generation of expression profiles for visceral adipose tissue may serve as a reference for future physiological and pathophysiological studies concerning obesity (Yang *et al.,* 2003), comparisons of differential expression profiles in fat depots and in lean and obese individuals may be much more informative in determining specific obesity causing genes.

Differential expression profiles from subcutaneous and visceral fat depots have also been generated as a means to elucidate obesity candidate genes, and to determine the genetic factors influencing metabolic differences between the two depots. Profiles generated using Affymetrix human U13A chips revealed a total of 347 transcripts that differ in expression between the two fat depots (Vohl *et al.,* 2004). These include genes involved in *Wnt* signaling, lipolytic stimuli, cytokine secretion, as well as *CEPBA* and *HOX* genes. Furthermore, the gene encoding phospholipid transfer protein (*PLTP*) was shown to be up-regulated in visceral when compared to subcutaneous adipose tissue of obese individuals (Linder *et al.,* 2004). Evidence suggests that expression of *PLTP* in visceral adipose tissue influences plasma concentrations of PLTP, which has been associated with plasma HDL and insulin resistance (Riemens *et al.,* 1998). Although correlations between the expression of genes in visceral and subcutaneous depots is high (Vohl *et al.,* 2004), the detection of numerous transcripts differentially expressed between depots supports the idea that the two depots may function in different ways with respect to obesity.

Studies comparing expression profiles of visceral adipose tissue between lean and obese individuals have yielded numerous obesity candidate genes with highly diverse

functional roles. Genes involved in lipolysis activation were down-regulated in visceral adipose tissue from obese individuals, while genes involved in lipolysis repression were up-regulated (Gomez-Ambrosi *et al.,* 2004). This suggests a genetic influence on the rate of lipolysis that differs between lean and obese people. Furthermore, genes involved in processes such as cell proliferation, immune response, angiogenesis, metabolism, and signal transduction may play a role in the etiology of obesity as they have also been shown to be differentially regulated between visceral adipose tissue from lean and obese persons (Gomez-Ambrosi *et al.,* 2004). Visceral adipose tissue expression profiles have also shown that complement proteins are up-regulated in obese persons, which suggests that obesity may result, in part, from an innate immune response in the adipose tissue (Gabrielsson *et al.,* 2003).

The role of subcutaneous adipose tissue gene expression in the etiology of obesity has not been rigorously studied. However, it is accepted that this depot is more sensitive to the antilipolytic effects of insulin (Linder *et al.,* 2004), and has higher levels of leptin expression (Vohl *et al.,* 2004), which may indicate a role for this depot in the development of obesity and related metabolic disorders. Investigations into genes expressed in subcutaneous adipose tissue have yielded numerous obesity candidate genes that, similar to the visceral depot, display a wide range of biological functions. These include regulation of gene expression, cell structure and motility, cell signaling, cell division, and cell defense (Gabrielsson, 2000).

Recently, changes in expression profiles of subcutaneous adipose tissue in response to a very low caloric diet (VLCD) have been generated in lean and obese women. Two distinct clusters were revealed, one cluster of genes up-regulated by the

VLCD, and one that was repressed by VLCD (Clement *et al.,* 2004). The fact the gene expression in SCAT can be induced or repressed in response to alterations in energy balance provides supporting evidence of a role for this depot in the development of obesity. However much work is still needed regarding SCAT and its role in the pathophysiology of obesity. First, we lack information concerning the comprehensive gene expression profile of SCAT in both lean and obese humans at baseline, which is critical for further studies. Second, can the gene expression profiles generated from subcutaneous adipose tissue define lean and obese individuals at baseline? Although Clement and associates show two distinct clusters of genes that are deregulated in response to VLCD, there is no data presented as to possible clustering of genes at baseline.

Chapter 1.7: Thesis rationale.

The main goal of the current work was to assay gene expression in the subcutaneous adipose tissue of lean and obese young men. This allows for insight into the pathophysiology of obesity by comparing lean and obese profiles and deducing how they may affect adipose tissue function. The generation of gene expression profiles at baseline will also provide a platform for further studies involving nutritional intervention. That is, we can assay gene expression in response to overfeeding or underfeeding, and compare profiles to those at baseline. Furthermore, we plan to determine if a gene expression profile exists that can accurately define an individual as lean or obese. The current study will be used to define this profile, which can then be tested using a second, independent cohort.

Another goal of the current work was to determine if genetic variation in the Cocaine and Amphetamine Regulated Transcript (*CART*) gene is associated with obesity or obesity related phenotypes in the Newfoundland population. This gene codes for a peptide which is produced predominantly in the hypothalamus and can regulate food intake in animal models (reviewed by Hunter *et al.*, 2004). As the number of samples in this study is small and the data and interpretation limited, this will be presented as an appendix to this thesis (**Appendix 1**). **Appendix 2** contains an abstract for a publication in the American Journal of Clinical Nutrition, of which I am a co-author. That work involves the comparison of two methods for measuring body fat content in humans.

Chapter 2: Materials and Methods

Chapter 2.1: Subject recruitment for microarray study.

A total of 16 male volunteers between the ages of 20-27 were recruited from the Canadian province of Newfoundland and Labrador (NL). Subjects were healthy without metabolic or endocrine disorders, and were not on medication for lipid metabolism. All subjects reported having a stable weight (±5 pounds) within the previous six months. This study was approved by the Ethics Committee of Memorial University of Newfoundland, and all participants gave written informed consent to participate. Subjects were defined as lean or obese based on body fat percentage (%BF), as assessed by dual energy X-ray absorptiometry (DXA) after a 12 hour fast. Lean individuals were defined as ≤ 20 %BF, while obese individuals were defined as ≥ 25 %BF (Bray *et al.*, 1998).

Chapter 2.2: Blood sampling and serum analysis

A fasting blood sample was taken from all volunteers for the current study. Blood samples were taken immediately before the adipose tissue biopsy. Serum concentrations of glucose, triglycerides (TG), total cholesterol, and HDL cholesterol (HDL-C) were measured using Synchron reagents and performed on an Lx20 (Beckman Coulter Inc, CA., U.S.A) in the biochemistry labs of the St. John's Healthcare Corporation. LDL-cholesterol (LDL-C) was calculated using the following formula: (total cholesterol) – (HDL-C) – (TG/2.2). Serum insulin levels were measured on an Immulite immunoassay analyzer (DPC, CA., U.S.A). The homeostasis model assessment (HOMA) was used to estimate insulin resistance (HOMA-R). Serum was stored at -80^0 C. Data obtained that

were not normally distributed were transformed using the natural log for subsequent analysis (ie. TG, insulin, and HOMA-R).

Chapter 2.3: Adipose tissue biopsy

Subcutaneous adipose tissue samples were obtained from eight lean and eight obese males following a 12 hour fast. Adipose tissue was removed from the sub-umbilical region. A local anesthetic was used, which was composed of 10 cc of lidocaine in dilute bupivacaine (40cc of 0.25% bupivacaine in 250 cc of normal saline). Approximately 4 g of subcutaneous adipose tissue was removed by physicians, and immediately flash frozen in liquid nitrogen, and stored in liquid nitrogen until further analysis.

Chapter 2.4: RNA isolation and preparation

Approximately 500 mg of adipose tissue was homogenized using a rotor-stator homogenizer (Brinkmann Instruments, NY. U.S.A.), and total RNA isolated from adipose tissue using RNeasy lipid tissue midi kit (Qiagen, CA., U.S.A). This process involves chloroform separation of DNA, RNA, and organic components, followed by binding of the RNA to a silica gel membrane for purification. While the RNA was bound to the membrane, an additional DNase digestion was performed to remove possible DNA contamination. RNA concentration and purity were determined spectrophotometrically (Eppendorf, Hamburg Germany), and integrity was assessed on a 2100 bioanalyzer (Agilent Technologies, CA., USA). For assessment of RNA integrity, approximately 10 ng of total RNA in 1 ul of RNase free water was used in each reaction.

Chapter 2.5: RNA amplification and labeling

Total RNA from the adipose tissue samples was amplified using a low input linear amplification kit (Agilent Technologies), based on the T7 linear amplification system, which has been validated for use in microarray experiments (Schneider *et al.,* 2004). 300 ng of total RNA was used for each amplification. The reaction mixture for this process was as follows: 4.0 ul of first strand buffer, 2.0 ul of 0.1 M DTT, 1.0 ul of 10 mM dNTP mix, 1.0 ul of MMLV-RT, and 0.5 ul RNase OUT per reaction. The reaction cycle was as follows: 2 hours at 40^0 C, 15 minutes at 65^0C, followed by 5 minutes at 4^0C. The end result of this process is cDNA synthesis from the total RNA input.

In separate parallel reactions, each amplified sample was labeled with either cyanine 3 (Cy3) or Cyanine 5 (Cy5) (Perkin Elmer, MA, U.S.A). Co-currently, amplified reference RNA (Stratagene, CA., U.S.A) was also labeled with Cy3 or Cy5. The reaction mixture was as follows: 15.3 ul of nuclease-free water, 20 ul of 4X transcription buffer, 6.0 ul of 0.1 M DTT, 8.0 ul of NTP mix, 6.4 ul 50 % PEG, 0.5 ul of RNase OUT, 0.6 ul of inorganic pyrophosphatase, and 0.8 ul of T7 RNA polymerase per reaction. The reaction was incubated at 40^0 C for 2 hours. Samples were then purified using RNeasy mini elute kit (Qiagen). The fundamental aspects of the amplification and labeling processes are summarized in **Figure 3**. Hybridization of amplified, labeled RNA samples was accomplished by use of Agilent's *in situ* hybridization kit.

Chapter 2.6: Array Hybridization and imaging

Each Cy3 or Cy5 labeled sample was competitively hybridized with a Cy5 or Cy3 labeled universal human reference RNA, to Agilent's 44K whole human genome chip. A

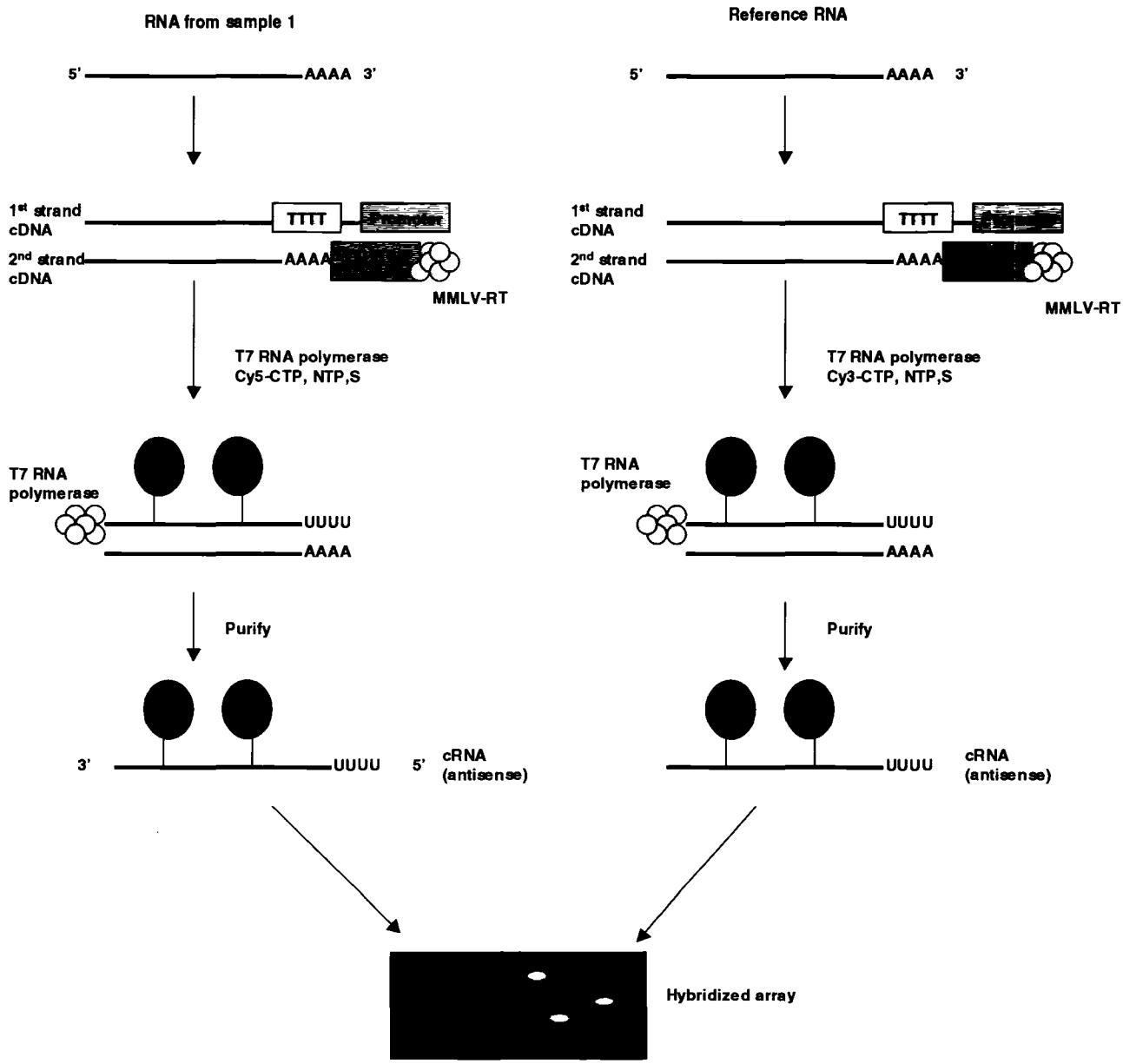

Figure 3: The amplification and labeling of RNA from adipose tissue. During the amplification process, total RNA is amplified into cDNA. An RNA copy is then made of each cDNA, and labeled using either Cy3 or Cy5. The two amplified, labeled samples are then competitively hybridized to the microarray.

total of 1.50 μg of amplified, labeled RNA was hybridized to each array; 750 ng of sample and 750 ng of reference RNA. Reverse hybridization was performed (one array hybridized with Cy3 labeled sample and Cy5 labeled reference RNA, and a second array hybridized with Cy5 labeled sample and Cy3 labeled reference RNA). Arrays were hybridized at 60^0C for 17.5 hours. Microarrays were washed with 1% saline-sodium citrate Buffer (SSC) for 3 minutes, then in 0.1% SSC for three minutes. A final 1 minute wash was completed, which contained an ozone scavenger dissolved in an acetonitrile solution.

All arrays were scanned with the ScanArray Express (Perkin Elmer, MA., U.S.A), with two lasers; one set at 550 nm that excites Cy3, and one set at 650 nm that excites Cy5. Both lasers were set to 80% power to avoid excess overexposed features. All arrays were quantified using Imagene software, version 5.6 (Biodiscovery Inc., CA., U.S.A.). Feature quality was determined using default parameters.

Chapter 2.7: Statistical Analysis

Raw data was determined for each array, and transformed for subsequent analysis using GeneSight lite version 5.6 (Biodiscovery Inc). First, local background intensities were subtracted, then the ratio of sample intensity to universal human reference RNA intensity was computed. Spots flagged for quality measures were deleted from each array individually. Briefly, spots where the signal was not at least twice that of the local background were deleted. Also, spots that were of an irregular shape, or had a reported signal intensity less than zero were also deleted. The remaining data (average of 21 989 spots per array) were transformed to a logarithmic scale (base 2), and then normalized

using Z-scores to ensure a normal distribution of data on all arrays. A mean value was determined for the two arrays for each sample, and was used in all analyses.

A paired design was used to minimize the potential influence of confounding factors in the current study. Each obese volunteer was paired with a lean volunteer based on physical characteristics such as age, height, physical activity levels, smoking status and alcohol consumption. That is, a mean ratio for each obese volunteer was compared directly to a mean ratio for the designated paired lean individual.

Stringent criteria were used to determine genes that had significantly increased or decreased expression in obese adipose tissue when compared to lean. First, genes were selected on the basis that an overall average increase or decrease of 1.5 fold existed between the eight pairs. A total of 11 134 genes fulfilled this criterion. Furthermore, only genes which displayed this regulation pattern in at least 5 out of the 8 pairs were included for further analysis. This criterion ensures that a gene is not called significant because of a large signal difference between a small number of pairs. A total of 5361 genes remained and were tested for significance using paired t tests. A p value of 0.01 or less was taken to be statistically significant, leaving a total of 318 genes. Using this level of significance, we estimate the maximum false discovery rate (FDR) will not exceed 8.4%. An analysis of the estimated FDR at different levels of significance is seen in **Table 2**. SPSS for windows, Version 11.5 was used for the paired statistical analysis.

Table 2: Comparison of false discovery rates at different levels of significance

Level of significance	Number of genes called significant	False discovery rate
$p \leq 0.001$	28	10.0%
$p \leq 0.005$	102	12.0%
$p \leq 0.01$	318	8.4 %
$p \leq 0.05$	917	14.5 %

FDR calculated using the following formula: [{(N)(Y)/2} / Z] x 100%, where N is the number of genes being compared, Y is the level of significance, and Z is the number of genes called significant at level Y.

Chapter 2.8: Analysis of gene ontology

An analysis of gene ontology was undertaken to determine if any set of genes involved in similar biological processes, molecular function, or found in a particular cellular component were over represented in our list of significant differentially regulated genes. This was accomplished by comparing our list of significantly up- or down-regulated genes with the list of all genes on the Agilent whole human genome chip (up and down-regulated genes were compared separately to the reference list). The expected number of genes for a specific ontology term was compared to the observed using a chi-square test, with a p value ≤ 0.01 taken to be significant. This was performed using Gene ontology Tree Machine software (http://genereg.ornl.gov/gotm).

Chapter 2.9: Cluster analysis

The 16 SCAT samples were clustered based on the expression ratio of selected gene sets to determine if any set exists that can accurately distinguish lean from obese adipose tissue. Hierarchical and K-means clustering (k=2) was undertaken using gene sets based on significance values (p values ≤ 0.001, ≤ 0.005, and ≤ 0.01). Specific gene sets based on ontology terms were also used for sample clustering. Euclidean distances were used for all clusters, based on average linkage. Genelinker Gold 2.0 (Improved Outcomes Software, ON. Canada) was used to perform all clustering analyses.

Chapter 3: Results

Chapter 3.1: Physical and biochemical characteristics of the subjects

Physical and biochemical characteristics of the subjects are shown in **table 3**. Obese subjects were defined as $\geq$ 25.5 %BF, while non-obese were defined as $\leq$ 20.0 %BF. Obese individuals showed increased levels of total cholesterol, LDL cholesterol, and triglycerides. This difference was statistically significant at the $p \leq 0.01$ level. As shown by the homeostasis assessment model for insulin resistance, obese volunteers were also more insulin resistant than lean volunteers, which was also significant at the $p \leq 0.01$ level

Chapter 3.2: Analysis of RNA integrity

To ensure that total RNA isolated from adipose tissue contained intact mRNA transcripts, we measured RNA integrality on a 2100 bioanalyzer. Representative electropherograms are shown in **Figure 4**. Two distinct peaks are shown in each electropherogram, indicating intact 28S and 18S ribosomal RNA. Thus, it can be inferred that mRNAs in each sample are intact as well.

Chapter 3.3: Characterization of significantly differentially expressed genes

Using a paired study design, we identified 318 genes and transcripts differentially expressed in SCAT from lean and obese persons at baseline. Of those 318, 232 (73.0%) have defined gene names, with 151 (65.1 %) of those 232 genes having associated biological processes defined by gene ontology terms. **Figure 5** shows the relative abundance of ontology terms associated with the differentially expressed genes. **Table 4** shows all the genes differentially expressed between lean and obese adipose

Table 3: Physical and biochemical characteristics of lean and obese subjects

Characteristic	Lean (n=8)	Obese (n=8)
Age	21.9 (20-25)	23.3 (21-27)
Height: (cm)	180.0 (171.2-189.6)	176.2 (166.8-186.5)
Weight: (kg)	74.7 (56.7-96.0)	94.5 (73.5-135.0)*
BMI (kg/m^2)	23.1 (17.9-30.3)	30.6 (22.2-43.8)*
%BF	14.9 (7.6-20)	30.5 (25.5-40.3)*
Insulin (pmol/L)	46.19 (28.6-78.6)	90.71 (34.5-175)
Glucose (mmol/L)	4.58 (1.9-5.2)	5.35 (4.5-6.5)
TG (mmol/L)[1]	0.78 (0.42-1.11)	1.12 (0.79-1.55)*
Total Chol (mmol/L)	3.82 (2.87-5.41)	4.85 (4.01-5.61)*
HDL-C (mmol/L)	1.36 (0.94-1.52)	1.48 (0.95-3.6)
LDL-C (mmol/L)	2.11 (1.29-3.26)	2.87 (1.59-3.68)*
HOMA-R[1]	1.31 (0.48-2.49)	3.13 (0.96-5.26)*

Data presented as mean, range in parenthesis

[1] statistical testing performed on log transformed data (base e)

* indicates means are statistically different between lean and obese, $p \leq 0.01$, student t test.

HOMA-R: homeostatic model for insulin resistance

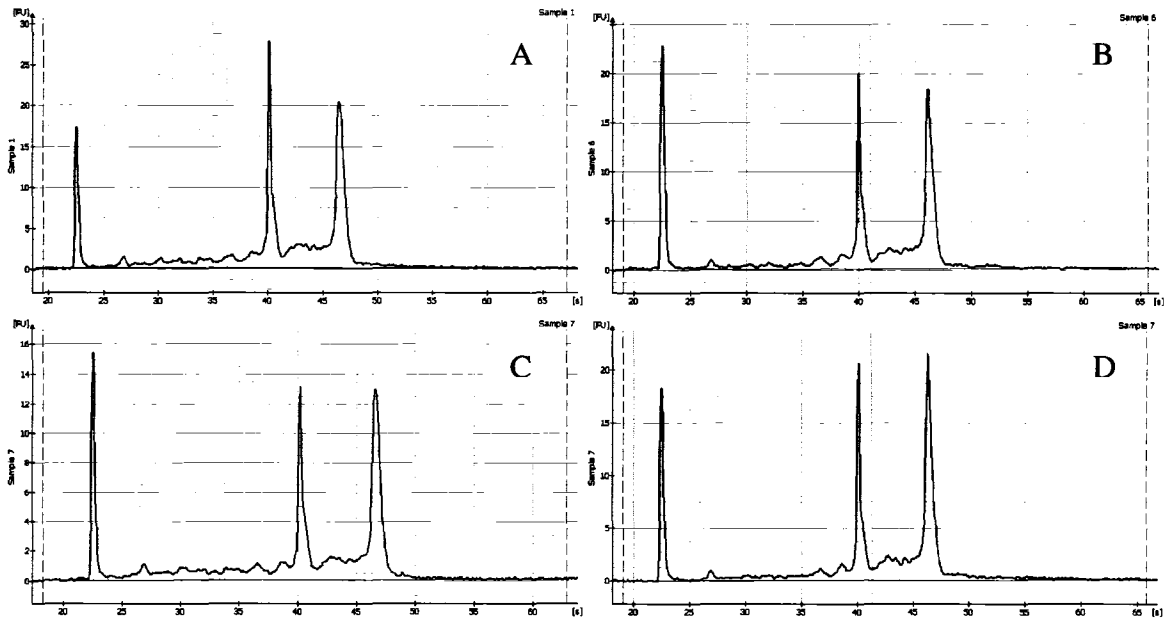

Figure 4: Representative electropherograms of total RNA isolated from adipose tissue. A and B represent lean samples, while C and D represent obese samples. In each panel, the three peaks (from left to right) represent the marker, 18s RNA, and 28s RNA. The two distinct peaks for ribosomal RNA are indicative of intact total RNA.

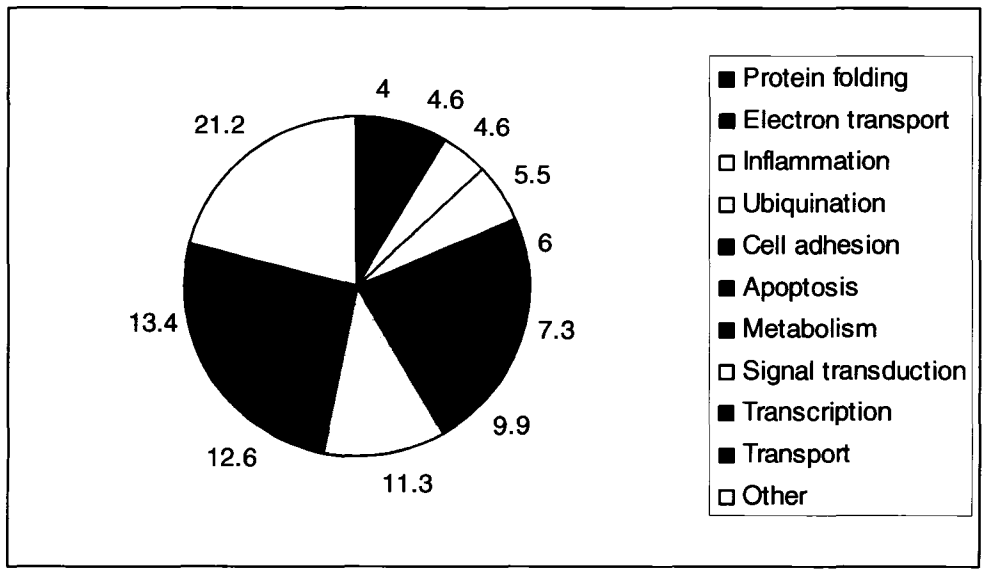

Figure 5: Relative abundance of Gene ontology terms (biological process) associated with 232 differentially expressed genes between obese and lean SCAT.

Table 4: All differentially expressed genes with defined names, organized by biological process.

HUGO name	Description	Fold Change*	p-value
Transport			
SLC25A13	solute carrier family 25, member 13	0.53	0.0036
SLC25A22	solute carrier family 25, member 22	0.50	0.0041
SLC9A3†	solute carrier family 9, isoform 3	0.31	0.0110
RABGEF1†	RAB guanine nucleotide exchange factor	0.54	0.0009
SEC15L1†	SEC15-like 1 (S. cerevisiae)	0.53	0.0051
MRPL45†	mitochondrial ribosomal protein	0.44	0.0051
FBXL5†	F-box and leucine-rich repeat protein 5	0.62	0.0116
ATP5S	ATP synthase, mitochondrial F0 complex, subunit s	0.61	0.0141
CECR2	cat eye syndrome chromosome region, candidate 2	0.50	0.0110
FLJ43855	similar to NA^+- and CL^--dependent creatine transporter	0.51	0.0066
LAX	hypothetical protein FLJ20340	0.55	0.0080
TLOC1	translocation protein 1	0.48	0.0068
KIAA0528	KIAA0528 gene product	0.42	0.0063
RAB14†	RAB14, member RAS oncogene family	0.57	0.0075
SLC16A3	solute carrier family 16, member 3	2.11	0.0109
LOC136306	Hypothetical protein LOC136306	3.43	0.0057
TNPO1	transportin 1, transcript variant 1,	2.78	0.0100
LOC203427	similar to solute carrier family 25, member 16	10.58	0.0057
TSAP6†	Dudulin 2	1.74	0.0071
NUPL1	nucleoporin like 1	2.55	0.0013

ERO1LB	ERO1-like beta	4.18	0.0034
Electron transport			
NDUFB5	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5	0.38	0.0015
NDUFB6	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6	0.39	0.0006
C6orf107	chromosome 6 open reading frame	0.58	0.0120
MGC25181	hypothetical protein MGC25181	0.51	0.0144
IL4I1	interleukin 4 induced 1, transcript variant 2,	6.10	0.0107
IVD†	isovaleryl Coenzyme A dehydrogenase	3.89	0.0089
Apoptosis			
SMNDC1†	survival motor neuron domain containing 1	0.40	0.0072
SIRT1†	silent mating information regulation 2 homolog1	0.38	7.01E-05
PAX3	paired box gene 3	0.34	0.0120
PRODH†	proline dehydrogenase (oxidase)	0.49	0.0056
APLP1	amyloid beta (A4) precursor-like protein 1	2.92	0.0121
PHF17	PHD protein Jade-1 (JADE1), transcript variant S	2.51	0.0061
API5	apoptosis inhibitor 5	2.27	0.0120
IER3†	immediate early response 3	2.35	0.0027
ERCC2	excision repair cross-complementing group 2	6.08	0.0012
ITGB2†	integrin, beta 2, macrophage antigen 1 beta subunit	15.25	0.0129
ROCK1†	Rho-associated, coiled-coil containing protein kinase 1	2.10	0.0096
Transcription			
TARDBP	TAR DNA binding protein	0.59	0.0095
IRX5	Unknown	0.52	0.0069
ZHX2	zinc fingers and homeoboxes 2	0.34	0.0140
GTF3C2	general transcription factor IIIC, polypeptide 2, beta	0.67	0.0140

SOX17	SRY (sex determining region Y)-box 17	0.56	0.0003
SNAPC4	small nuclear RNA activating complex, polypeptide 4,	0.63	0.0060
CREB3L3	cAMP responsive element binding protein 3-like 3	0.67	0.0043
EED	embryonic ectoderm development, transcript variant 1	0.50	0.0084
RBPSUH[†]	recombining binding protein suppressor of hairless	0.49	0.0110
ZNF295	zinc finger protein 295	0.48	0.0112
ZNF302	zinc finger protein 302	0.53	0.0054
ZNF160	zinc finger protein 160	0.58	0.0080
ZFY	zinc finger protein, Y-linked	0.42	0.0049
ODAG	ocular development-associated gene	0.58	0.0073
JDP2	jun dimerization protein 2	8.50	0.0033
NKX6-1	NK6 transcription factor related, locus 1	10.43	0.0143
SBZF3	zinc finger protein SBZF3	7.81	0.0114
ZNF541	zinc finger protein 541	3.72	0.0124
BRSK2	serine/threonine kinase 2	1.78	0.0118
Cell adhesion			
LRRN5	leucine rich repeat neuronal 5, transcript variant 1	0.47	0.0011
NID2[†]	nidogen 2 (osteonidogen)	0.67	0.0055
DCBLD1	discoidin, CUB and LCCL domain containing 1	0.54	0.0081
AZGP1	alpha-2-glycoprotein 1	0.39	0.0005
MYBPC1	myosin binding protein C, slow type, transcript variant 1	8.68	0.0049
SCARF2	scavenger receptor class F, member 2 transcript variant 1	2.53	0.0006
WISP2	WNT1 inducible signaling pathway protein 2	2.49	0.0089
GPLD1	glycosylphosphatidylinositol specific phospholipase D1	2.49	0.0072
CYR61[†]	cysteine-rich, angiogenic inducer, 61	2.82	0.0048

Signal transduction			
RAB18†	RAS oncogene family, member 18	0.66	0.0087
RAB33B	member RAS oncogene family , member 33B	0.45	0.0047
HTR1B5	hydroxytryptamine (serotonin) receptor 1	0.48	0.0144
GPR109B†	G protein-coupled receptor 109B	0.41	0.0005
NPB	preproneuropeptide B	0.63	0.0076
MAP4K4	mitogen-activated protein 4 kinase 4	0.43	0.0094
GRB10†	growth factor receptor-bound protein 10	0.58	0.0041
CAMKK1	calcium/calmodulin-dependent protein kinase kinase 1	0.46	0.0020
SHFM3†	split hand/foot malformation (ectrodactyly) type 3	0.64	0.0111
HTR1D5	hydroxytryptamine (serotonin) receptor 1D	0.33	0.0040
EFNB2†	ephrin-B2	0.54	0.0073
PPP2CB†	protein phosphatase 2 , beta isoform	0.49	0.0071
PPP2R2A†	protein phosphatase 2, regulatory subunit B	0.64	0.0062
PRKAR2A†	cAMP dependant protein kinase, regulatory subunit alpha	9.47	0.0050
OR51B4	olfactory receptor, family 51, subfamily B, member 4	8.36	0.0037
SAG	S-antigen; retina and pineal gland (arrestin)	4.36	0.0063
FLJ14249	HS1-binding protein 3	4.29	0.0050
Immunity/Defense response			
MST1R	macrophage stimulating 1 receptor	0.51	0.0046
YY1	YY1 transcription factor	0.44	0.0150
PLA2G7	phospholipase A2, group VII	15.74	0.0074
ATRN†	attractin transcript variant 2	4.16	0.0028
DEFB118	defensin, beta 118	6.96	0.0074
CXCL2	chemokine (C-X-C motif) ligand 2	5.00	0.0018
CR2†	complement component (3d/Epstein Barr virus) receptor 2	1.62	0.0098

Metabolism/Lipolysis			
PPP1CA†	protein phosphatase 1, catalytic subunit, alpha transcript	0.52	0.0087
PRKAR2B†	cAMP dependant protein kinase, regulatory subunit beta	0.31	0.0018
AGL	amylo-1, 6-glucosidase, 4-alpha-glucanotransferase,	0.48	0.0070
PC†	pyruvate carboxylase , transcript variant A	0.40	0.0026
BCKDHA†	branched chain keto acid dehydrogenase E1, alpha	0.44	0.0123
ENDOG	endonuclease G	0.62	0.0067
FASN†	fatty acid synthase	0.63	0.0032
DLST	dihydrolipoamide S-succinyltransferase	0.52	0.014
FAHD2A	fumarylacetoacetate hydrolase domain containing 2A	0.65	0.0098
ENPP6†	ectonucleotide pyrophosphatase/phosphodiesterase 6	5.98	0.0103
AGXT	alanine-glyoxylate aminotransferase	4.44	0.0094
FUK	fucokinase	2.00	0.0009
C14orf175	chromosome 14 open reading frame 175	10.44	0.0088
AGPAT4	1-acylglycerol-3-phosphate O-acyltransferase 4	2.79	0.0035
ACATE2†	acyl-Coenzyme A thioesterase 2, mitochondrial	1.87	0.0104
Protein Folding			
HSPD1†	heat shock 60kDa protein 1, transcript variant 1	0.32	0.0057
TTC1†	tetratricopeptide repeat domain 1	0.50	0.0078
DNAJB6†	DnaJ (Hsp40) homolog, subfamily B, member 6	0.50	0.0023
FKBP5	FK506 binding protein 5	0.59	0.0124
HCG3	HCG3 gene	0.58	0.0139
PFDN1†	prefoldin 1	0.65	0.0086
Protein Ubiquination/Ubiquination Cycle			
TRIM4	tripartite motif-containing 4, transcript variant alpha,	0.37	0.0016
ITCH	itchy homolog E3 ubiquitin protein ligase	0.65	0.0116

FBXO30	F-box protein 30	0.51	0.0105
CNOT4	CCR4-NOT transcription complex, subunit 4	0.56	0.0062
USP48	ubiquitin specific protease 48	0.60	0.0107
USP25†	ubiquitin specific protease 25	0.54	0.0029
TRIM17	tripartite motif-containing 17	4.08	0.0101
KIAA0804	KIAA0804 protein	4.62	0.0079
Amine biosynthesis			
CTBP2†	C-terminal binding protein 2	0.59	0.0113
TMLHE	trimethyllysine hydroxylase, epsilon	0.59	0.0004
DHFR	dihydrofolate reductase	0.62	0.0056
Miscellaneous			
TSPYL3	TSPY-like 3 (TSPYL3)	0.39	0.0133
PALMD	palmdelphin	0.51	0.0057
PGPEP1	pyroglutamyl-peptidase I	0.61	0.0095
TIMP4†	tissue inhibitor of metalloproteinase 4	0.48	0.0010
ADSSL1†	adenylosuccinate synthase like 1, transcript variant 2	0.44	0.0028
PES1†	pescadillo homolog 1, containing BRCT domain	0.56	0.0065
NEDD5	neural precursor cell expressed, develop. down-regulated 5	0.60	0.0076
C1qTNF7	C1q and tumor necrosis factor related protein 7	0.46	0.0005
TMPIT†	transmembrane protein induced by TNF alpha	0.37	0.0056
CTGLF1	centaurin, gamma-like family, member 1	0.55	0.0025
C20orf141	chromosome 20 open reading frame 141	0.54	0.0149
TTLL3†	tubulin tyrosine ligase-like family, member 3	0.50	0.0135
NOP5/58	nucleolar protein NOP5/NOP58	0.64	0.0004
MST4†	Mst3 and SOK1-related kinase	0.52	0.0149

ZCCHC11	zinc finger, CCHC domain containing 11	0.63	0.0126
FLJ23588	CAP-binding protein complex interacting protein 1	0.61	0.0069
FHOD3	formin homology 2 domain containing 3	0.29	0.0107
IDI2	isopentenyl-diphosphate delta isomerase 2	0.52	0.0025
YT521	splicing factor YT521-B	0.52	0.0031
MRPS31[†]	mitochondrial ribosomal protein S31	0.44	0.0115
HS6ST3	heparan sulfate 6-O-sulfotransferase 3	3.64	0.0132
OLFM1	olfactomedin 1, transcript variant 2	4.75	0.0090
MRC1	mannose receptor, C type 1	2.03	0.0021
RNPEP	arginyl aminopeptidase (aminopeptidase B)	18.13	0.0103
ACTR3	ARP3 actin-related protein 3 homolog	3.77	0.0129
DUSP7	dual specificity phosphatase 7	3.89	0.0089
KIAA1008[†]	KIAA1008 protein	8.60	0.0069
NAPSA	napsin A aspartic peptidase	6.22	0.0072
TTLL4	tubulin tyrosine ligase-like family, member 4	13.69	0.0147
No defined biological process			
AZI2	5-azacytidine induced 2	0.44	0.0004
OSRF	osmosis responsive factor	0.42	0.0033
TUSC1	tumor suppressor candidate 1	0.50	0.0033
CGI-62	CGI-62 protein	0.39	0.0022
FLJ22794	FLJ22794 protein	0.44	0.0034
FLJ20699	hypothetical protein FLJ20699	0.49	0.0033
LOC92270	hypothetical protein LOC92270	0.60	0.0034
HMGN1	high-mobility group nucleosome binding domain 1	0.59	0.0039
METTL2	methyltransferase like 2	0.44	0.0048

TORC2	transducer of cAMP response element-binding protein 2	0.58	0.0131
FLJ10241	hypothetical protein FLJ10241	0.57	0.0144
ITPKC	inositol 1,4,5-trisphosphate 3-kinase C	0.46	0.0149
KIAA0182	KIAA0182 protein	0.57	0.0134
KIAA1164	hypothetical protein KIAA1164	0.64	0.0049
FLJ11235	hypothetical protein FLJ11235	0.67	0.0131
KLHL15	kelch-like 15 (Drosophila)	0.35	0.0061
LOC133619	hypothetical protein MGC12103	0.46	0.0131
NOL7	nucleolar protein 7, 27kDa	0.65	0.0062
FLJ13291	hypothetical protein FLJ13291	0.58	0.0065
MESP1	mesoderm posterior 1	0.25	0.0075
MR-1	myofibrillogenesis regulator 1	0.46	0.0036
HSPC129	hypothetical protein HSPC129	0.54	0.0035
LOC51234	hypothetical protein LOC51234	0.50	0.0024
FLJ38716	hypothetical protein FLJ38716	0.48	0.0015
PRICKLE2	prickle-like 2 (Drosophila)	0.38	2.64E-05
TMEM22	transmembrane protein 22	0.47	0.0003
LOC440234	similar to hypothetical protein	0.57	0.0079
LOC341333	similar to Heterogeneous nuclear ribonucleoprotein A1	0.54	0.0087
MGC42493	hypothetical protein MGC42493	0.55	0.0092
LOC339210	hypothetical protein LOC339210	0.51	0.0101
FLJ10246	hypothetical protein FLJ10246	0.64	0.0101
LOC56181	hypothetical protein RP1-317E23	0.51	0.0113
HSPC159	HSPC159 protein	0.57	0.0122
KIAA0892	KIAA0892 protein	0.67	0.0080
ZNF575	zinc finger protein 575	0.47	0.0005

ELP4	elongation protein 4 homolog	0.67	0.0017
LSM6	LSM6 homolog, U6 small nuclear RNA associated	0.56	0.0019
C10orf24	chromosome 10 open reading frame 24	0.52	0.0056
CPAMD8	C3 and PZP-like, alpha-2-macroglobulin domain	0.62	0.0068
RTN4RL2	reticulon 4 receptor-like 2	0.64	0.0072
PLEKHJ1	pleckstrin homology domain, family J member 1	0.43	0.0074
PRKCL2	protein kinase C-like 2	0.57	0.0077
CPNE4	copine IV	0.40	0.0086
CLN8†	ceroid-lipofuscinosis, neuronal 8	0.64	0.0092
NUDT14	nucleoside diphosphate linked moiety X-type motif 14	0.52	0.0092
ADHFE1	alcohol dehydrogenase, iron containing, 1	0.61	0.0095
PSPC1	paraspeckle component 1	0.54	0.0098
BAGE	B melanoma antigen	0.45	0.0098
ZNF395	unknown	0.52	0.0107
LENG4†	leukocyte receptor cluster (LRC) member 4	0.54	0.0119
C20orf141	chromosome 20 open reading frame 141	0.54	0.0149
C5orf18†	chromosome 5 open reading frame 18	0.50	0.0060
PRRG3	proline rich Gla (G-carboxyglutamic acid) 3	0.48	0.0122
FLJ11301	hypothetical protein FLJ11301	2.45	0.0064
HCA112	hepatocellular carcinoma-associated antigen 112	1.57	0.0073
LOC389903	similar to Taxol resistant associated protein 3 (TRAG-3)	2.13	0.0081
PVR	poliovirus receptor	5.33	0.0009
MUM1L1	melanoma associated antigen (mutated) 1-like 1	4.40	0.0058
C20orf40	chromosome 20 open reading frame 40	3.25	0.0044
KIAA0635	KIAA0635 protein	2.47	0.0081
KIAA0907	KIAA0907 protein	2.39	0.0031

KIAA1720	KIAA1720 protein	4.77	0.0094
LOC92345	hypothetical protein BC008207	8.67	0.0048
RGAG1	retrotransposon gag domain containing 1	5.26	0.0101
TMED4	transmembrane emp24 protein transport domain 4	6.10	0.0107
FLJ38507	colon carcinoma related protein	4.38	0.0144
GR6	putative GR6 protein	2.98	0.0144
NRG4	neuregulin 4	3.33	0.0047
FLJ22582	hypothetical protein FLJ22582	2.34	0.0112
FLJ20195	hypothetical protein FLJ20195	3.95	0.0113
DOK3	docking protein 3	2.49	0.0131
PIWIL4	piwi-like 4 (Drosophila)	4.28	0.0129
GBL	G protein beta subunit-like	12.80	0.0124
UNC93B1	unc-93 homolog B1	3.61	0.0007
ACP5[†]	acid phosphatase 5, tartrate resistant	4.01	0.0050
KIAA0853	KIAA0853	3.39	0.0051
KIAA1919	KIAA1919	6.91	0.0056
C9orf138	chromosome 9 open reading frame 138	11.36	0.0087
C20orf22	chromosome 20 open reading frame 22	13.49	0.0098
SAMSN1	SAM and SH3 domain, and nuclear localisation signal,	15.47	0.0099
RTN4IP1	reticulon 4 interacting protein 1	3.16	0.0119

* Fold change presented as obese/lean

significance testing performed using paired-t tests

[†] indicates previous reports of adipose tissue expression

tissue as defined by biological processes. Genes involved in transport and transcription were the most abundant, comprising 13.4% and 12.6% of the genes with defined ontologies, respectively. Genes involved in signal transduction and metabolism were also abundant, comprising 11.3% and 9.9% of genes with defined ontologies, respectively. Furthermore, only 51 of the genes with defined names have been previously shown to be expressed in human adipose tissue. Thus, we report a total of 181 defined genes that, to our knowledge, have not been previously reported as expressed in adipose tissue. A total of 87 potential genes without defined names were significantly differentially expressed between lean and obese individuals. Of these 87 potential genes, 33 were up-regulated in obese subjects (**Table 5**), while 54 were down-regulated in obese subjects (**Table 6**). These potential genes represent EST's and open reading frames.

Chapter 3.4: Gene ontology (GO) analysis

Gene ontology analysis revealed an over-representation of genes involved in specific biological processes as differentially expressed between lean and obese individuals. These processes include cell adhesion, apoptosis, cellular morphogenesis, inflammatory response, and cell growth (**Figure 6**), which were up-regulated in obese adipose tissue. Similarly, **Figure 7** shows that genes involved in ATP coupled electron transport, NADH dehydrognease activity, and carrier activity which includes both hydrogen and sodium ion transporters, amine biosynthesis, and unfolded protein binding proteins were statistically over-represented in our list of down-regulated genes in obese adipose tissue. With respect to cellular component ontology terms, genes

Table 5: Potential genes without defined names, that are up-regulated in obese adipose tissue

Systematic Name	Fold change*	p-value	Systematic Name	Fold change*	p-value
AK096606	1.72	0.0002	BC017202	3.89	0.0089
AW316654	7.18	0.0011	THC2107966	53.57	0.0092
THC2131432	2.11	0.0020	THC2165289	1.78	0.0099
A_24_P831005	2.36	0.0021	A_23_P9707	12.21	0.0099
A_23_P157695	11.06	0.0032	THC2051081	7.07	0.0102
BC004382	22.06	0.0035	A_24_P307395	1.50	0.0103
A_24_P517355	3.43	0.0037	AK024304	119.69	0.0104
AF086548	4.14	0.0044	A_32_P179199	5.11	0.0105
BU731317	1.98	0.0048	A_24_P409772	1.67	0.0110
AB002439	15.20	0.0052	AK026328	3.72	0.0110
A_24_P644556	16.45	0.0069	A_32_P18300	9.15	0.0117
THC2141858	10.87	0.0073	AK022227	12.80	0.0124
A_24_P578437	24.96	0.0076	THC2086431	3.47	0.0124
X61177	3.62	0.0082	AF007131	2.89	0.0130
THC2175701	2.25	0.0086	ENST00000333662	5.18	0.0136
AF278760	3.03	0.0087	AK055716	9.04	0.0148
THC2105601	2.49	0.0040			

*Fold change presented as obese/lean

Significance testing performed using paired t tests

Table 6: Potential genes without defined names that are down-regulated in obese adipose tissue.

Systematic Name	Fold change*	p-value	Systematic Name	Fold change*	p-value
A_32_P86517	0.30	0.0004	AF119848	0.36	0.0083
BC038098	0.64	0.0011	BC010544	0.47	0.0086
AL832534	0.54	0.0011	A_23_P51269	0.45	0.0086
ENST00000297317	0.60	0.0017	NP511100	0.56	0.0087
THC2088849	0.59	0.0087	A_32_P175557	0.64	0.0092
A_32_P120818	0.56	0.0019	THC2173367	0.63	0.0092
A_24_P846755	0.33	0.0020	A_24_P914102	0.52	0.0093
AK026896	0.46	0.0021	AK000380	0.43	0.0094
A_24_P24332	0.56	0.0026	ENST00000328411	0.50	0.0100
BC009800	0.63	0.0027	BX101146	0.29	0.0106
A_32_P200092	0.61	0.0031	THC2135324	0.62	0.0106
THC2088623	0.46	0.0034	BC016022	0.51	0.0110
A_24_P556328	0.60	0.0043	BC046172	0.47	0.0112
CR609588	0.66	0.0048	A_24_P927902	0.32	0.0113
AF086388	0.41	0.0048	AK091744	0.52	0.0115
THC2174703	0.60	0.0054	A_24_P24786	0.45	0.0123
BE537483	0.46	0.0055	AF086527	0.67	0.0129
BC036435	0.64	0.0058	AK056401	0.44	0.0130
A_23_P39251	0.38	0.0067	BC030112	0.51	0.0132
A_32_P95502	0.66	0.0070	THC2209865	0.58	0.0134

A_24_P307443	0.49	0.0070	BC017590	0.58	0.0139
AK001936	0.39	0.0071	A_24_P384239	0.37	0.0141
A_24_P916423	0.39	0.0075	THC2131163	0.64	0.0141
A_24_P938357	0.29	0.0077	THC2050410	0.46	0.0149
A_24_P477048	0.31	0.0078	A_24_P516728	0.52	0.0081
ENST00000311218	0.50	0.0080	THC2077398	0.55	0.0045
A_23_31348	0.51	0.0065	A_24_P882309	0.51	0.0110

*Fold change presented as obese/lean

Significance testing performed using paired t tests

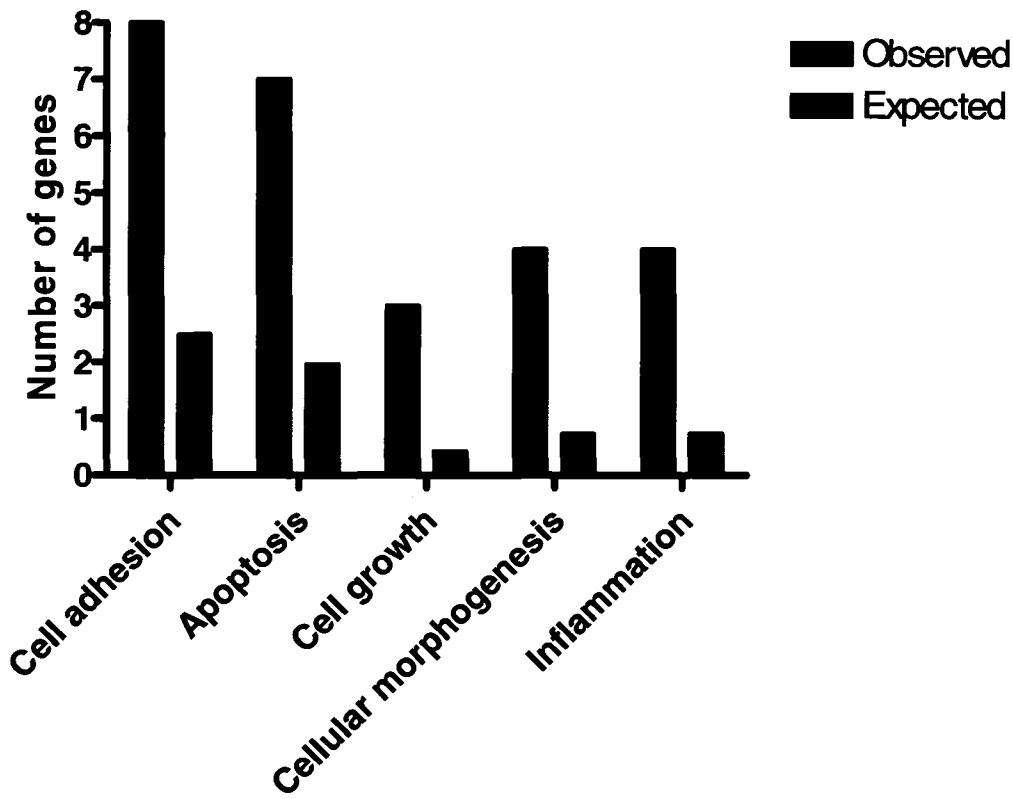

Figure 6: Gene classifications statistically over-represented in obese (up-regulated). Gene ontology classifications based on biological process ontology terms only. Ontology classifications are not mutually exclusive; any gene can exist in more that one category. Difference between observed and expected are statistically significant ($P \leq 0.01$), based on chi-squared analysis. http://genereg.ornl.gov/gotm. Expected values calculated from the number of genes associated with an ontology term on the Agilent whole human genome chip.

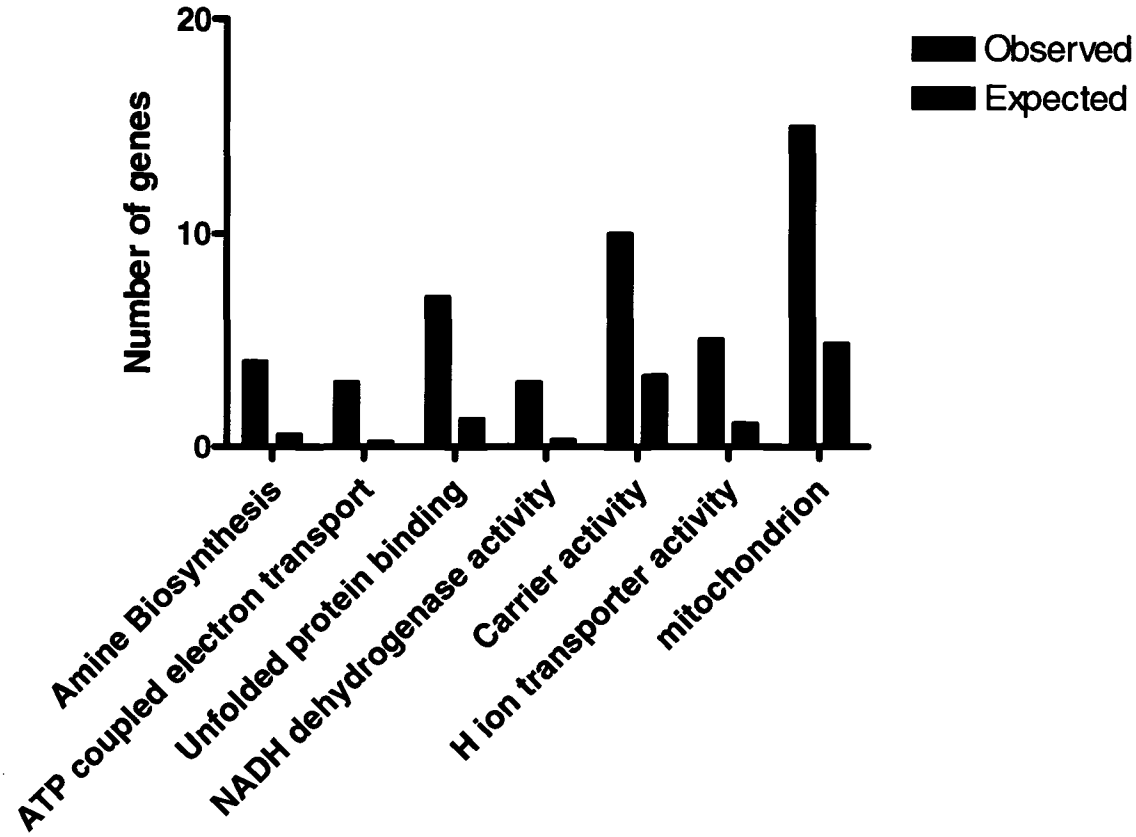

Figure 7: Gene classifications statistically over-represented in SCAT from obese subjects (down-regulated). Gene ontology based on biological process (Amine biosynthesis, ATP coupled electron transport), cellular component (mitochondrion), and molecular function (all others). Ontology classifications are not mutually exclusive; any gene can exist in more that one category. Difference between observed and expected are statistically significant ($P \leq 0.01$), based on chi-squared analysis. http://genereg.ornl.gov/gotm. Expected values calculated from the number of genes associated with an ontology term on the Agilent whole human genome chip.

with functions in the mitochondria were over-represented in our list of down-regulated genes.

Chapter 3.5: Supervised clustering of genes and samples

Supervised clustering was undertaken to determine which, if any, set of gene expression values could accurately distinguish between lean and obese individuals. Hierarchical and K-means clustering was first performed on gene sets based on significance values. Thus, genes with p values $\leq$ 0.001, 0.005, 0.01, and 0.05 were clustered as separate gene sets. Using this approach, the clustering algorithms could not group obese samples separately from the lean samples with a high degree of specificity (data not shown).

Supervised clustering was also performed using gene sets based on ontology terms. Specific gene sets were built using genes that had statistically over- represented ontology terms. Using this rationale, we identified a set of 27 genes that clustered obese samples separately from the lean samples with a high degree of specificity. Using a K-means clustering approach (K=2), all obese samples were clustered separately from the lean samples, indicating a specificity of 100% (**Figure 8**). However this specificity was reduced using a hierarchical clustering algorithm. Using this approach, two main clusters were generated, one containing seven lean and one obese sample and the other containing seven obese and one lean sample, resulting in a specificity of 87.5% (**Figure 9**). All genes contained in this cluster, and associated gene ontology terms are shown in **table 7**.

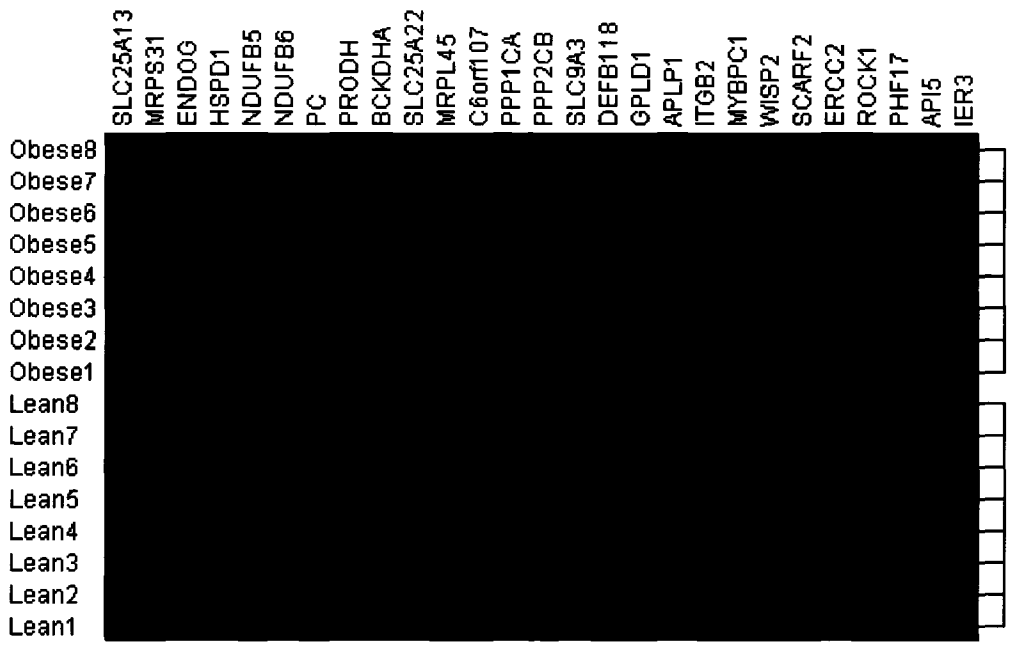

Figure 8: K means (K=2) clustering of samples using a 27 gene set comprised of genes with similar ontology terms. Red, black, and blue indicates expression ratio (sample/reference) above the median, equal to, and below the median respectively. All obese samples clearly cluster separately from lean.

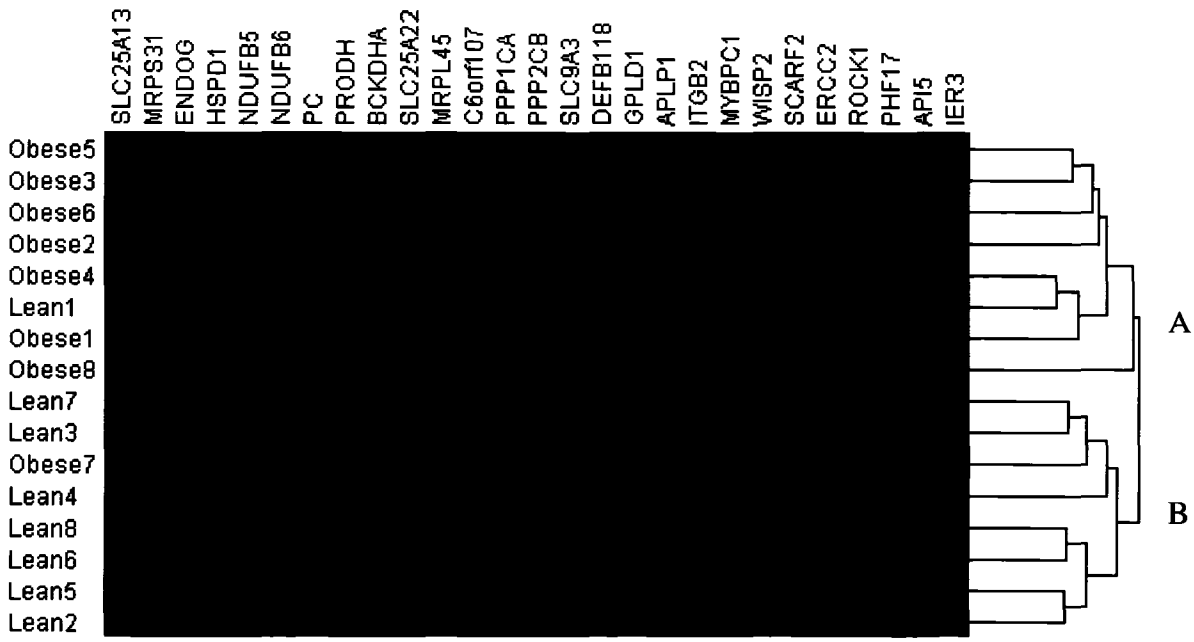

Figure 9: Hierarchical clustering of samples using a 27 gene set comprised of genes with similar ontology terms. Red, black, and blue indicates expression ratio (sample/reference) above the median, equal to, and below the median respectively. 2 main clusters are generated, one containing seven obese samples and one lean (A), and the other containing seven lean samples and one obese (B).

Table 7: Genes contained in the 27 gene set cluster that accurately distinguishes between obese and non-obese young men.

Gene symbol	Gene name	Associated GO term
SLC25A13	solute carrier family 25, member 13	transport, mitochondria
MRPS31	mitochondrial ribosomal protein S31	mitochondria
ENDOG	endonuclease G	mitochondria
HSPD1	heat shock 60kDa protein 1	protein folding, mitochondria
NDUFB5	ubiquinone 1 beta subcomplex, 5	electron transport, mitochondria
NDUFB6	ubiquinone 1 beta subcomplex, 6	electron transport, mitochondria
PC	pyruvate carboxylase	metabolism, mitochondria
PRODH	Proline dehydrogenase	Apoptosis, mitochondria
BCKDHA	branched chain keto acid dehydrogenase E1	metabolism, mitochondria
SLC25A22	solute carrier family 25, member 22	transport, mitochondria
MRPL45	mitochondrial ribosomal protein	transport, mitochondria
C6ORF107	chromosome 6, open reading frame 7	electron transport, mitochondria
PPP1CA	protein phosphatase 1, catalytic subunit	metabolism, lipolysis
PPP2CB	protein phosphatase 2 beta isoform	signal transduction
SLC9A3	solute carrier family 9, member A3	transport, mitochondria
DEFB118	defensin, beta 118	immunity, defense response
GPLD1	glycosylphosphatidylinositol phospholipase D1	cell adhesion
APLP1	amyloid beta (A4) precursor-like protein 1	apoptosis
ITGB2	integrin, beta 2, macrophage antigen 1	apoptosis, cell adhesion
MYBPC1	myosin binding protein C, slow type	cell adhesion
WISP2	WNT1 inducible signaling pathway protein 2	cell adhesion
SCARF2	scavenger receptor class F, member 2	cell adhesion

ERCC2	excision repair cross-complementing group 2	apoptosis
ROCK1	Rho-associated, coiled-coil containing kinase 1	apoptosis
PHF17	PHD protein Jade-1	apoptosis
API5	apoptosis inhibitor 5	apoptosis
IER3	immediate early response 3	apoptosis

Chapter 4: Discussion

Chapter 4.1: Numerous biological processes may be involved in the pathophysiology of obesity.

Numerous studies have been conducted that examine the gene expression profile of adipose tissue, but most have been limited by the number of genes and/or ESTs contained on the microarray itself. Here, for the first time, we report the expression profile of subcutaneous adipose tissue and how it differs between obese and non-obese young men, using whole human genome microarrays. As a result, we have not only been able to confirm trends discovered in previous microarray studies, but have also discovered novel biological trends and obesity candidate genes that can serve as a basis for future obesity related research.

For many years, the control of the rate of lipolysis and lipogenesis has been the main avenue of obesity related research. However many other processes are now thought to be involved, including, inflammation (Clement *et al.,* 2004) apoptosis (Aubin *et al.,* 2004; Duff *et al.,* 2005), and cell adhesion (Abella *et al.*, 2004; Eguchi *et al.,* 2004). By comparing our list of differentially expressed genes with all the genes on the Agilent 44K array, we have identified a statistical over-representation of genes involved inflammation, apoptosis, cell adhesion and cellular morphogenesis, as well as others. Genes involved in these processes were found to have increased expression in the subcutaneous adipose tissue of obese individuals, indicating potential roles for these processes, and the genes involved, in the development of obesity. Furthermore, genes with an ontology classification of inflammation, apoptosis, cell adhesion, or cellular morphogenesis were included in the set of genes that correctly groups obese from non-obese subjects. This

cluster will be tested in future investigations to determine if it can accurately predict obesity in a second cohort of subjects, and to determine if these expression values change in response to nutritional challenges. Thus, all of the genes contained in this cluster represent possible obesity candidate genes for further research.

Chapter 4.2: Mitochondrial function and ATP synthesis may be deregulated in young men.

The role of mitochondrial function and cellular ATP concentration has not been rigorously studied with respect to obesity. However, perilipin null mice, which have a lean phenotype and are resistant to diet induced obesity, demonstrate increased expression levels of genes involved in ATP synthesis (Castro-Chavez *et al.,* 2003). Our results indicate a reduction in the expression of a number of genes with functions in the mitochondria, four of which are directly involved in ATP coupled electron transport. Specifically, we observed a 2.6 fold decrease in NDUFB6 and NDUFB5, which comprise 2 of the ubiquinone oxidoreductase complex I subunits of the mitochondrial electron transport chain. Numerous subunits of this complex are up-regulated in the lean perilipin null mouse, but not NDUFB5 or NDUFB6 in particular. We also observed a 1.7 fold decrease of C6orf107 in obese adipose tissue. The product of this gene is also thought to be involved in ATP coupled electron transport based on its proposed protein structure (http://genereg.ornl.gov/gotm), but no mechanism has yet been elucidated. Furthermore, in obese individuals, we also observe a decrease of expression of ATP5S, which codes for the ATP synthase 5S subunit of the mitochondrial F0 complex. It remains to be determined whether this reduction in the expression of genes involved electron transport

represent a primary effect leading to the obese state, or a secondary effect that results due to increased adiposity.

Numerous other genes with functions in the mitochondria were also down-regulated in obese individuals. Most of these genes code for transport proteins, such as solute carriers. It is difficult to determine what role, if any, these genes may play in the pathophysiology of obesity based on the current study, however many of the genes that have functions in the mitochondria were included in the cluster of genes we have used to distinguish between obese and non-obese individuals. These genes include the four genes mentioned earlier that are involved in ATP coupled electron transfer, as well as many of the mitochondrial transport proteins. This provides strong support to the theory that mitochondrial function and cellular energy production may play a role in the development of obesity.

Previous studies have indicated that mitochondrial function and ATP generation may affect the rate of lipolysis (reviewed by Rossmeisl *et al.,* 2004), and thus the status of adiposity in a given individual. The AMP activated protein kinase (AMPK) plays an important role in coupling cellular energy production to lipolysis. This enzyme responds to changes in the AMP/ATP ratio, and inhibits lipolysis in adipocytes (Winder *et al.,* 1999). An increase in the AMP/ATP ratio (more AMP, less ATP), increases the activity of AMPK by promoting phosphorylation by an unknown AMPK kinase (Winder *et al.,* 1999). Although we did not observe any differential expression with respect to this gene, we did observed a down regulation of two subunits of protein phosphatase 2A (PP2A) in obese individuals. PP2A is known to facilitate the interaction of regulatory and catalytic subunits of AMPK, thus keeping it in the inactive state (Gimeno-Alcaniz, *et al.,* 2003). A

decrease in the expression of the genes encoding PP2A may represent a mechanism by which AMPK activity is increased in obese young men, thereby inhibiting lipolysis. Further work in this area could include an analysis of the ratio of inactive to active AMPK protein in lean and obese individuals, which could add credibility to this theory.

Chapter 4.3: PKA induced lipolysis may be increased to compensate for excess adiposity in obese young men.

We also detected a number of genes that are involved in the typical cAMP activated protein kinase (PKA) regulation of lipolysis, as differentially expressed in adipose tissue, including two regulatory subunits of PKA itself. One regulatory subunit (PRKAR2B), also know as RII-beta showed a decrease in obese subjects. Another regulatory subunit (PRKAR2A), also known as RII-alpha, showed an increase of expression in obese subjects. It is difficult to determine what affect this differential regulation has on the activity of PKA based on the current study, however it is possible that differential expression of regulatory subunit isoforms may change the enzymes activity.

In support of this hypothesis, it has been shown that RII-beta knock out mice have a lean phenotype and are resistant to diet induced obesity (Planas *et al.,* 1999). An absence of the RII-beta subunit can be compensated for by an increase in the expression of RI-alpha, which more readily binds cAMP. This leads to lipolytic activation at lower levels of cAMP. Thus, these mice also exhibit an increase in the basal rate of lipolysis (Planas *et al.,* 1999). We have shown a reduced expression of RII-B in the adipose tissue of obese humans, and hypothesize that this indicates a compensatory mechanism of

lipolytic induction in response to increased adiposity. Support for this theory can also be gained through the use of animal models. For example, when RII-β is deleted in the obese, leptin deficient *ob/ob* mouse, body weight is decreased and energy expenditure is increased (Newhall *et al.,* 2005). This shows that knocking out RII- β, or possibly decreasing its expression can counteract symptoms of obesity. The effect of increased RII-α expression is not as clear, as this subunit has not been implicated in the pathophysiology of obesity, and there is no data as to its ability to bind cAMP relative to other regulatory subunits.

In addition, our results show the catalytic subunit of protein phosphatase 1 (PPP1CA), has reduced expression in obese individuals. PPP1CA dephosphorylates perilipin (Clifford *et al.,* 1998), a protein that protects the lipid droplet from lipolytic enzymes. Dephosphorylation of perilipin promotes association with the lipid droplet, and thus a decrease in PPP1CA would favor a situation whereby lipolysis is increased. A reduction in the expression of PPP1CA in obese individuals could also represent a secondary, or compensatory mechanism to increase the rate of lipolysis in the presence of increased adiposity. This gene is also a member of our 27 gene cluster, which adds further support for its role in the pathophysiology of obesity.

In addition to PKA's direct role in lipolysis activation, this enzyme can also affect nuclear gene transcription, which can have numerous implications on energy balance and the development of obesity. PKA mainly affects gene transcription by phosphorlyation of the cyclic AMP response element binding protein (CREB) (Zhang *et al.,* 2005). In animal models, loss of CREB function results in protection against diet induced obesity (Tsuchida *et al.,* 2005). We have detected reduced expression of CREB 3-like-3

(CREB3L3), and the transducer of CREB (TORC2) in the adipose tissue of obese individuals. This down regulation of CREB may act as a compensatory mechanism to protect against further triglyceride accumulation in obese individuals, assuming that the function of CREB is conserved between mice and humans.

Chapter 4.4: Numerous genes that may influence the rate of lipolysis.

It has been well documented that insulin and insulin-like growth factor (IGF) signaling inhibits lipolysis through the cAMP/PKA pathway (Large *et al.,* 1998). We have identified GRB10 as differentially expressed. GRB10 is an adapter protein that serves to destabilize the insulin and the IGF receptors (Vecchione *et al.,* 2003). The expression of GRB10 was reduced in obese individuals, which would favor a situation of increased insulin signaling, and therefore inhibition of lipolysis. Thus, this may represent a primary effect that may lead to increased adiposity.

Recent evidence shows that the SIRT1 gene may play an important role in the pathophysiology of obesity. This gene has mainly been studied for its role in caloric restriction induced longevity, and is believed to interact with the insulin/IGF pathway in animal models (Tissenbaum *et al.,* 2001). However it is now known that SIRT1 can mobilize fat in white adipose tissue (Picard *et al.,* 2004), thereby providing a protective effect against obesity. We have detected a reduced expression of the SIRT1 gene in obese individuals. Moreover, this difference was highly significant ($P = 0.00007$). This would favor a situation of reduced fat mobilization in obese individuals. Thus the SIRT1 gene represents a good candidate for further work in the field of obesity.

In the present study, numerous other genes that may have important functions in the development of obesity were found to be differentially expressed between lean and obese individuals. One such gene is the nicotinic acid receptor, GPR109B, which we found to be down-regulated in obese adipose tissue. Nicotinic acid has been used for many years as an effective treatment for lowering serum lipid concentrations (Karpe *et al.,* 2004), and is believed to affect the rate of lipolysis. However the mechanism is still poorly understood. As well, we detected an increased expression of the gene encoding the 1-acylglycerol-3-phosphate O-acyltransferase 4 protein (AGPAT4). Although there is very little data concerning the role of this gene in obesity, another gene of the same family (AGPAT2) has been implicated in a rare form of congenital lipodystrophy in humans. Loss of function mutations in this gene cause a complete absence of adipose tissue and susceptibility to type 2 diabetes (Gomes *et al.,* 2004), but whether AGPAT4 influences the state of obesity remains to be determined.

Chapter 4.5: Summary of findings

In summary, we have identified 114 significantly up-regulated and 204 down-regulated genes and transcripts in obese SCAT, when compared to non-obese SCAT. We have identified 181 genes that have never before been shown to be expressed in adipose tissue. This provides novel obesity candidate genes for further study. Furthermore, gene ontology analysis revealed an over-representation of genes involved in a specific range of biological process, which opens many new avenues for obesity related research. As well, we have discovered a set of 27 genes that accurately distinguishes between obese and

non-obese adipose tissue. The genes contained in this cluster all have ontology terms that are over-represented in our list of significantly differentially expressed genes, indicating potential roles for these genes, and the processes they are involved in the development of obesity.

With respect to biological processes that are believed to be involved in the development and/or pathophysiology of obesity, our results indicate that ATP production within the SCAT mitochondria may be deregulated in obese young men, and that this may have implications on whole body energy balance. Furthermore, we detected numerous genes involved in the control of lipolysis as differentially expressed between obese and non-obese SCAT. The regulation patterns of most of these genes indicate that the rate of lipolysis may be increased in obese young men, possibly representing a secondary effect to compensate for increased adiposity.

Chapter 5: Future directions

One of the main goals of the current work was to create a database of differential gene expression between obese and non-obese young men at baseline. The design of this study allows for further work involving adipose tissue gene expression profiles. Because mRNAs from the adipose tissue samples were competitively hybridized to our arrays with a standard reference RNA, we can now introduce nutritional challenges to our volunteers, and directly compare the results to the gene expression profiles at baseline. Moreover, any treatment can be introduced such as overfeeding, underfeeding, or acute exercise, and as long as the mRNAs from those adipose tissue samples are hybridized to an array with the same reference RNA, the results can be directly compared to baseline values.

Furthermore, we have identified a set of genes that accurately distinguishes obese from non-obese young men. This cluster of genes can be tested in further work with a second, independent set of samples to ensure the legitimacy of this result. As well, this cluster can be tested in response to nutritional challenges, to determine if the set of 27 genes can distinguish between the response to overfeeding, underfeeding, or acute exercise, in obese and non-obese individuals.

The use of real time polymerase chain reaction would add an important experiment based on the current findings. This method could be used to quantify the levels of expression of any given gene, and could be used to verify a sub-set of our results from the microarray experiments. Specifically, the genes we identified that distinguish

between obese and non-obese should be tested to ensure that the regulation patterns we identified via microarray are indeed correct.

One of the trends we identified in our data is that numerous genes with functions in the mitochondria, many of which are involved in the process of ATP coupled electron transport, have reduced expression in obese individuals. We hypothesize that AMPK may be involved in coupling reduced ATP production to the rate of lipolysis in adipose tissue. However this inference is based only on findings in other studies, and the fact that we identified an inhibitor of AMPK as having reduced expression in obese individuals. One crucial experiment that could clarify whether or not AMPK is involved in this process would be to measure the amount of AMPK protein, and activated AMPK protein (phosphorylated AMPK) via western blot. If AMPK is indeed involved in coupling ATP production to lipolysis in adipose tissue, one would expect increased levels of activated AMPK in obese adipose tissue, as AMPK is known to inhibit lipolysis (Winder *et al.*, 1999)

In addition, our lab is currently doing a large scale population based association study, where single nucleotide polymorphisms (SNPs) are genotyped and associated with measures of obesity and obesity related disorders. Previously, this study has focused on known obesity candidate genes known from the literature. As we have now identified 318 genes that are differentially expressed between obese and non-obese adipose tissue, these genes can serve as obesity candidate genes for the association study. This will allow for the discovery of new obesity causative genes that have not been published elsewhere to date.

In summary, the current study has found many novel aspects of the genetic control of obesity, through the comparison of obese and non-obese expression profiles. Our results indicate a state of decreased expression of genes involved in ATP production. Our results also suggest that the process of lipolysis plays an important role in the pathophysiology of obesity. This process may be inhibited in obese individuals, but compensatory mechanisms such as a reduction in the expression of the R2-B regulatory subunit may act to increase TG hydrolysis in the presence of increased adiposity. Furthermore, we have defined a gene expression cluster that predicts the obese state with a high degree of accuracy. This cluster contains genes involved in lipolysis, as well as other biological processes that may also be involved in the development of obesity. Most importantly, the discovery of 181 novel obesity candidate genes provides valuable targets for further obesity and pharmaceutical based research.

Although the current work adds much insight to the pathophysiology and etiology of obesity, there are limitations. Because the gene expression of adipose tissue was assayed at baseline, it is difficult to determine primary changes in gene expression as opposed to secondary changes in gene expression. That is, what changes are pathogenic, and may result in increased adiposity, and which changes are secondary due increased adiposity. In other words, for any change in expression for a given gene, it is difficult to determine if that change in a cause or an effect. Further work may help to distinguish between the two. One such experiment would be to assay the gene expression in adipose tissue in "pre-obese" individuals. "Pre-obese" individuals would be defined as people who are overweight, but not yet obese, and have a family history of obesity. This would

allow for a "snapshot" of a gene expression profile that may be primarily responsible for the development of obesity.

In order to reduce the effect of confounding factors in the current work, we used male volunteers only, with a narrow age range. However this limits the interpretation of the results as the expression of genes that influence obesity, or any disease or condition, can be influenced by age and gender. This study will soon be repeated using female volunteers, and volunteers of an older age range to determine what genes may influence the status of adiposity in these subsets of the population, and how their profiles differ from those of young males. As well, due to the high cost of microarray studies, most work published to date involves a low sample number. We have used a total of 8 pairs, or 16 individuals, in the current work. Although this is larger than most microarray studies conducted on adipose tissue, it is still relatively small and limits the statistical power of our study.

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Appendix 1: CART gene association study

The Cocaine-Amphetamine regulated transcript (CART) is a known obesity candidate gene. As part of the authors' thesis work, a population based association study was undertaken to determine if sequence variation in the CART gene is associated with obesity and/or obesity related phenotypes. As the number of subjects used in the study is small, and the study incomplete, a complete discussion of the material and results were not included in this thesis. Briefly, two single nucleotide polymorphisms located near the CART gene were genotyped in a total of 527 Newfoundlanders. These included an A/G SNP in the 5' untranslated region of the gene, and a C/T SNP in the 3' untranslated region of the gene. Fasting blood samples were taken for analysis of serum components and isolation of genomic DNA. Body composition was assayed using dual energy X-ray absorptiometry (DXA). The following represents primary data and basic statistical analysis for the CART gene association study.

Results

Physical characteristics of the subjects involved in the association study are shown in **supplementary table 1**. When analyzed by gender, men were significantly younger than women, and had a lower percentage of body fat. However, men had a significantly higher BMI when compared to women. Men were taller and heavier than women on average, and had higher serum levels of glucose. Women had significantly higher levels of serum HDL-C and TG.

The frequency of the minor allele for the A/G SNP in the 5' region of the CART gene was 0.42. The frequency of the minor allele for the C/T SNP in the 3' region of the

CART gene was 0.15. Both SNP's were in Hardy-Weingberg equilibrium as assessed by Chi-squared analysis. As little difference between genders was observed with the associations, analysis of allele and genotype associations are reported in all volunteers combined unless otherwise stated.

As shown in **supplementary table 2**, no significant differences were seen between physical and biochemical characteristics and the three possible genotypes for the 5' A/G SNP.

Similarly, as shown in **supplementary table 3**, no differences were seen between the three genotypes for the 3' C/T SNP and measures of obesity, however minor allele homozygotes do have significantly lower levels of fasting serum insulin. However, fasting levels of glucose remain normal, as is the measure of insulin resistance (HOMA-R), so the biological significance of this trend remains unclear.

Supplementary table 1: Physical and biochemical characteristics of the subjects for CART gene association analysis.

Characteristic	All subjects (n=527)	Women (n=409)	men (n=118)
Age	42.18 (19-62)	42.7 (19-62)	39.8 (19-61)*
%BF	34.69 (4.6-59.9)	36.7 (4.6-59.9)	24.6 (7.4-41.6)*
BMI	26.21 (16.98-54.27)	26.0 (17.0-54.3)	27.4 (19.9-43.3)*
Height	164.3- (134-198)	162.1 (135-178)	175.0 (157-198)*
Weight	70.87 (45.4-156.8)	68.1 (45.4-156.8)	83.7 (54.7-136.5)*
Insulin	72.8 (15.7-720.0)	72.3 (15.7- 720)	75.5 (22.9-301.0)
Glucose	5.1 (2.2- 7.0)	5.0 (3.0-10.8)	5.3 (2.2-14.4)*
HDL-C	1.52 (0.65-3.15)	1.59 (0.79-3.15)	1.24 (0.65-2.21)*
LDL-C	3.17 (1.02-7.61)	3.18 (1.02-7.16)	3.14 (1.31-5.69)
TG	1.28 (0.27-5.88)	1.50 (0.31-4.7)	1.23 (0.27-5.88)*
HOMA-R	2.4 (0.5-22.8)	2.3 (0.5-22.8)	2.5 (0.5-10.6)

Data presented as mean, (range in parentheses)

* indicates statistically significant difference between males and females, student t-test

Supplementary table 2: Genotypic association between the 5' A/G SNP and measures of obesity and related phenotypes.

Characteristic	minor allele homozygotes	heterozygotes	major allele homozygotes
N	99	226	157
% Body Fat	35.0 ± 8.6	34.8 ± 9.1	34.0 ± 8.4
BMI	26.2 ± 5.3	26.4 ± 5.3	26.3 ± 4.6
Glucose	5.17 ± 1.33	5.11 ± 0.79	4.98 ± 0.51
Insulin[1]	74.8 ± 45.3	73.7 ± 60.5	73.6 ± 43.3
HDL	1.53 ± 0.35	1.52 ± 0.33	1.54 ± 0.42
LDL	3.17 ± 0.77	3.22 ±0.90	3.17 ± 0.84
TG[1]	1.32 ± 0.88	1.29 ± 0.83	1.24 ± 0.79
HOMA-R[1]	2.44 ± 1.83	2.34 ± 1.70	2.41 ± 2.20

Data presented as mean ± SD

No significant differences, as assessed by one-factor ANOVA, corrected for multiple testing using Bonferroni t-test.

[1]transformed using the natural Log for significance testing

Supplementary table 3: Genotypic associations between the 3' C/T SNP and measures of obesity and related phenotypes.

Characteristic	minor allele Homozygotes	heterozygotes	major allele heterozygotes
N	13-14	137-158	375-444
% Body Fat	35.4 ± 7.6	34.7 ± 9.3	34.6 ± 8.7
BMI	26.8 ± 7.2	26.3 ± 4.8	26.1 ± 4.9
Glucose	4.9 ± 0.4	5.0 ± 0.9	5.1 ± 0.8
Insulin[1]	46.3 ± 21.4*	69.8 ± 45.4	74.9 ± 53.7
HDL	1.44 ± 0.26	1.56 ± 0.40	1.53 ± 0.38
LDL	3.27 ± 0.66	3.21 ± 0.87	3.17 ± 0.86
TG[1]	1.07 ± 0.60	1.25 ± 0.90	1.30 ± 0.81
HOMA-R[1]	2.16 ± 1.20	2.30 ± 1.66	2.39 ± 1.97

Data presented as mean ± SD

* significant from all other genotypes, $p \leq 0.01$, one-factor ANOVA

[1]transformed using the natural Log for significance testing

Appendix 2: Published manuscript

As part of my graduate project, I also collected data for another study, which assessed the differences between two methods of body fat estimation. For this study, we compared body fat estimation by bioelectrical impedance analysis (BIA) to that of a standard method, dual energy X-ray abosrptiometry (DXA). The main finding of this study was that the bias introduced by BIA is dependant on the adiposity of the individual. This study was published in the American Journal of Clinical nutrition, of which the abstract is presented below. As the second author, I was responsible for data collection, statistical analysis, and editing the manuscript.

Abstract

Background: Bioimpedance analysis (BIA) is widely used in clinics and research work to measure body composition. However the results of BIA validation with reference methods are contradictive, and there is little data about the influence of adiposity on measurement of body composition by BIA.

Objective: To determine the effect of gender and adiposity on the validity of BIA predicted %Fat when compared to that of dual energy X-ray absorptiometry (DXA).

Design: 591 healthy subjects were recruited in Newfoundland and Labrador. %Fat was predicted using BIA and compared to dual energy X-ray absorptiometry. Methods agreement was assessed by Pearson correlation, and Bland and Altman analysis. Differences of percent body fat among groups based on gender and adiposity was performed using one-way ANOVA and corrected using Bonferroni.

Results: Correlation between BIA and DXA reaches 0.88 for the whole population, 0.78 for men, and 0.85 for women. The mean %Fat determined by BIA (32.89 ± 8.0) was significantly lower than that by DXA (34.72 ± 8.70). BIA significantly overestimated %Fat in lean subjects and underestimated %Fat in overweight subjects. The cutoffs are gender specific. BIA overestimated by 3.0% and 4.4 % when %Fat is <15 in men and <25 in women, and underestimated by 4.3% and 2.7% when %Fat > 25 in men and >33 in women.

Conclusions: BIA is a good alternative to estimate percent body fat when subjects are within a healthy range of %Fat. BIA tends to overestimate %Fat for lean subjects and underestimate %Fat in obese subjects.

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