UNDERSTANDING THE ETIOLOGY OF OBESITY: A MULTI-FACETED APPROACH

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Understanding the etiology of obesity: A multi-faceted approach

by

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Abstract

Obesity, caused by an excessive accumulation of body fat due to a chronic energy surplus, is a serious public health concern with numerous comorbidities. It is a complex disease with many factors contributing to its manifestation; it is thought that obesity results from the action of multiple genes in combination with lifestyle and environmental factors. At the current time, only a fraction of the genes involved in obesity have been identified. The aims of this thesis were first, to characterize the obesity phenotype in the Newfoundland population and second, shed light on its genetic etiology. This goal was achieved using data from two different studies – the large scale, population-based CODING (Complex Diseases in the Newfoundland Population: Environment and Genetics) Study and an intervention-based, 7-day overfeeding study.

We have shown that body mass index (BMI) misclassifies adiposity status in nearly one-third of individuals compared to the more accurate reference method, dual energy X-ray absorptiometry (DXA). Furthermore, we found that approximately half of obese subjects were metabolically healthy when using DXA criteria, which was significantly higher than previous reports using BMI. Among BMI-defined normal weight individuals, higher body fat percentage (%BF) determined using DXA was associated with a 3-fold increased risk of cardiometabolic disease. To further understand the genetic etiology, a candidate gene, genetic association approach was utilized. We identified two SNPs (rs10882280 and rs11187545) within *RBP4*, a newly discovered adipokine, that were associated with increased serum HDL cholesterol but no other

obesity-related parameter. No significant associations were observed between genetic variation in another novel adipokine, *NAMPT*, and parameters of glucose and lipid metabolism, obesity, or systemic inflammation. We also sought to explore the response of lean and obese subjects to a 7-day hypercaloric diet. We found that RBP4 was not regulated by the overfeeding challenge but could serve as a predictor of insulin resistance in lean subjects. In addition, 45 novel obesity candidate genes have been identified that were regulated by the nutritional challenge; of these, six were differentially expressed between lean and obese and as such, represent the most promising targets for downstream work related to obesity.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

%BF	Body fat percentage
%TF	Trunk fat percentage
BIA	Bioelectric impedance analysis
B2M	Beta-2-microglobulin
BMI	Body mass index
CODING Study	Complex Diseases in the Newfoundland Population: Environment and Genetics Study
CTSC	Cathepsin C
Cy3	Cyanine 3
Cy5	Cyanine 5
DBP	Diastolic blood pressure
DXA	Dual energy X-ray absorptiometry
FDR	False discovery rate
FTO	Fat mass and obesity gene
GLUT4	Glucose transporter 4
GO	Gene Ontology
GWAS	Genome wide association study
HOMAβ	Homeostasis model assessment for beta-cell function
HOMA-IR	Homeostasis model assessment for insulin resistance
hsCRP	High-sensitivity C-reactive protein
IL-6	Interluekin-6
IRS2	Insulin receptor substrate 2
KEGG	Kyoto Encyclopedia of Genes and Genomes
LD	Linkage disequilibrium
MAF	Minor allele frequency
MHO	Metabolically healthy but obese
MONW	Metabolically obese but normal weight
NAD	Nicotinamide adenine dinucleotide
NAMPT	Nicotinamide phosphoribosyltransferase
NL	Newfoundland
PDC	Pyruvate dehydrogenase complex
PDK4	Pyruvate dehydrogenase kinase, isozyme 4
RBP4	Retinol binding protein 4
SBP	Systolic blood pressure
SCD	Stearoyl-CoA desaturase
SNP	Single nucleotide polymorphism
T2D	Type 2 diabetes
TALDO1	Transaldolase 1
TF	Transferrin
TG	Triacylglycerol
WHO	World Health Organization

Co-Authorship Statement

This thesis consists of seven published articles, six of which I am first author. For each manuscript where I am primary author, I participated in study design and data collection. Specifically, I aided with recruitment of volunteers, performing DXA scans, obtaining adipose tissue samples in collaboration with a surgeon, and delivering food to volunteers in the overfeeding study. I had a lead role in all laboratory work including plasma/serum isolation, DNA/RNA isolation of blood and adipose tissue, hsCRP measurements, SNP genotyping, and microarray experiments. I performed the majority of data analyses and wrote the initial draft of each manuscript. I am listed as second author for Chapter 2.1 ("Comparison of the classification of obesity by BMI versus dual energy x-ray absorptiometry in the Newfoundland population"). For this chapter, I aided in data collection and analyses, as well as drafting the manuscript in collaboration with the primary author.

1

Introduction

1.1 Prevalence and consequences of obesity

Obesity, a condition of excessive body fat, is one of the most serious public health problems facing the world today. Obesity rates amongst developed countries have increased substantially in the past three decades and the disease is now affecting millions globally. Specifically, the World Health Organization (WHO) estimated that at least 400 million adults are obese worldwide and projected this number to nearly double by 2015 (1). In Canada, the growing trend towards increased body weight is especially concerning as the number of overweight people increased from 27% to 34% between 1985 – 2003 while the prevalence of obesity nearly tripled during the same time period (2). Currently, over 23% of Canadians are classified as obese according to body mass index (BMI) criteria (≥ 30.0 kg m^{-2}; ref 3). Of particular importance, obesity rates in the province of Newfoundland and Labrador (NL) are approximately 12% higher than the national average indicating a significant disease burden in our province (Figure 1).

The consequences of excess body fat are numerous and include type 2 diabetes (T2D; ref 4), cardiovascular disease (5), hypertension (6), stroke (7), dyslipidemia (8), and certain types of cancer (9). Furthermore, obesity has been found to decrease quality of life (10) and overall life expectancy (11). As a result, this disease and its associated comorbidities are placing a large burden on our already overwhelmed health care system (12). In particular, a recent systematic review of the worldwide costs associated with obesity estimated that between 0.7% and 2.8% of total health care expenditures are attributable to this disease (13). Moreover, obese individuals have significantly higher

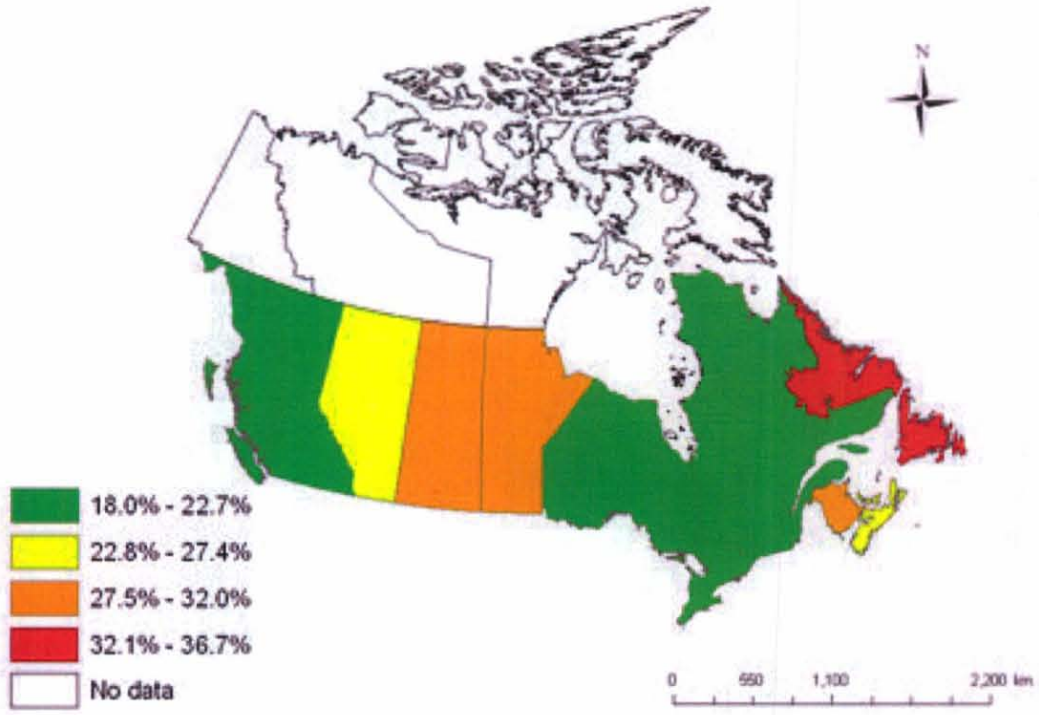

Figure 1.1 Map of the prevalence of obesity in Canadian Adults according to body mass index (BMI $\geq$ 30.0 kg m^{-2}) (CCHS 2.2; ref 14)

(30%) medical costs compared to normal weight individuals (13). Taken together, these data indicate that the growing obesity epidemic is an alarming trend that warrants further study to understand the issue.

1.2 Defining obesity

Obesity is defined as an excessive amount of body fat and is commonly assessed using BMI, calculated as a person's weight divided by their height squared. According to the WHO, BMI-defined adiposity status is classified as follows: underweight (>18.5 kg m^{-2}), normal weight (18.5 – 24.9 kg m^{-2}), overweight (25.0 – 29.9 kg m^{-2}) and obese (>30.0 kg m^{-2}) (15). In addition, the obese category is further divided into class I (30.0 – 34.9 kg m^{-2}), class II (35.0 – 39.9 kg m^{-2}), and class III (>40.0 kg m^{-2}). However, it is the amount of body fat and not necessarily the amount of excess body weight that determines an individual's health risks and as such, BMI has come under criticism for its inability to differentiate between lean tissue and fat. Furthermore, BMI does not measure fat distribution and we now know that central adiposity plays a major role in the development of obesity-related disease. As a result, recent work has focused on identifying tools that are better able to predict disease risk. In terms of anthropometric measurements, waist circumference is a valuable method that indirectly measures intra-abdominal fat, a known contributor to cardiovascular and other obesity-related diseases. A number of studies have demonstrated that waist circumference is a better predictor of health risk and ultimately death compared to BMI (16-17). Presently, waist

circumference measurements ≥ 102 cm for men and ≥ 88cm for women indicate higher health risks according to clinical guidelines (18). Although anthropometric classifications such as BMI and waist circumference have proven useful in large-scale population studies, they are not without limitations. For instance, both lack sensitivity and specificity when applied to individuals (19). Specifically, individuals with the same BMI can have vast differences in the amount of body fat and vice versa (20). Likewise, large interindividual variation in visceral fat exists in individuals with the same waist circumference (21). These data suggest that anthropometric measurements alone are insufficient for making any conclusive statements regarding an individual's health risk.

As it is the amount of excess fat relative to lean tissue that conveys health risk, obesity is more accurately defined according to body fat percentage (%BF). For more direct measurements of %BF other instruments are often employed, some more expensive than others. Bioelectric impedance analysis (BIA) is a common method used to estimate %BF owing to its low cost and portability. Numerous studies have investigated the accuracy of BMI as an index of obesity compared to %BF measurements determined using BIA. Romero-Corral et al demonstrated that BMI has limited ability to accurately predict BIA-defined obesity, particularly for those in the overweight range, (25.0 – 29.9 kg m^{-2}) (20). Although BIA is considered a superior method compared to BMI, even more accurate instruments exist for the measurement of body fatness. Underwater weighing has long been used as the traditional standard (22) however air-displacement plethysmography and dual energy X-ray absorptiometry (DXA) have recently been employed as two new reference methods (23-25). Although more precise in their

measurements, the use of these methods is limited due to their high cost and inaccessibility. Nonetheless, confirmation of anthropometric indicators of obesity, namely BMI, is required by a large population-based study using these more accurate body composition measures.

Despite the fact that obesity is characterized by the presence of excess body fat, this does not always imply or reliably predict ill health. As such, current obesity classification systems based solely on BMI, waist circumference, or %BF are not always an accurate reflection of obesity-associated disease risks, comorbidities, or quality of life. In this regard, recent work has focused on classification of subgroups of obesity whose definitions not only include measurements of body composition, but also a number of cardiometabolic abnormalities including hypertension, abnormal lipid profiles, insulin resistance, and systemic inflammation. Specifically, it has now been recognized that not all obese individuals display clustering of the aforementioned metabolic and cardiovascular risk factors (metabolically healthy but obese {MHO}; ref 26). Moreover, not all lean individuals are void of these risk factors; in fact, a significant proportion of normal weight subjects display a metabolic profile similar to what is associated with being overweight or obese (metabolically obese but normal weight {MONW}; ref 26). This has led to the identification of different subtypes of obesity as outlined in Figure 1.2. Despite long-standing clinical awareness, characterization of both of these phenotypes is still in its infancy. It is known that both of these subtypes of obesity are well represented; MHO individuals account for approximately 30% of the obese population while MONW individuals account for up to 25% of normal weight subjects (27-29). Presently,

Metabolically Obese Normal Weight (MONW)

- Normal BMI
- Hypertension
- Low insulin sensitivity
- Low HDL, High TG
- Systemic inflammation

Metabolically Healthy

- Normal BMI
- Normal blood pressure
- High insulin sensitivity
- High HDL, Low TG
- Low inflammation

Metabolically Healthy Obese (MHO)

- High BMI
- Normal blood pressure
- High insulin sensitivity
- High HDL, Low TG
- Low inflammation

Metabolically Abnormal Obese

- High BMI
- Hypertension
- Low insulin sensitivity
- Low HDL, High TG
- Systemic inflammation

Figure 1.2 Subtypes of obesity and their metabolic characteristics

these subtypes have been defined according to BMI criteria and as stated above, more accurate methods for determining %BF exist. As such, confirmation of the prevalence of these subtypes using DXA measurements as well as further characterization of their association with body fat is necessary.

1.3 Etiology of obesity

Body weight regulation involves a complex set of factors, including environmental, endocrine, and genetic influences that ultimately control the balance between energy intake and expenditure. Obesity has been attributed to an energy imbalance in which energy intake exceeds energy expenditure resulting in increased fat stores in the body. The complexity of body weight regulation imparts a considerable challenge to obesity researchers in understanding the etiology of this disease. However, identifying the environmental, endocrine, genetic and/or combination of these factors that contribute to the chronic state of energy imbalance evident in western society will ultimately aid in developing effective prevention and treatment strategies.

1.3.1 Environmental factors contribute to the growing prevalence of obesity

Although susceptibility towards obesity is in part determined by genetic factors, an obesity-promoting or obesigenic environment is normally necessary for its phenotypic expression (30). The rapid weight gain in the population over the last three decades is

largely due to the changing environment (31). We currently live in a world that promotes energy consumption and discourages energy expenditure. There are a number of factors that play a role in the overconsumption of energy including the ease at which good-tasting, inexpensive, energy-dense foods are available and the serving of these foods in large portions (31). Food is also heavily advertised, and it has become acceptable to eat food everywhere (32). Other environmental factors play a role in the reduction of physical activity levels in recent years which has led to a decrease in total energy expenditure. These include reductions in jobs requiring physical labor, reduction in energy expenditures at school and in daily living, as well as an increase in time spent engaging in sedentary activities such as watching television, playing video games and surfing the Web (31). As well, communities are built in such a way that promotes driving and not walking (32). Taken as a whole, the 20th century has been a transition period characterized by important environmental changes that influence diet and activity habits (33). Technological advancements have resulted in an easier way of life for most people including pre-packaged and fast food, a reduction in daily physical labor, and increased time for leisure activities. While some may perceive this changing environment as advantageous, it has ultimately contributed to the current obesity epidemic.

1.3.2 The genetic contribution to obesity

Although the growing trend towards increased caloric intake and decreased physical activity plays a significant role in the obesity epidemic, it has become evident

that genetics contributes to a person's risk as well. It is well established that mutations in genes that encode proteins involved in appetite regulation are responsible for many Mendelian disorders in which obesity is a major phenotype. Such monogenic forms of obesity include Prader-Willi syndrome (which involves a mutation or deletion of the paternally contributed chromosome 15q11-q13) (34), and Bardet Biedl syndrome (associated with mutations in at least twelve different loci) (35). Obesity can also result from single gene mutations in genes involved in appetite regulation such as the leptin gene (36), leptin receptor gene (37), and the melanocortin-4 receptor gene (*MC4R*; ref 38) as well as many others. Although there is ample information available on the genetics of these mutations in the literature, such syndromic forms of obesity are quite rare in the general population. According to the most recent version of the human obesity gene map, only 176 human obesity cases due to single-gene mutations in 11 different genes have been reported in the literature (39).

Although great strides have been made in the identification of genes involved in rare forms of obesity, this has not translated to an explanation of the underlying genetic etiology of the common form of obesity. Common obesity is a complex multifactorial disease meaning that inter-individual variation is thought to be a result of the action of multiple genes and the environment (40). In this model, many genes have a small influence on the adiposity status of a given individual. Although the overall genetic contribution to BMI is quite high (ranging between 40-70%) (41), evaluating the contribution of any single gene to human obesity is difficult, with the exception of specific mutations with a very low frequency in the population, such as those mentioned

above. According to the latest installment of the Obesity Gene Map, more than 600 genes, markers, and chromosomal regions have been associated with obesity phenotypes (39).

Initial studies regarding the genetics of common obesity were mostly family-based genome-wide linkage scans. Although successful in identifying genetic variants causing rare monogenic disease, linkage studies proved less successful when applied to multifactorial diseases. Positional cloning based on linkage results has identified a small number of possible candidates, including glutamate decarboxylase 2 (*GAD2*; ref 42), ectonucloetide pyrophosphatase/phosphodiesterase 1 (*ENPP1*; ref 43), and solute carrier family 6 (amino acid transporter), member 14 (*SLC6A14*; refs 44-45). A recent meta-analysis of 37 published linkage studies containing over 31,000 individuals did not detect strong evidence for linkage for BMI at any locus (46). Recently, genome wide association studies (GWAS) have identified a number of additional candidates including the fat mass and obesity (FTO) gene, the first common obesity gene (47-48). A common variant in *FTO* was unequivocally associated with BMI and increased risk for obesity simultaneously by two groups. Specifically, variation in *FTO* was estimated to account for approximately 1% of the total heritability of BMI. Although this is a relatively small effect on total adiposity, countless other groups have confirmed these initial findings (49-51). Additional candidates have since been identified through GWAS including variants in or near *MC4R*, *NCP1*, *MAF*, *PTER*, *KCTD15*, *MTCH2*, *NEGR1*, *SH2B1*, and *TMEM18* (52-55). At the current time, however, more work is required to elucidate further genes

and pathways involved in the genetic etiology of obesity as well as reveal their biological role in this disease.

1.3.3 Gene-Environment interactions in the etiology of obesity

The inability to provide simple genetic answers regarding the obesity phenotype is not unusual among common, complex diseases. This is due to the fact that multiple factors can influence the pathogenesis of the disease including interactions among many genes, as well as interactions between the environment and genes. Considering gene-environment interactions is thought to be important as it allows for full evaluation of the relationship between environmental and genetic components that contribute to obesity. In simple terms, a gene-environment interaction effect can be described as the differential response or adaptation to an environmental factor, such as a change in energy balance, depending on the genotype of the individual (56). Perhaps the most well-known example of this is the substantial difference in obesity rates between different populations of Pima Indians. Although they have a similar genetic make-up, those living in the restrictive environment of the remote Mexican Sierra Madre Mountains have a much lower prevalence of obesity and T2D than those living in the obesigenic environment of Arizona (57). Specifically, Mexican Pimas had an average BMI of 24.9 kg m^{-2} while Arizona Pima Indians had an average BMI of 33.4 kg m^{-2}. This study suggests that despite similar genetic predisposition among both populations, a more traditional lifestyle characterized by a healthier diet and greater energy expenditure may protect against the

development of obesity. Furthermore, it has also been documented that various nutrients can modulate gene expression and thus influence the impact of these variants on the development of complex diseases, such as obesity (58). In addition, overfeeding studies in monozygotic twins have demonstrated significant gene-diet interaction effects; the within-pair response to a positive energy balance is much greater than the between-pair response for a number of parameters including total body weight, %BF, and estimated subcutaneous fat (59-60). This has also been established in negative energy intervention twin studies (61-62).

Presently, only a small fraction of the genetic contribution to obesity has been identified. This is in part due to the complex interplay between genetic and environmental factors that likely masks the effect of specific genetic variants. As such, gene-environment interactions will become increasingly important to consider as the environment becomes more conducive to the development of obesity. Moving forward, it will be important to use our current knowledge of obesity genetics to describe the inter-individual variation in the response to various environmental factors, such as overfeeding, to provide additional information regarding the pathogenesis of this disease. The goal of this type work will be to develop more effective prevention and treatment options based on one's personalized genetic background to ultimately improve overall quality of life for these patients.

1.4 Adipose tissue plays an active role in the regulation of energy balance

Adipose tissue is a specialized connective tissue that acts as a major storage site for fat in the form of triacylglycerol (TG). The amount of TG stored within adipocytes is an accurate reflection of the imbalance between energy intake and energy expenditure, integrated over a long period of time. Uncontrolled expansion of adipose tissue as a result of a chronic positive energy balance leads to obesity and because of this it has been extensively studied for the role it plays in the phenotypic expression of this disease. Moreover, adipose tissue is now recognized as an active endocrine organ that influences body weight regulation through control of both hunger and satiety signals. Specifically, adipose tissue is both the target organ for regulatory signals from other parts of the body while at the same time sending out signals to act on metabolism as well as other physiological processes. As a result of this complex network, adipose tissue influences metabolic activity at many other sites as well, including skeletal muscle, liver and the brain.

Over the past 15 years, significant advancements have been made in our understanding of the adipocyte as a secretory cell. It is now known that a number of hormones are released from adipose tissue in response to various physiological cues. These hormones, collectively called adipokines, are involved in a number of metabolic and inflammatory processes and play an important role in maintaining energy homeostasis. In addition, endocrine dysfunction at the level of the adipocyte is thought to play a crucial role in the development of obesity. At the current time, more than two

dozen adipokines have been identified as being expressed from various adipose tissue depots including leptin, adiponectin, visfatin, and retinol binding protein 4 (RBP4) as well as various inflammatory cytokines such as tumor necrosis factor α, interleukin-6 (IL-6), and monocyte chemoattractant protein-1 among others. Recent work has addressed the role that each of these plays in the development of obesity.

Leptin, the first adipokine discovered in 1994 (63), is a 16 kDa anorexigenic peptide that acts on the hypothalamus to regulate body weight by inhibiting food intake and stimulating energy expenditure (64-65). Both nonsense and missense mutations in the leptin and leptin receptor genes induce hyperphagia and obesity in both animal models (66) and humans (36, 67) although the prevalence of these disorders is quite rare. Circulating levels of leptin increase with obesity due to resistance to the hormone (68); furthermore, leptin levels increase in response to overfeeding and decrease with starvation (69). In addition, leptin is involved in the regulation of the reproductive system and onset of puberty (70). Moreover, testicular steroids decrease leptin concentrations (71) while ovarian steroids increase levels (72). Other physiological roles for leptin include modulating the T-cell immune response, stimulating the proliferation of T-helper cells, and increasing production of pro-inflammatory cytokines through regulation of different immune cells (64,73).

Adiponectin is another well characterized 30 kDa adipokine that has been implicated in the regulation of body weight and glucose metabolism. In animal models, adiponectin null mice have reduced insulin sensitivity (74-75) while adiponectin

overexpression in *ob/ob* mice confers remarkable metabolic improvements (76). In humans, circulating levels of this hormone are decreased in obesity-induced insulin resistance (77-78). In addition, a wide array of diseases, including stroke, coronary heart disease, steatohepatitis, nonalcoholic fatty liver disease, and a number of cancers, have been associated with decreased adiponectin levels (79).

Although less known, visfatin and RBP4 are two newly discovered adipokines that have garnered recent attention for their potential role in the development of obesity and related disorders. Of particular interest to our lab, these hormones both play fundamental roles in modulating glucose homeostasis, albeit in opposite directions. Visfatin, previously called pre-B colony enhancing factor, is expressed in many different cells and tissues, and was first identified as a protein involved in inflammatory processes, particularly, B-cell maturation (80). More recently, visfatin was found to be predominantly expressed in visceral adipose tissue, from which the name visfatin was derived (81). Initial rodent studies suggested that visfatin had insulin-mimetic properties; injection of visfatin in mice lowered blood glucose while mice with a mutation in the visfatin gene had higher glucose levels (81). Although promising, subsequent studies failed to confirm this initial finding and as a result, the paper was later retracted (82). In 2007, Revollo et al demonstrated that visfatin instead has nicotinamide adenine dinucleotide (NAD) biosynthetic activity and is in fact a circulating form of nicotinamide phosphoribosyltransferase (NAMPT), an essential enzyme in the NAD biosynthetic pathway (83). In particular, haplodeficiency and chemical inhibition of visfatin/NAMPT in mice resulted in impaired glucose tolerance and caused defects in glucose-stimulated

insulin secretion in pancreatic islets *in vivo* and *in vitro*. Human studies have been more conflicting. Although some have demonstrated associations between serum visfatin and T2D (84), insulin resistance (85) and obesity (86), others have not (87-88). Furthermore, it has been shown that visfatin is not responsive to PPARγ agonists (89). As a result, the role that visfatin plays in maintaining glucose homeostasis in humans is not clear.

In 2005, RBP4 was identified as an adipose tissue-derived circulating factor that was highly expressed in adipose tissue-specific glucose transporter 4 (GLUT4) knockout (adip-*Glut4*$^{-/-}$) mice (90). GLUT4 facilitates the transport of glucose across plasma membranes into both skeletal muscle cells and adipocytes. Although skeletal muscle is the major site of insulin-stimulated glucose uptake, studies have shown that insulin resistant states such as obesity, metabolic syndrome, and T2D result in a down-regulation of GLUT4 in adipose tissue but not in skeletal muscle as would be expected. This led to the hypothesis that there must be a circulating factor secreted from adipose tissue in response to the down-regulation of GLUT4 that signals impaired glucose uptake in skeletal muscle resulting in peripheral insulin resistance. Yang et al identified RBP4 as such a factor in six insulin resistant mouse models of insulin resistance (90). Moreover, they found that transgenic mice overexpressing *Glut4* had lower circulating RBP4 and enhanced insulin sensitivity. In addition, *Rbp4*$^{-/-}$ mice also demonstrated elevated insulin sensitivity. Conversely, transgenic mice expressing human RBP4 and wild-type mice injected with recombinant human RBP4 had elevated insulin levels and insulin resistance. When adip-*Glut4*$^{-/-}$ mice were injected with rosiglitazone, a commonly prescribed drug for T2D that binds to peroxisome proliferator-activated receptor (PPAR) γ thereby

increasing insulin sensitivity, RBP4 levels were normalized. Furthermore, the synthetic retinoid fenretinide, which increases urinary excretion of RBP4, resulted in lower RBP4 levels and ameliorated insulin resistance as well as glucose intolerance in diet-induced obese mice. The authors tried to delineate a possible molecular mechanism and found that RBP4 altered insulin sensitivity in part by affecting insulin signaling in muscle through a reduction in both insulin-stimulated phosphoinositide-3-kinase activity and tyrosine phosphorylation of insulin receptor substrate-1. As well, the authors found that RBP4 indirectly induced expression of a gluconeogenic enzyme, phosphoenolpyruvate kinase, in the liver of mice injected with human RBP4. Moreover, RBP4 treatment increased basal glucose production and reduced the effectiveness of insulin in suppressing glucose production in rat hepatocytes. From these studies the authors concluded that in addition to its other endocrine functions, adipose tissue also acts as a glucose sensor. Specifically, impairment of GLUT4 in adipose tissue, such as that seen in adip-*Glut4*$^{-/-}$ mice, leads to secretion of RBP4 from adipocytes and a subsequent decline in glucose uptake into skeletal muscle as well as stimulation of glucose production by liver, ultimately increasing blood glucose levels and triggering systemic insulin resistance (Figure 1.3).

Following this initial discovery, human studies ensued, however, the findings have been more ambiguous. Although some have shown that serum RBP4 is increased in obese subjects (90-91), patients with T2D (91-92), and in lean subjects with a family history of T2D (91), others have failed to corroborate this (93). One study found no association between serum RBP4 and insulin sensitivity in older subjects but a weak

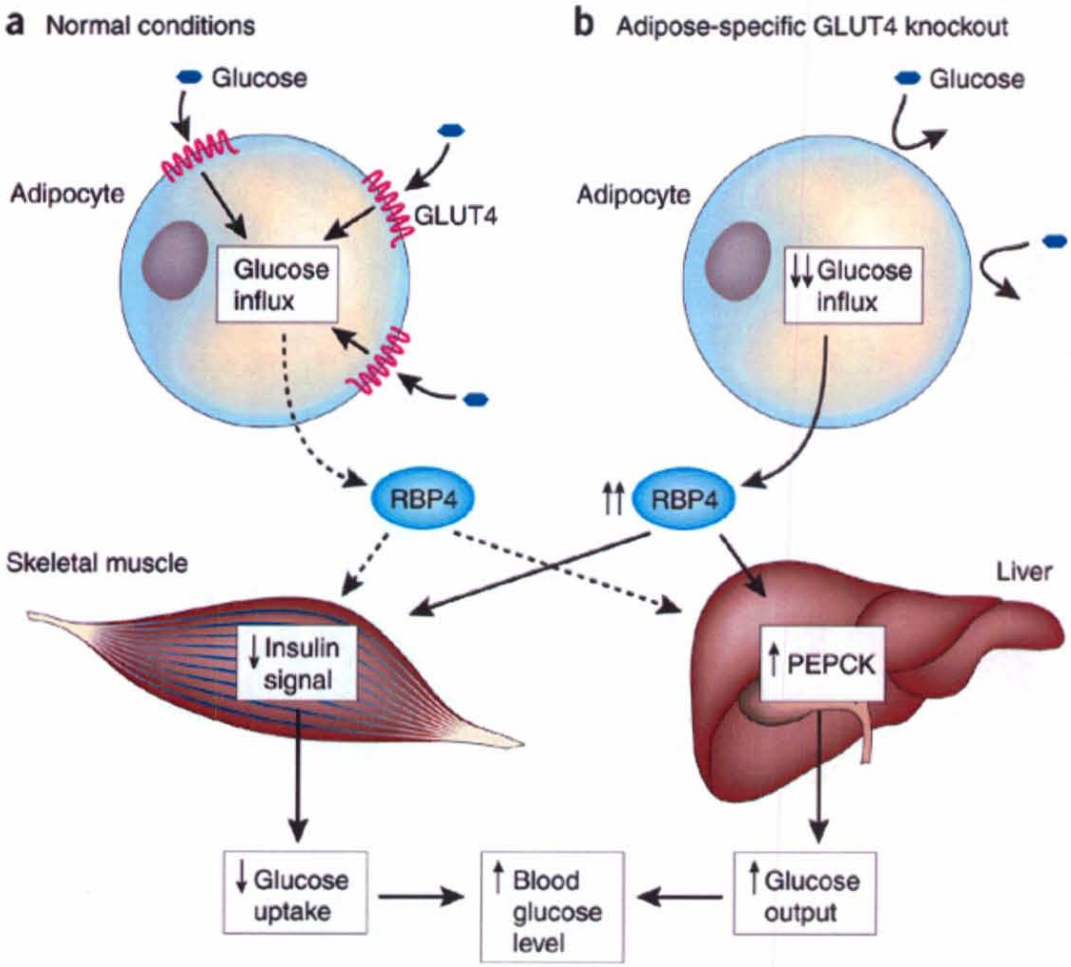

Figure 1.3 RBP4 modulates glucose metabolism in skeletal muscle and liver through down-regulation of GLUT4 in adipose tissue (95)

association in younger subjects, suggesting age-related differences in the regulation of this adipokine (94). Although some have found a decrease in circulating RBP4 in response to the hypoglycemic drugs, thiazolidinediones, in agreement with the initial animal studies (96-97), others have failed to confirm these findings (98-99). At the current time, more work is needed to discern a definitive role for RBP4 in modulating glucose homeostasis in humans.

1.5 Approaches to understanding the etiology of obesity

1.5.1 Genetic studies: The role of association studies and gene expression profiling in identifying novel obesity candidate genes

In choosing the most appropriate approach to take when trying to elucidate the genetic factors involved in the etiology of obesity, it is important to emphasize that one technique alone cannot fulfill the task of identifying all disease-causing genes. Each method has its own unique advantages and it is therefore critical for researchers to combine a number of different techniques to fully exploit the data available in their respective labs. In this thesis, two common molecular methodologies were combined to increase our chances of identifying novel obesity candidates. First, a candidate gene association approach was utilized to assess the involvement of two novel adipokines in the development of obesity-related traits. Second, global gene expression profiling of subcutaneous adipose tissue using DNA microarray technology was utilized to identify genes involved in the inter-individual response to an overfeeding challenge.

Genetic association studies are often employed to identify causative genes in complex diseases, such as obesity. They assess correlations between genetic variants and trait differences on a population scale (100) and have been successful in identifying many genetic risk factors for common diseases. Specifically, the candidate-gene association approach has been widely used for the study of obesity. This approach can be defined as the study of genetic influences on a complex trait by identifying variants, such as single nucleotide polymorphisms (SNPs), in or near a candidate gene that may have a role in the etiology of the disease (101). Selection of obesity candidate genes is typically based on prior knowledge of their known physiological role in pathways related to food intake, energy expenditure, as well as glucose and lipid metabolism. In addition, candidate genes may also be chosen on the basis of previous evidence of association with obesity-related traits in other populations. Candidate gene studies take advantage of both the increased statistical efficiency of association analysis of complex diseases and the biological understanding of the phenotype, tissues, genes, and proteins that are likely to be involved in the disease (101).

To date, candidate gene association studies have been successful in elucidating a number of variants in known genes that are associated with obesity-related phenotypes. For example, there is mounting evidence that missense mutations in genes involved in monogenic obesity are associated with the common form of obesity. Indeed, non-synonymous variants in both *LEP* and *LEPR* have been associated with adult (102-104) and childhood (105) obesity. Furthermore, the common SNP -11391G>A, located in the promoter region of the adiponectin gene (*ADIPOQ*), results in increased expression and

circulating levels of this protein and is associated with severe childhood and adult obesity in French Caucasians (106). Moreover, this has been replicated in other studies of adult obesity (107-108). Additional genes identified through this approach include *CNR1* (109), *DRD2* (110-111), *HTR2C* (112-113), and *MAOA* (114). There are, however, numerous examples of associations that cannot be replicated, which has led to skepticism regarding the utility of this approach. Explanations for this include poor study design, small sample size, poorly matched control group, incorrect assumptions about the underlying genetic architecture of the population and misinterpretation of data (100). In addition, different environmental conditions between different studies represent a problem for replication of initial findings. However, as long as care is taken in the choice and analysis of candidate genes and SNPs, these problems can be overcome with a well designed study.

A novel approach to identifying causative genes in complex diseases is genome-wide screening using microarray technology. There are different kinds of microarrays including, but not limited to, DNA, protein and tissue arrays. DNA microarrays consist of a collection of cDNA or oligonucleotide probes, commonly representing single genes, arrayed on a chip. DNA microarrays can be used for two main purposes: 1. genome wide association studies using SNP arrays, and 2. genome wide screening of gene expression in specific tissues thought to be involved in disease pathogenesis. SNP arrays are a recent development and allow for whole-genome assessment of variants associated with common diseases (115). Although GWAS using SNP array technology have been successful in identifying and replicating a number of variants associated with increases in

BMI (116), waist circumference (53), and total body weight (117), this technology is not feasible in most labs as a result of the astronomical cost and resources required to complete these studies. This is mostly due to the large number of subjects required to provide sufficient statistical power and reach genome-wide significance. Expression arrays provide simultaneous information on mRNA expression of the entire human genome in just one experiment. This allows for comparison of expression between different experimental conditions (ie. lean vs obese) providing a more comprehensive understanding of gene function, regulation, as well as interactions between different genes (118).

The first study employing DNA microarrays for the study of obesity was published by Soukas et al in 2000 (119). In these experiments, expression of approximately 6500 murine genes was compared between adipose tissue of *ob/ob* mice and wild-type lean controls. Since that initial experiment, global gene expression profiling has been performed in numerous other studies, involving both animal models and human tissues. These data have provided us with a wealth of information regarding the differential gene expression profiles induced by obesity. Moreover, the application of DNA microarray technology has led to the discovery of novel obesity candidates including RBP4, as mentioned previously. As such, gene expression arrays represent a fruitful means by which additional obesity candidate genes can be identified.

1.5.2 Overfeeding studies: The unique role of a positive energy challenge in revealing the underlying molecular mechanisms of obesity

Maintenance of body weight and body composition results from efficient control between substrate intake and utilization; an imbalance between these two results in either weight loss or gain, depending on the direction. Disruption of this highly regulated system through positive or negative energy balance interventions provides researchers with the ability to investigate the means employed by humans under stressful nutritional situations to counteract the energy imbalance (120). Overfeeding studies in particular provide a means in which both genetic and biochemical changes that would be evident with extended overeating can be investigated. This is important as obesity is largely caused by a chronic energy surplus. By mimicking this in a laboratory setting, overfeeding interventions allow for a better understanding of the differences between lean and obese individuals in response to a positive energy challenge, ultimately revealing the underlying mechanisms involved in the predisposition towards obesity. The ingestion of excess energy can help delineate adaptive mechanisms of a metabolic nature. An overfeeding study might allow the unravelling of subtle metabolic mechanisms, which are difficult to discover under maintenance conditions.

The concept of overfeeding studies dates back to the late sixties, at which time Sims and colleagues reported differences in weight gain among a group of lean individuals undergoing prolonged overfeeding (121). While some subjects gained a significant amount of body fat, others were able to maintain their initial weight. This

initial study was followed up in the 1980s by a series of highly-controlled overfeeding studies in monozygotic twins (59-60, 122). The authors reported remarkable within pair similarity in a number of biomarkers including body composition, energy expenditure, and plasma lipids indicating that genetic background plays a large role in determining the response to nutritional interventions. Other more recent studies have also investigated the nutritional regulation of various adipokines. Nonetheless, the number of overfeeding studies that have been performed is still limited. As a positive energy balance is the fundamental cause of the rising prevalence of obesity, performing additional well-controlled overfeeding studies on human subjects is essential.

1.6 Rationale

Given the complexity and heterogeneity of obesity, this thesis sought to further understand the etiology of this disease from a number of different perspectives including epidemiological, genetic and nutritional viewpoints. The main goals of the current work were three-fold:

First, we wanted to characterize our population and clarify differences between previously reported definitions of obesity. Using a large number of subjects from the CODING (Complex Diseases in the Newfoundland Population: Environment and Genetics) Study, we first attempted to evaluate the accuracy of BMI as an estimate of body fat compared to a more accurate reference method, DXA. As the utility of BMI as a marker of obesity has long come into question, we examined the discrepancy between

BMI-defined adiposity status compared to %BF-defined adiposity (Chapter 2.1) in the NL population. Building on this, we then expanded our definition of obesity to include recently identified subtypes (MONW and MHO) based on markers of cardiometabolic dysregulation and again, evaluated the discrepancy between BMI- and DXA-defined adiposity (Chapter 2.2). To further categorize MONW individuals in particular, we investigated the association between DXA-defined %BF and cardiometabolic risk factors among a large number of BMI-defined normal weight subjects (Chapter 2.3).

The second aim of this thesis was to utilize the candidate gene association approach to assess the influence of common variants in two obesity-related genes, *NAMPT* (Chapter 3.1) and *RBP4* (Chapter 3.2), on adiposity-associated traits including markers of insulin resistance, lipid metabolism, and systemic inflammation. As mentioned previously, both of these genes encode novel adipokines (visfatin and RBP4, respectively) with possible involvement in obesity and body weight regulation. Furthermore, recent association studies involving these genes have been controversial; while some have demonstrated a relationship between genetic variation and the variables mentioned above, others have not. In the majority of these reports, sample sizes were either quite small, or statistical analyses questionable likely contributing to the controversy. Using our uniquely homogenous, large-scale cohort from the CODING Study, we attempted to clarify this issue.

The third and final goal of this thesis was to explore the nutritional regulation of adipose tissue from both a genetic and endocrine point of view. To do this, subjects

underwent a 7-day overfeeding challenge consisting of 50% carbohydrates, 35% fat, and 15% protein (± 5%) to mimic the daily diet in North America. As obesity is a chronic state of energy surplus, mimicking this energy imbalance in a lab setting allowed us to investigate the endocrine and genetic changes that would be evident with extended overeating. Although this has been heavily studied in animals under conditions of a high fat diet, these findings have not always translated well to human physiology. The majority of studies examining energy homeostasis in humans have been negative energy balance interventions, induced either through exercise or caloric restriction. As a positive energy balance is the fundamental cause of obesity, it is imperative to explore the inter-individual differences in weight gain induced through overfeeding to better understand the underlying molecular mechanisms of this disease. In this regard, we initially examined the nutritional regulation of RBP4 in response to a 70% hypercaloric diet (Chapter 4.1). The objective of this study was to gain a greater understanding of the role RBP4 plays in the development of insulin resistance during overfeeding. In the second overfeeding study, we sought to examine gene expression profiles of subcutaneous adipose tissue in lean and obese young men in response to a 40% hypercaloric diet in hopes of identifying novel obesity candidate genes that would otherwise go undetected (Chapter 4.2). By investigating the response of both a novel adipokine as well as gene expression profiles of subcutaneous adipose tissue under conditions of a positive energy challenge, the ultimate goal of both these studies was to provide further insight into the regulation of adipose tissue metabolism to provide valuable candidates for future work related to obesity.

2

Defining obesity: Use of dual energy x-ray absorptiometry and markers of cardiometabolic dysregulation

2.1

Comparison of the classification of obesity by BMI versus dual energy x-ray absorptiometry in the Newfoundland population

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Introduction

The incidence of obesity has increased substantially over the past three decades and is now one of the most important public health concerns with worldwide incidence at over 300 million people (123). Chronic health problems associated with obesity are numerous and include T2D, heart disease, hypertension, and certain types of cancer (124). As the incidence of obesity increases, the need for accurate measurements of adiposity is becoming increasingly important to allow for appropriate diagnosis and treatment. BMI has been the dominant index used to measure obesity owing to its simplicity and low cost however it has recently come under criticism as it fails to account for a number of adiposity related-factors including age, gender, and ethnicity. Reference methods such as DXA, air-displacement plethysmography, and underwater weighing provide a more accurate indication of %BF (22-25), which is one of the fundamental links between obesity and its associated disease risk.

The use of BMI for the classification of adiposity status and disease risk is based on epidemiological associations of BMI with morbidity and mortality (125-126). Despite this, numerous studies have produced evidence that BMI has limited ability to accurately predict body composition as evidenced by sizeable differences between BMI estimated body fat and densitometrically determined body fat (127-130). Furthermore, the relationship between BMI and %BF has been shown to vary with age, sex and ethnicity (131-133). It is therefore essential to identify how well BMI criteria match more accurate reference methods based on %BF and to what extent major factors such as age, gender

and adiposity distort the accuracy of BMI. In the current study, we investigated differences between BMI-determined adiposity status and DXA to evaluate the accuracy of BMI. At the present time, there is little systematic data available in Canada regarding the accuracy of BMI compared to a standard reference method such as DXA, at the population level. The objectives of our study were as follows: 1) Determine the accuracy of BMI classifications compared to %BF classifications measured by DXA; 2) Determine if discrepancies between BMI and DXA are gender- and age-specific; 3) Identify whether an individual's current adiposity status (i.e. overweight or obese) can affect the size of error in their BMI.

Methods and Procedures

Subjects

Subjects (n = 1712) were recruited from an ongoing large scale nutritional genetics study of human complex diseases called the CODING Study (134-136). As BMI and %BF criteria are specific to individuals $\geq$ 20 years old, we excluded all participants below this age limit (21 individuals) leaving us with a cohort of 1691 subjects (1321 females, 370 males). All volunteers were from the Canadian province of NL. Each individual completed a screening questionnaire that included information regarding physical characteristics, dietary habits, and physical activity levels. Inclusion criteria in the present study were as follows: 1) between the ages of 20 and 79 years old; 2) at least third generation Newfoundlander; 3) healthy, without any serious metabolic,

cardiovascular, or endocrine disease. All subjects provided written and informed consent, and the Human Investigation Committee of the Faculty of Medicine, Memorial University of Newfoundland approved the study.

Measurements of BMI and %BF

All measurements were performed following a 12 hour fasting period. Subjects were weighed to the nearest 0.1 kg in standardized light clothes and without shoes on a platform manual scale balance as previously described by us (Health O Meter Inc., Bridgeview, IL) (134-136). Height was measured using a fixed stadiometer to the nearest 0.1 cm. BMI was calculated as a person's weight in kilograms divided by their height in meters squared. Waist and hip circumference were measured to the nearest 0.1 cm using a flexible metric measuring tape while the participant was in a standing position. Waist circumference was measured as the horizontal distance around the abdomen at the level of the umbilicus and hip circumference was measured as the largest circumference between the waist and thighs. Waist-to-hip ratio was calculated as waist circumference divided by hip circumference.

Whole body composition measurements including fat mass, lean body mass and bone mineral densities were measured using DXA Lunar Prodigy (GE Medical Systems, Madison, WI, USA). DXA is a relatively new reference method used to determine body composition that produces an accurate measurement of all adipose tissue within the body with a low margin of error. For this reason DXA is considered to be one of the most

accurate measurements of adiposity and is commonly used as a standard compared to less accurate field methods such as BMI. Measurements were performed on subjects following the removal of all metal accessories, while lying in a supine position as previously described (134-136). %BF was determined as a ratio of fat mass over total body mass (including bone mineral densities) using the manufacturer's software (Version 4.0). Quality assurance was performed on our DXA scanner daily as recommended by the manufacturer. Briefly, the procedure involves scanning a calibration block and functional tests of diagnostic parameters. If any of these tests fail on two consecutive occasions, the user is instructed to contact the manufacturer. The typical CV was 1.3% during the study period.

Statistical Analysis

All data are reported as mean ± SD. Prior to performing any statistical analyses, subjects were classified according to adiposity status using both BMI and %BF criteria. Subjects were classified using BMI as underweight (< 18.5 $kg \cdot m^{-2}$), normal weight (18.5 - 24.9 $kg \cdot m^{-2}$), overweight (25.0 – 29.9 $kg \cdot m^{-2}$), or obese (30.0 $kg \cdot m^{-2}$) according to criteria from the WHO. Subjects were grouped according to %BF based on criteria recommended by Bray that is both age and gender specific (Table 2.1) (137). Differences in physical characteristics between men and women were assessed using Student's t-test. Differences in adiposity classification between BMI and DXA were analyzed on the following three levels:

1. Discrepancy analyses between BMI and DXA within gender.

   Men and women were separated into adiposity classifications according to BMI and %BF criteria. The number of subjects grouped into each adiposity category by both methods was calculated as a percentage of the total number of participants. Differences in percentages between BMI- and %BF-defined adiposity status were analyzed within gender using Chi-Square analyses.

2. Discrepancy analysis by age group.

   BMI-defined adiposity classifications were compared to %BF criteria among different age groups to investigate the effect of age on BMI accuracy. Women were separated into four groups according to their age (20-29.9, 30-39.9, 40-49.9, 50+) and analysis repeated as above. Due to the small number of men in our cohort, similar analysis could not be performed as the number in each cell (four age groups by four weight groups) was too small for effective comparison.

3. Ranges of percent body fat based on BMI cutoffs.

   In order to study the range of %BF found in each BMI category, subjects were grouped by BMI into adiposity groups and then %BF averages for each BMI group were calculated along with minimum and maximum values.

SPSS version 16.0 (SPSS Inc., Chicago, IL) was used for all analyses. Statistical analyses were two-sided and a p value < 0.05 was considered to be statistically significant.

Table 2.1 Percentage body fat (%BF) cut-off points for women and men[1].

	Women (%BF)				Men (%BF)			
Age (yrs)	**Underweight**	**Normal Weight**	**Overweight**	**Obese**	**Underweight**	**Normal Weight**	**Overweight**	**Obese**
20-39	<21	21-32	33-38	39+	<8	8-20	21-25	26+
40-59	<23	23-34	35-40	41+	<11	11-22	23-28	29+
60-79	<25	25-37	38-42	43+	<13	13-24	25-30	31+

[1]Adapted from Bray (137).

Results

Physical characteristics of the subjects

Physical characteristics for female and male participants are shown in Table 2.2. The subjects' ages ranged from 20-76.8 years old. Male subjects were 3.0 years younger than women on average. Men were also 15.5 kg heavier and 13.3 cm taller compared to women and had BMI measurements 1.2 units higher which reflect averages seen in similar studies (138). Although BMI values were higher in men, women had greater waist-to-hip ratios and increased %BF and trunk fat percentage (%TF).

General discrepancy analyses by gender

Significant discrepancies between BMI and %BF criteria were identified in both women and men. Of the 1321 women included in our study, BMI classified 44.2% as normal weight while DXA classified only 29.6% as normal weight (Figure 2.1). Among obese women there was again a large discrepancy between the two methods. According to BMI criteria, 20.3% of women in our cohort were obese however according to %BF criteria 37.1% of women were obese. As a result, BMI classified 14.6% more women as normal weight and 16.8% less women as obese compared to %BF criteria determined by DXA ($p<0.001$). Classification of underweight and overweight women was similar between the two methods (underweight: BMI 1.2%, DXA 2.2%; overweight: BMI 34.2%, DXA 30.9%). A total discrepancy of 34.7% was found between the two methods in women.

Table 2.2 Physical characteristics of female and male subjects (n = 1691)[1].

Variables	Women n = 1321	Men n = 370	*t*-test *p* value[2]
Age (yrs)	44.2 ± 10.6 (20, 76.8)	41.2 ± 13.4 (20, 76)	<0.001
Weight (kg)	69.6 ± 14.2 (39.5, 156.8)	85.1 ± 14.5 (53.6, 149.4)	<0.001
Height (cm)	162.1 ± 5.8 (135.0, 186.7)	175.4 ± 6.4 (157.0, 198.0)	<0.001
BMI (kg m^{-2})	26.5 ± 5.2 (16.0, 54.3)	27.7 ± 4.5 (16.8, 50.4)	<0.001
Waist (cm)	90.7 ± 14.3 (62.7, 168.0)	98.1 ± 12.1 (63.7, 148.8)	<0.001
Hip (cm)	102.6 ± 11.6 (76.5, 172.0)	101.5 ± 9.1 (69.0, 145.1)	<0.001
Waist-to-hip ratio	0.88 ± 0.07 (0.64, 1.24)	0.97 ± 0.06 (0.74, 1.20)	<0.001
Body fat (%)	37.7 ± 7.3 (4.6, 59.9)	25.5 ± 7.5 (5.6, 47.6)	<0.001
Trunk fat (%)	38.8 ± 8.6 (3.9, 64.3)	30.3 ± 8.7 (4.7, 53.1)	<0.001

[1]All values are mean ±SD; minimum and maximum values are in parentheses.

[2] Women significantly different from men according to Student's t-test ($p<0.001$).

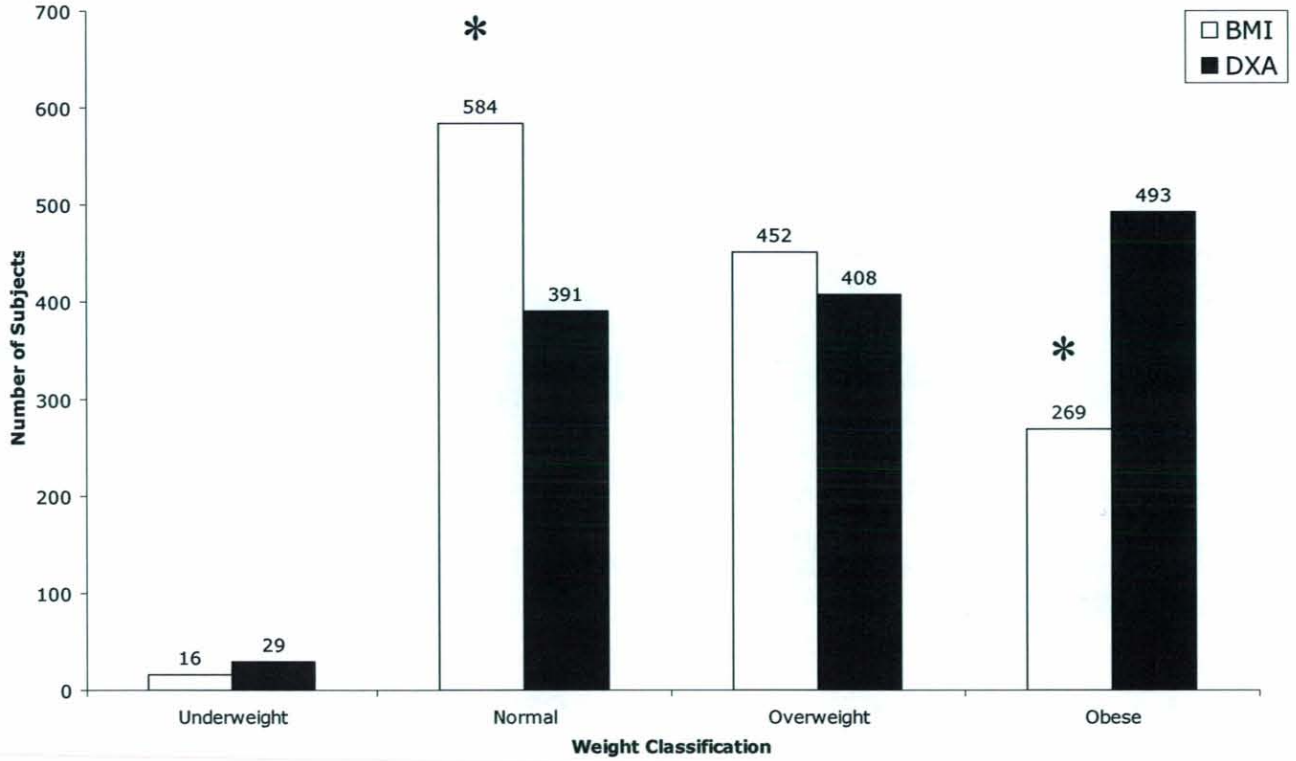

Figure 2.1 Comparison of BMI- and DXA-defined weight classifications in women according to body size (n = 1321). * p < 0.001 for difference between the two methods according to Chi Square analyses.

Of the 370 men included in this study, BMI and %BF classifications were similar for normal weight individuals (BMI 28.9%, DXA 31.6%; Figure 2.2). Among overweight and obese men, significant differences were evident in adiposity classification among the two methods. BMI categorized 45.7% of men as overweight and 24.9% as obese while DXA classified only 28.1% of men as overweight and 38.4% as obese. BMI classified 17.6% more men as overweight and 13.5% less men as obese compared to %BF criteria based on DXA measurements ($p<0.001$). A total discrepancy of 35.2% was discovered between the two methods in men.

Discrepancy analyses by age group

After separation of the female cohort into groups based on age, similar discrepancies were evident between BMI- and %BF-defined adiposity status across all four age groups (Table 2.3). There was a significant discrepancy between the two methods for normal weight and obese women across all age groups ($p < 0.001$). The discrepancy between BMI and DXA-determined %BF ranged from 11.5% to 18.9% for normal weight women and 13.3% to 22.5% for obese women. Women in their 20s demonstrated the largest discrepancy between BMI and %BF for the normal weight group and women in their 30s had the largest discrepancy in the obese group. Women in their 40s demonstrated the smallest discrepancy between the two methods among the four age groups. The discrepancies found in the female cohort for underweight and

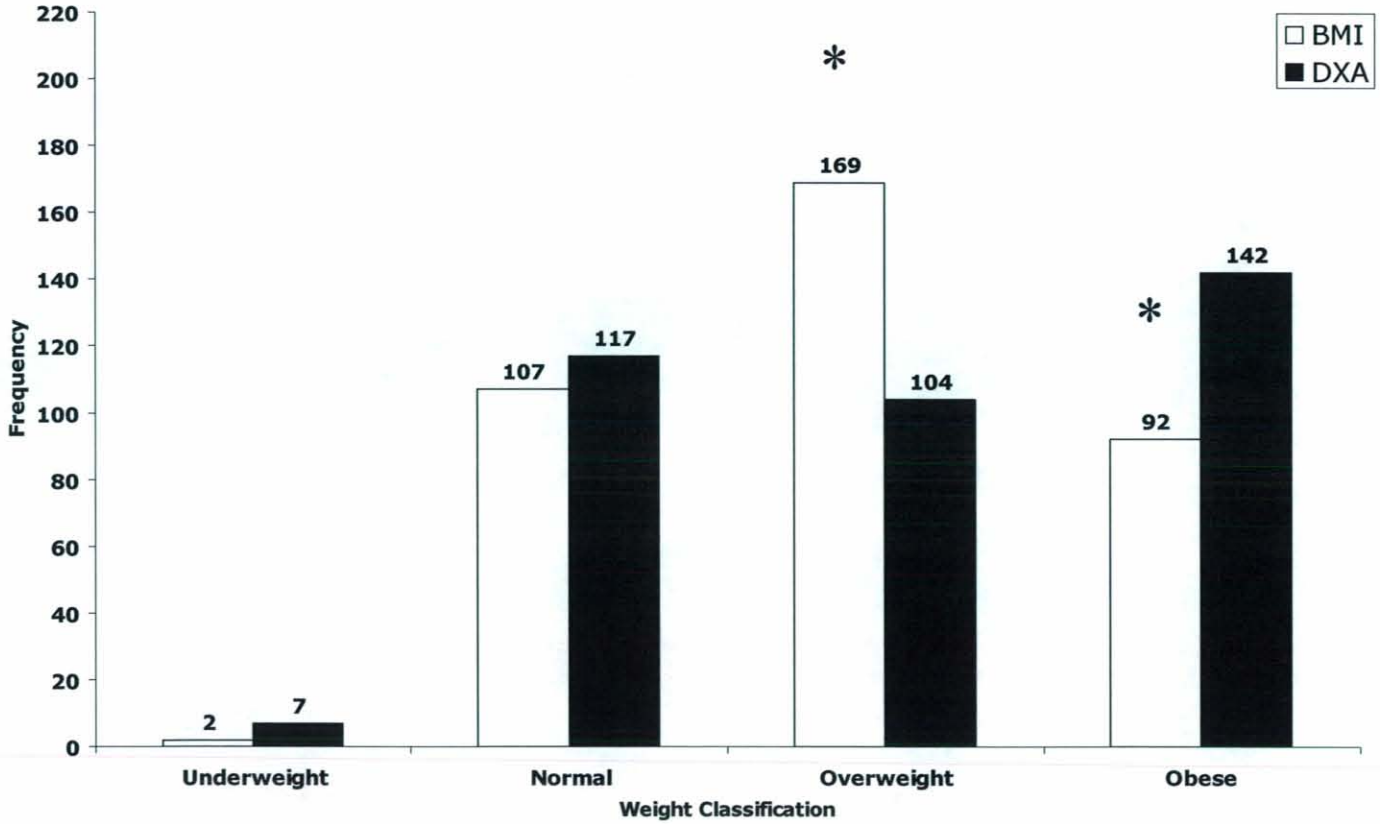

Figure 2.2 Comparison of BMI- and DXA-defined weight classifications in men according to body size (n = 370). * p < 0.001 for difference between the two methods according to Chi Square analyses.

Table 2.3 Percent discrepancies between BMI and DXA weight classifications in women according to age (n = 1321)[1].

	Age Group														
	20-29.9 n = 164			**30-39.9** n = 204			**40-49.9** n = 513			**50+** n = 440			**Overall** n = 1321		
	BMI	DXA	**%D[2]**	BMI	DXA	**%D**	BMI	DXA	**%D**	BMI	DXA	**%D**	BMI	DXA	**%D**
Underweight	4	9	**3.0**	4	3	**0.5**	5	13	**1.6**	3	4	**0.2**	16	29	**1.0**
Normal weight	100	69	**18.9[3]**	100	63	**18.1[3]**	214	155	**11.5[3]**	170	104	**15.0[3]**	584	391	**14.6[3]**
Overweight	43	35	**4.9**	64	56	**3.9**	187	170	**3.3**	158	147	**2.5**	452	408	**3.3**
Obese	17	51	**20.7[3]**	36	82	**22.5[3]**	107	175	**13.3[3]**	109	185	**17.3[3]**	269	493	**16.8[3]**

[1]BMI and DXA values are raw numbers of women classified into each adiposity group.

[2]%D, Percent discrepancy between BMI- and DXA-defined weight classification; calculated as:

| percentage of women grouped into BMI category - women grouped into DXA category |.

[3]Significant difference between BMI- and DXA-defined weight classification according to Chi Square analyses ($p < 0.001$).

overweight BMI classifications compared to %BF were not significant for all age groupings.

Error range in classification by BMI

Figure 2.3 shows the variation in %BF according to BMI categories for men and women. A large range of error indexed by %BF was found in each BMI category for both genders. A total of 251 obese women (determined by DXA) were misclassified as either normal weight (n = 42) or overweight (n = 209) by BMI criteria. There was a wide range in %BF for BMI-defined normal weight and overweight women (4.6 – 51.1% and 14.8 – 51.8%, respectively). Overweight women (DXA) were also misclassified as underweight and normal weight according to BMI criteria. Normal weight and underweight women (DXA) were misclassified as overweight and normal weight, respectively. This suggests that BMI misclassifies female subjects across all four adiposity classifications. The data among men was similar. A total of 73 obese men (determined by DXA) were misclassified as normal weight (n = 7) or overweight (n = 66) according to BMI criteria. The range in %BF for BMI-defined normal weight and overweight men was 5.6 – 31.2 % and 10.8 – 41.3%, respectively. Although the misclassifications were bi-directional, BMI tended to under-classify the majority of subjects.

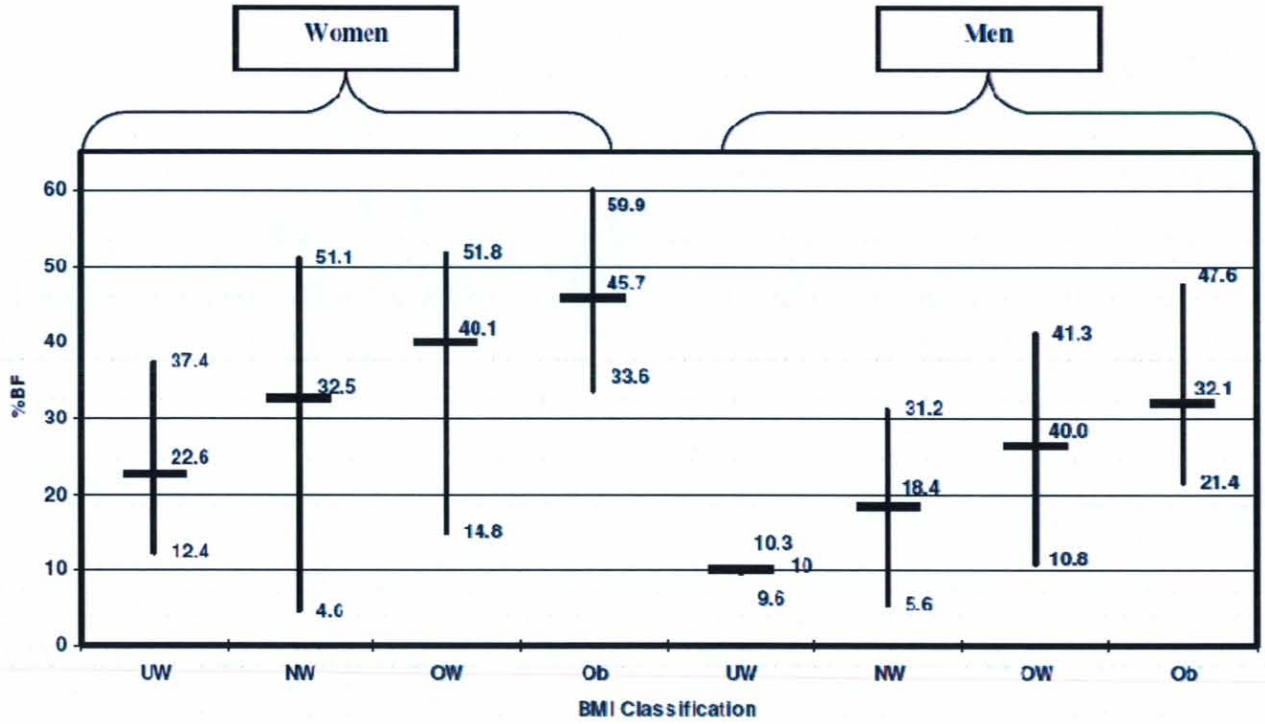

Figure 2.3 Percentage body fat (%BF) variations among women and men according to BMI classification. %BF, percentage body fat; UW, underweight; NW, normal weight; OW, overweight; Ob, obese. Data is presented as mean and minimum/maximum values for %BF.

Discussion

Our study, involving a large sample from the NL population, demonstrates the limited ability of BMI to accurately estimate adiposity. One of the major findings in the present study is that there is a large discrepancy between BMI- and DXA-defined adiposity status that is both gender and age specific. Over one third of women and men were misclassified by BMI criteria compared to %BF criteria determined by DXA. A significant proportion of obese individuals were misclassified as either normal weight or overweight by BMI criteria. This poses serious health consequences on a population level as the opportunity to intervene and reduce health risk in these individuals is lost. Overall, BMI had the poorest ability to predict true adiposity in normal weight and obese women, and in overweight and obese men. Furthermore, this misclassification was influenced by age, with younger women (under 40 years old) demonstrating the largest discrepancy between the two methods. We also found significant inter-subject variability in %BF for any given BMI value.

The ability of BMI to define adiposity status has been repeatedly questioned. It has previously been shown that BMI is not accurate at predicting adiposity status in the normal to mildly obese range (20, 139-140) as well as in severely obese individuals (141). In particular, BMI was not accurate at predicting obesity in individuals with a body mass less than 80 kg compared to %BF determined by DXA (140). Similarly, a significant number of people with a BMI below 30 kg m^{-2} were actually obese when classified by %BF determined by Bioelectric Impedance Analysis (BIA) (139). A more recent study, involving a large multiethnic sample from the US population found BMI to

have limited diagnostic performance, especially in those with a BMI < 30 kg m^{-2} (20). Despite BMI-defined obesity having good specificity when compared to BIA-defined obesity, BMI had low sensitivity, missing nearly half of %BF-determined obese people (20). These findings suggest that BMI may not be accurate at assessing adiposity status in normal weight and overweight individuals. Our study included all ranges of BMI and %BF (16.0 – 54.3 $kg.m^{-2}$ and 4.6 - 59.9 %, respectively) and revealed a higher discrepancy for each of these adiposity categories.

We also observed gender differences in the discrepancy between the two methods. Although there was good agreement between BMI and DXA for overweight women, BMI had limited ability to predict the correct adiposity classification for normal weight and obese women. In men, however, the greatest discrepancy was evident in the overweight and obese groups. BMI has a better correlation with lean mass compared to %BF in men but not in women (20) which may explain why there was a greater discrepancy between BMI and %BF-defined adiposity status in overweight men but not in overweight women. Furthermore, males demonstrate a linear relationship between BMI and %BF while females demonstrate a curvilinear relationship (141) which may explain why we observed a high discrepancy between BMI- and DXA-defined normal weight women but not men. Gender differences in body composition are a profound physiological phenomenon however standard WHO BMI criteria do not accommodate for this. Our results suggest that this problem needs addressing. A re-adjustment of obesity criteria to include accommodations for gender differences will increase the accuracy of BMI to predict adiposity in both males and females.

We also analyzed our data after stratifying females according to age groups. The largest discrepancy between BMI and DXA weight classifications was evident in women under the age of 40 while there was moderate agreement between the two methods in older women. These results are surprising as previous studies have found that the diagnostic performance of BMI diminishes as age increases (20), likely due to an increase in the ratio of fat mass to fat free mass that is evident with age (142). Further studies are warranted to address the potential mechanism surrounding this phenomenon. Obesity criteria based on %BF are age specific however, BMI criteria are identical across all age groups. From our results it is apparent that BMI cannot accurately reflect age-related changes in adiposity.

Our analysis was originally performed using %BF criteria from earlier publications by Dr. Bray. Bray's original obesity criteria (defined as BF > 25 % in men and BF > 33% in women) lacked any adjustment for age or ethnicity (143). Using these criteria, we found that approximately 72% of obese females and 54% of obese males were misclassified as normal weight or overweight according to BMI criteria. Our current results indicate that the new Bray body fat classifications (Table 1) are a better fit to BMI criteria, however a significant margin of error still remains between the two methods. It is evident that age, gender and ethnicity-specific criteria are necessary for more accurate BMI calculations that reflect %BF.

The findings from our study highlight the importance of exercising caution when defining adiposity status using BMI criteria. Although previous studies have

demonstrated similar trends, most have small sample sizes (144-145) or have used less accurate methods to estimate %BF such as BIA or skin fold thickness (20, 139, 146). To the best of our knowledge, this is the first study of its kind to demonstrate a discrepancy between BMI- and DXA- defined adiposity in a large cohort containing both men and women of all different age groups. Nevertheless, our study is not without limitations. Other methods to measure adiposity, such as BIA, are cheaper and easier to use compared to DXA, despite its reported limitations. Although DXA is considered to be one of the more accurate measurements for %BF, it is not without its own limitations. Lean tissue determined by DXA contains water as its dominant component, therefore differences in hydration may affect the calculation of body fat and hence, may have also contributed to the discrepancy between BMI and DXA adiposity measurements. Past studies have shown, however, that the effect of hydration on fat mass is not significant (147). All subjects fasted (no food or water) for 12 hours prior to having a DXA scan performed to control for differences in hydration therefore this should not have any significant effect on our results. Our study was also limited in the number of male participants and ethnic groups. Future studies investigating the discrepancy between BMI- and DXA-defined adiposity are warranted in a larger male cohort and in other populations.

In summary, we compared BMI adiposity classifications to DXA-determined adiposity classifications based on %BF in 1691 adult Newfoundlanders. BMI misclassified 34.7% of women and 35.2% of men into an incorrect adiposity category. BMI misclassifications were also influenced by age, with the largest discrepancy observed in women under 40 years old. Our findings support previous research and

demonstrate the necessity to revise current BMI criteria to include such confounding factors as age, gender and ethnicity (141, 148-149). Further research is needed to help alleviate these problems so that BMI can continue to be used in everyday health appraisals. Using the current BMI criteria can be dangerous as it may misdiagnose obese individuals as normal weight and result in missed opportunities to intervene and reduce disease risk. For these reasons, we recommend that caution should be taken when BMI is used in scientific research as well as in clinical practice.

2.2

The prevalence of metabolically healthy obese subjects defined by BMI and dual energy x-ray absorptiometry

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Introduction

Obesity, which is commonly measured using BMI, is closely associated with a number of metabolic and cardiovascular risk factors including high fasting glucose (150), hypertension (151), dyslipidemia (152) and high C-reactive protein levels (153). Recently however, studies have indicated that the disease risks associated with excess adiposity may not be uniform. For example, a subset of normal weight individuals display a metabolic profile similar to what is often associated with being overweight or obese (27, 154). Conversely, a subset of obese individuals appears to be resistant to the development of obesity-related metabolic complications (26-28, 155). Interestingly, the metabolic profile of obese individuals who are metabolically healthy is similar to that of young, lean individuals (156).

Although it has been reported that approximately one-third of obese adults are metabolically healthy (26-27), the majority of these studies have classified obesity using BMI criteria. Recently, the utility of BMI as an accurate measure of adiposity has come under scrutiny compared to reference methods such as DXA (24, 140, 157). Specifically, we have shown that BMI tends to misdiagnose approximately one-third of obese individuals as normal weight or overweight (157). As BMI and DXA likely represent different physiological entities, their relationship with obesity-related risk factors may differ. This point was acknowledged in a recent editor's correspondence regarding the study performed by Wildman et al. (27), where it was stated that the use of BMI as an index of obesity in the context of their study had serious limitations (158). Thus, there is

an immense need for data regarding this issue using more accurate measurements of body fat.

In the current study, we were interested in investigating the prevalence of cardiometabolic abnormalities in normal weight, overweight and obese individuals using both BMI and %BF criteria (determined using DXA). Our goal was to determine if the prevalence of these phenotypes is similar to what has been reported in the literature when using a more accurate index of adiposity. At the present time, little is known regarding the differences in the prevalence of cardiometabolic risk factors among individuals classified using BMI versus %BF adiposity classifications. The objectives of the study were as follows: 1. Compare the prevalence of cardiometabolic abnormalities in a large cohort using BMI versus %BF criteria; and 2. Determine if the discrepancy in the prevalence of these metabolic abnormalities is influenced by gender.

Materials and Methods

Subjects

Subjects (n = 1981) were recruited from an ongoing large scale nutrigenomics study (CODING Study) (134-136, 157, 159). Participants ≤ 19 years old were excluded as BMI and %BF criteria are specific to those 20 years of age and older (n = 29). All underweight individuals were excluded as well (n = 45) leaving a final sample size of 1907 (n = 1464 women, n = 443 men). All volunteers were from the Canadian province

of NL. Inclusion criteria were as follows: 1) between the ages of 20 and 79 years old; 2) at least third generation Newfoundlander; 3) healthy, without any serious metabolic, cardiovascular or endocrine diseases; and 4) not pregnant at the time of the study. The Human Investigation Committee of the Faculty of Medicine, Memorial University of Newfoundland approved the study and all subjects provided written and informed consent.

Measurements of BMI and %BF

All measurements were performed following a 12 hour fasting period. Subjects were weighed to the nearest 0.1 kg in standardized light clothes and without shoes on a platform manual scale balance as previously described by us (Health O Meter Inc., Bridgeview, IL) (134-136, 157). Height was measured using a fixed stadiometer to the nearest 0.1 cm. BMI was calculated as a person's weight in kilograms divided by their height in meters squared. Waist and hip circumference were measured to the nearest 0.1 cm using a flexible metric measuring tape while the participant was in a standing position. Waist circumference was measured as the horizontal distance around the abdomen at the level of the umbilicus and hip circumference was measured as the largest circumference between the waist and thighs.

Whole body composition measurements including fat mass, lean body mass and bone mineral densities were measured using dual-energy X-ray absorptiometry (DXA) Lunar Prodigy (GE Medical Systems, Madison, WI, USA). Measurements were

performed on subjects following the removal of all metal accessories, while lying in a supine position as previously described (134-136, 157). Percent body fat (%BF) was determined as a ratio of fat mass over total body mass (including bone mineral densities) using the manufacturer's software (Version 4.0). Subjects were classified as NW, OW or OB according to %BF based on criteria recommended by Bray (Table 2.1) (137).

Serum measurements

Blood samples were taken from all subjects in the morning, following a 12-hour fasting period. Serum was stored at -80 °C for subsequent analyses. Serum concentrations of glucose, TG, total cholesterol, and HDL cholesterol were performed on an Lx20 analyzer (Beckman Coulter Inc., CA, USA) using Synchron reagents. LDL cholesterol was calculated using the following formula: (total cholesterol) – (HDL cholesterol) – (TG/2.2) which is reliable in the absence of severe hyperlipidemia. Serum insulin levels were measured on an Immulite immunoassay analyzer (DPC, CA, USA) and the homeostasis model assessment was used to measure insulin resistance (HOMA-IR) and β-cell function (HOMAβ) (160). High-sensitivity C-reactive protein (hsCRP) was measured by nephelometry according to the manufacturer's protocol (Beckman Coulter Inc).

Definition of Metabolically Benign and Metabolically Unhealthy Phenotypes

For the present study, six cardiometabolic abnormalities were considered (elevated TG and glucose levels, decreased HDL cholesterol levels, insulin resistance, hypertension, and elevated hsCRP). Because there are no standard criteria to categorize metabolically healthy and abnormal individuals, we performed our analyses using the definition recommended by Wildman et al (ref 27; Table 2.4). Subjects were classified as metabolically abnormal if they had ≥ 2 cardiometabolic abnormalities listed in Table 2.4 and metabolically healthy if they did not fulfill the above criteria (0 or 1 cardiometabolic abnormality). Further analyses were performed using additional criteria for the metabolically healthy phenotype based on the definition for the metabolic syndrome (161), criteria recommended by Karelis et al. (162), as well as an insulin resistance cut point (metabolically healthy, lower quartile of HOMA index; metabolically abnormal, upper quartile of HOMA index; ref 163).

Statistical Analyses

Data are presented as mean (SD). Prior to performing any statistical analyses, subjects were grouped according to adiposity status using both BMI and %BF criteria. Based on their BMI, subjects were classified as normal weight (18.5 – 24.9 kg m^{-2}), overweight (25.0 – 29.9 kg m^{-2}) or obese (> 30 kg m^{-2}) according to criteria from the WHO (Table 2.1; ref 143). Subjects were also grouped according to %BF based on criteria recommended by Bray that are both age and gender specific (Table 1; ref 137).

Table 2.4 Methods used to define the metabolically healthy phenotype.[1]

Method	Definition
Wildman et al (27)	Less than two criteria: Blood pressure ≥ 130/85 mm Hg TG ≥ 1.7 mmol/L HDL-C: Men < 1.03 mmol/L Women < 1.30 mmol/L Glucose ≥ 5.6 mmol/L HOMA-IR > 4.27 (ie. 90th percentile) hsCRP > 7.89 mg/L (ie. 90th percentile)
NCEP ATP III MetS (161)	Two or less metabolic criteria: Blood pressure ≥ 130/85 mm Hg TG ≥ 1.7 mmol/L HDL-C: Men < 1.03 mmol/L Women < 1.30 mmol/L Glucose ≥ 5.6 mmol/L Waist Circumference: Men < 102 cm Women < 88 cm
Karelis et al (162)	Meeting four out of five criteria: HOMA-IR ≤ 2.7 TG ≤ 1.7 mmol/L HDL-C ≥ 1.3 mmol/L LDL-C ≤ 2.6 mmol/L hsCRP ≤ 3.0 mg/L
HOMA Index (163)	HOMA ≤ 1.27 (lowest quartile of HOMA index)

[1]Those not meeting the above criteria were classified as metabolically abnormal. TG, triacylglycerol; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model for insulin resistance; LDL-C, low-density lipoprotein cholesterol; hsCRP, high sensivity C-reactive protein; NCEP ATP III MetS, National Cholesterol Education Program Adult Treatment Panel III definition for metabolic syndrome.

Subjects' physical characteristics and metabolic profiles were calculated according to their adiposity status (based on %BF criteria) within each metabolic subgroup (metabolically healthy or metabolically abnormal) as well as overall. Differences in these characteristics among the three groups of subjects (normal weight, overweight, and obese) were analyzed within each metabolic subgroup using one-way ANOVA. The prevalence of the metabolically healthy and metabolically abnormal phenotypes are presented as the mean percentage within each adiposity group. The level of agreement between the prevalence of these phenotypes among normal weight, overweight, and obese subjects using BMI versus %BF criteria was assessed using a Kappa test. Differences within each adiposity group were assessed using Chi-Square analyses. This analysis was also repeated after stratifying subjects according to gender. SPSS version 16.0 (SPSS Inc., Chicago, IL) was used for all analyses. Statistical analyses were two-sided and a p value < 0.05 was considered to be statistically significant.

Results

Physical and Biochemical Parameters

Among the 1907 subjects in our study 40.4% were normal weight (n = 771), 37.6% were overweight (n = 716), and 22.0% were obese (n = 420) using BMI criteria. According to %BF criteria, which gives a more accurate measurement of adiposity compared to BMI (13), 31.5% were normal weight (n = 602), 30.8% were overweight (n

Table 2.5 Physical and biochemical characteristics of subjects according to adiposity (%BF) and metabolic status.[1]

	Metabolically Healthy			Metabolically Abnormal			
Variables	**Normal Weight (n = 456)**	**Overweight (n = 367)**	**Obese (n = 339)**	**Normal Weight (n = 146)**	**Overweight (n = 220)**	**Obese (n = 379)**	**Overall (n = 1907)**
Age (y)	40.2 (12.6)	43.3 (11.6)	43.2 (11.4)	47.1 (13.5)	48.8 (10.0)	46.0 (11.6)	44.0 (12.1)
Height (cm)	165.8 (7.7)	164.1 (7.8)	164.8 (8.2)	167.8 (9.6)	165.8 (8.4)	164.9 (8.6)	165.3 (8.3)
Weight (kg)	62.7 (8.9)	68.6 (10.3)[2]	79.8 (13.3)[2,3]	69.0 (13.1)	74.1 (11.4)[2]	87.5 (16.7)[2,3]	73.6 (15.3)
BMI ($kg*m^{-2}$)	22.7 (2.1)	25.4 (2.7)[2]	29.3 (4.2)[2,3]	24.4 (3.5)	26.8 (3.2)[2]	32.2 (5.3)[2,3]	26.9 (5.0)
Body Fat (%)	27.3 (6.3)	35.8 (5.0)[2]	42.3 (5.7)[2,3]	26.4 (7.0)	34.2 (6.1)[2]	42.0 (6.8)[2,3]	35.2 (8.7)
SBP (mm Hg)	116.4 (12.8)	118.2 (12.5)	120.7 (12.8)[2,3]	133.4 (18.3)	136.0 (17.7)	135.4 (19.1)	124.9 (17.4)
DBP (mm Hg)	75.8 (9.3)	76.7 (8.2)	78.9 (9.1)[2,3]	84.5 (12.2)	87.3 (12.4)	88.5 (12.8)[2]	81.1 (11.7)
TG (mmol/L)	0.89 (0.44)	1.01 (0.57)[2]	1.10 (0.60)[2]	1.42 (0.88)	1.66 (0.99)	1.71 (0.94)[2]	1.24 (0.79)
HDL-C (mmol/L)	1.58 (0.39)	1.51 (0.36)[2]	1.39 (0.36)[2,3]	1.52 (0.40)	1.45 (0.38)	1.39 (0.35)[2]	1.48 (0.38)
Glucose (mmol/L)	4.8 (0.5)	4.9 (0.4)[2]	5.0 (0.4 [2,3]	5.4 (1.0)	5.5 (1.3)	5.6 (1.4)	5.1 (0.9)
HOMA-IR	1.41 (0.68)	1.72 (0.75)[2]	2.13 (0.92)[2,3]	2.51 (2.38)	3.15 (5.77)	4.12 (4.26)[2,3]	2.46 (3.09)
hsCRP (mg/L)	1.46 (2.39)	2.02 (2.19)	3.10 (2.73)[2,3]	2.89 (3.82)	3.49 (3.92)	6.25 (6.60)[2,3]	3.15 (4.24)

[1]Data are presented as mean (SD). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triacylglycerol; HDL-C, HDL cholesterol; HOMA-IR, homeostasis model for insulin resistance; hsCRP, high-sensitivity C-reactive protein.

[2]$p < 0.05$ versus normal weight within metabolic subgroup

[3]$p < 0.05$ versus overweight within metabolic subgroup

= 587), and 37.7% were obese (n = 718). Age and height were statistically similar among the three adiposity groups (Table 2.5). Not surprisingly, weight, BMI, and %BF were all higher in the overweight and obese groups compared to normal weight individuals within each metabolic subgroup. Obese subjects had higher systolic and diastolic blood pressure compared to normal weight individuals within the metabolically healthy subgroup but no significant differences were observed with the metabolically abnormal subgroup. In terms of serum lipids, TG and HDL cholesterol were elevated in overweight and obese subjects in the metabolically healthy subgroup and in obese subjects in the metabolically abnormal subgroup compared to normal weight subjects. Fasting glucose levels and insulin resistance were higher in overweight and obese individuals compared to normal weight in the metabolically healthy subgroup but not in the metabolically abnormal subgroup. In terms of systemic inflammation, hsCRP was elevated in obese compared to normal weight individuals in both metabolic subgroups.

Discrepancy Analyses in the Prevalence of Metabolically Healthy and Metabolically Abnormal Phenotypes

In our cohort, a total of 1162 subjects were metabolically healthy and 745 were metabolically abnormal. When subjects were classified using BMI criteria, 77.6% of normal weight individuals, 58.8% of overweight individuals and 34.0% of obese individuals were metabolically healthy (Figure 2.4A). When subjects were classified using more accurate %BF criteria, 75.7% of normal weight individuals, 62.5% of

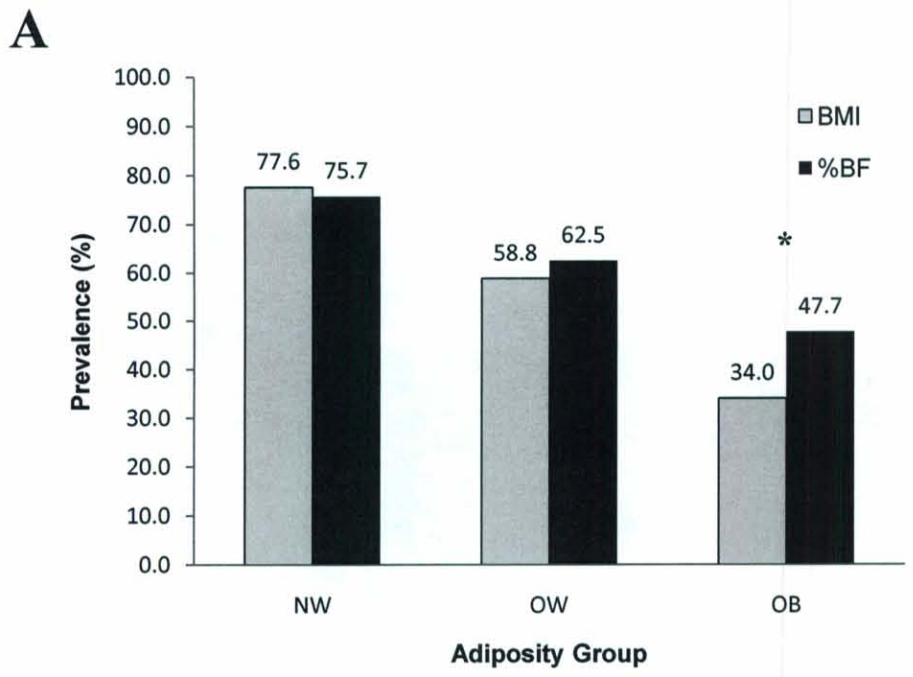
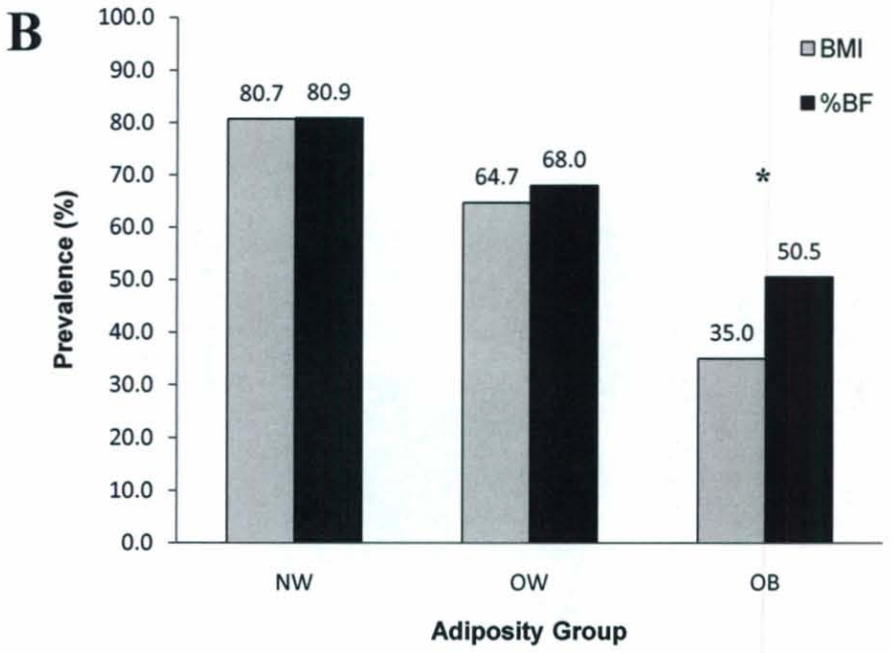


Figure 2.4 Prevalence of metabolically healthy phenotype within each adiposity group determined by BMI and %BF criteria. A. Overall (n = 1907); B. Women (n = 1464); C. Men (n = 443). NW, normal weight; OW, overweight; OB, obese; BMI, body mass index; %BF, body fat percentage. *$p < 0.05$ between BMI and %BF classifications.



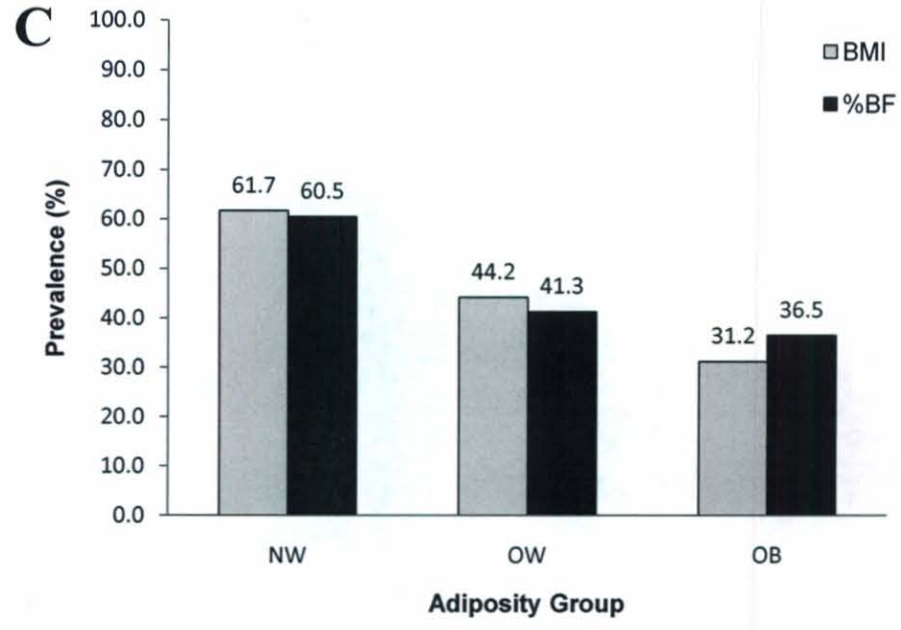


Figure 2.4 continued...

overweight individuals, and only 47.7% of obese subjects were metabolically healthy (Figure 2.4A). We found low agreement in the prevalence of the metabolically healthy (Kappa = 0.373) and abnormal (Kappa = 0.408) phenotypes between BMI and %BF adiposity groupings ($p < 0.001$). Specifically, there was no statistical difference in the discrepancy of these two phenotypes in normal weight (1.9%) and overweight (3.7%) subjects using BMI compared to %BF criteria. There was, however, a significant discrepancy (13.7%) among obese subjects classified using both methods ($p < 0.05$).

When subjects were classified as metabolically healthy using the additional definitions outlined in Table 2.4, comparable results were observed for the discrepancy analyses (Table 2.6). We found low agreement in the prevalence of metabolically healthy individuals across adiposity groups using BMI versus %BF classifications for all methods used to define the metabolically healthy phenotype. Specifically, there was a significant difference in the prevalence of metabolically healthy obese subjects but not normal weight or overweight for all definitions considered. Results were similar when subjects were stratified according to gender (data not shown).

The Influence of Gender on Discrepancy Analyses

We also sought to explore the influence of gender on the discrepancy between BMI and %BF classifications. In our cohort, 65.4% of women (n = 958) and 46.0% of men (n = 204) were metabolically healthy. For women, the prevalence of this phenotype was similar among normal weight individuals classified using BMI and %BF criteria

Table 2.6 Prevalence of metabolically healthy individuals among different weight classifications using various criteria to define the phenotype.[1]

	Wildman et al.		NCEP ATP III MetS		Karelis et al.		HOMA Index	
	BMI	**%BF**	**BMI**	**%BF**	**BMI**	**%BF**	**BMI**	**%BF**
NW	77.6	75.7	87.0	84.7	65.9	62.1	85.0	81.5
OW	58.8	62.5	58.0	64.6	38.5	48.4	48.8	59.2
OB	34.0	47.7[2]	29.5	44.7[2]	14.3	25.9[2]	7.8	22.0[2]

[1]Data presented as percentage (%) of metabolically healthy individuals in each adiposity group. BMI, body mass index; NW, normal weight; OW, overweight; OB, obese; NCEP ATP III MetS, National Cholesterol Education Program Adult Treatment Panel III definition for metabolic syndrome.

[2]$p < 0.05$ between BMI and %BF classifications according to Chi-Square analyses.

(80.7% vs. 80.9%, respectively) as well as overweight subjects (64.7% vs. 68.0%; Figure 2.4B). This was also true for normal weight and overweight men (Figure 2.4C). There was, however, a significant discrepancy in the prevalence of the metabolically healthy phenotype among obese women (Figure 2.4B). Although 35.0% of obese women fulfilled the criteria when they were defined using BMI, 50.5% did when using %BF to define obesity ($p < 0.05$). A similar trend was evident in men although the difference did not reach statistical significance (31.2% vs 36.5%).

As well, we chose to repeat the above analyses using waist circumference as a criterion for obesity as it is more closely related to metabolic risk factors in addition to being an appropriate method for field and clinical settings. Subjects were categorized into tertiles based on waist circumference within gender (lowest third, normal weight; middle third, overweight; upper third, obese). The discrepancy between the prevalence of the metabolically healthy phenotype among obese subjects using waist circumference compared to %BF was smaller than BMI in women but larger in men (data not shown). Specifically, 45.4% of obese women and 30.7% of men were metabolically healthy according to waist circumference measurements.

Discussion

Recently, a unique subset of obese individuals has been described that appear to be protected from the cardiometabolic abnormalities often associated with excess body weight (27-28). Although there has been a surge in research investigating this unique

phenotype, the majority of studies have used BMI as an index of obesity. As the utility of BMI to accurately estimate true adiposity has come into question (24, 140, 157), we sought to investigate the prevalence of this phenotype using more accurate %BF criteria. Among obese individuals, we found that nearly one-half were metabolically healthy using %BF criteria, which is significantly greater than what has previously been reported using BMI. This finding was evident regardless of gender although the discrepancy between the two indices of adiposity was smaller in men. Among normal weight and overweight individuals, the discrepancy was not significant between BMI and %BF classifications. We also applied additional definitions to characterize the metabolically healthy phenotype and found similar trends which further supports our findings. To the best of our knowledge, this is the first study of its kind to compare the prevalence of this group of individuals between BMI and %BF adiposity groups.

We recently found that approximately one third of men and women were misclassified into the incorrect adiposity group using BMI compared to %BF criteria (157). The discrepancy was greatest for obese individuals; approximately one quarter of our cohort was classified as obese according to BMI however 37-38% were in fact considered obese using %BF criteria. Others have shown similar discrepancies between BMI adiposity classifications and more accurate methods such as bioelectric impedance analysis (139) and DXA (140). As BMI cannot distinguish between fat tissue and lean tissue, its use as an index of obesity, particularly as it relates to obesity-associated disease, is questionable. Furthermore, BMI criteria are not gender or age specific and it is well known that there are gender differences in body fat (20, 141) and that body fat

increases with age, in spite of weight maintenance (sarcopenic obesity; ref 164). In a recent editor's correspondence, Lesser (158) pointed out these inherent flaws in the study by Wildman et al and stated that the serious limitations associated with BMI must be addressed to improve interpretation of their results. Indeed, our data show a large discrepancy between the prevalence of cardiometabolic abnormalities among obese individuals classified by BMI compared to %BF. Perhaps surprisingly, the prevalence of the metabolically abnormal phenotype was larger among obese individuals classified using BMI criteria. If %BF provides a more reliable measurement of true obesity, and cardiometabolic abnormalities such as those measured in our study are associated with an increase in adiposity, we would expect a higher prevalence of those abnormalities in obese individuals classified using %BF. The reason for this is unclear and further studies are required to understand this phenomenon. We also compared the prevalence of cardiometabolic abnormalities between waist circumference measurements (divided into thirds, according to gender) and %BF. Although the discrepancy between waist circumference and %BF was smaller in women compared to BMI vs. %BF, it was larger in men. This indicates that there are still important differences in the prevalence of cardiometabolic abnormalities when using more accurate reference methods to define obesity compared to field methods.

In the current study, we were most interested in determining if the prevalence of metabolically benign obesity was similar using BMI and %BF criteria and how this compared to what has previously been reported in the literature. When using BMI to define obesity, we found that approximately one-third of obese individuals in the NL

population were metabolically healthy which is similar to what has been found in previous studies. For example, among 5440 participants studied in the National Health and Nutrition Examination Surveys 1999-2004 (a multi-ethnic sample that is representative of the US population), 31.7% of obese individuals were metabolically healthy which is comparable to the prevalence observed in our study (27). These percentages are also in line with those found in earlier studies. Stefan et al found that 25% of their German sample were obese yet metabolically healthy (high insulin sensitivity and low intima-media thickness of the common carotid artery) (28). Among a white, Italian sample, 27.5% of obese subjects were metabolically healthy (normal lipid profile, blood pressure, electrocardiograms as well as low white blood cell counts and plasma fibrinogen levels) (29) while 20% of participants in the Bruneck Study were obese yet metabolically healthy (absence of impaired glucose tolerance, dyslipidemia, hyperuricemia and/or hypertension) (165). Interestingly, when we applied more accurate %BF criteria to define obesity, we observed a much higher percentage (47.7%) of obese individuals that were metabolically healthy. As %BF defined by DXA is considered a superior method to estimate obesity (25, 127), our data demonstrate that an even greater percentage of obese individuals may in fact have a normal metabolic profile compared to what has been reported in the literature. Further studies are needed to validate our findings.

Although the prevalence of body size phenotypes has been investigated in a number of studies, there is no clear consensus regarding the correct definition to classify an individual as metabolically healthy or abnormal. In the current study, we chose to use

the definition recommended by Wildman et al as we feel it gives the most comprehensive picture of an individual's metabolic status. By using this definition, which includes components of the metabolic syndrome, insulin resistance, as well as systemic inflammation (measured using circulating hsCRP levels), we have obtained a more thorough picture of metabolic health and broader, more complete definitions of body size phenotypes. Previous studies have relied solely on either insulin resistance cut points (28, 163, 166) or the metabolic syndrome definition (167). Interestingly, when we applied these criteria to our data, as well as the definition recommended by Karelis et al (162), similar results were obtained. It appears that there is a significant discrepancy in the proportion of metabolically healthy obese subjects between BMI and %BF classifications regardless of the criteria used to define this phenotype.

Although we provide comprehensive data investigating the influence of the index of obesity used in determining the prevalence of metabolically healthy and abnormal phenotypes, our study is not without its limitations. We recognize the fact that %BF measurements determined using DXA are not usually feasible in the clinical setting due to high cost and availability. Therefore, similar studies should be performed exploring the difference in the prevalence of the metabolically healthy phenotype among obese individuals classified using other field methods, such as BIA. Furthermore, although DXA is considered a more reliable measure of body fat compared to BMI, its use involves certain assumptions including uniform hydration of lean tissue (147). To account for this, all subjects fasted for 12 hours prior to having a DXA scan performed to control for differences in hydration. As well, it should be noted that the %BF cut points

used in this study were developed based on BMI cut points and not their association with disease biomarkers and risk of mortality (137). The discrepancy in the prevalence of the studied phenotypes between BMI and DXA measures of obesity may be reduced if DXA cut points were established based on disease risk. Moreover, our study population was uniquely homogeneous and as such, limits generalization to other populations. Further studies are therefore required in other ethnic groups. While not necessarily a limitation, it is also important to note that we examined risk factors for disease in the current study and not the outcomes caused by these risk factors (cardiovascular disease, T2D, etc). Future studies should investigate differences in the prevalence of these diseases among obese individuals classified using BMI versus DXA measurements.

In summary, we compared the prevalence of cardiometabolic abnormalities (categorized as two phenotypes: metabolically healthy and metabolically abnormal) among normal weight, overweight and obese Newfoundlanders classified using BMI versus %BF criteria. The level of agreement was poor between the prevalence of metabolically healthy obese subjects classified using BMI compared to %BF determined using DXA. Our findings indicate that nearly one-half of obese subjects are metabolically healthy when classified using %BF measurements, which is significantly higher than what has previously been reported using BMI. This was evident regardless of gender, although the discrepancy was smaller in men. There were no significant differences in the prevalence of cardiometabolic abnormalities among normal weight and overweight individuals classified using BMI versus %BF. Further research is needed to investigate differences in the prevalence of body size phenotypes among obese

individuals using other indices of obesity such as bioelectric impedance analysis, air-displacement plethysmography, and underwater weighing.

2.3

Body fat percentage is associated with cardiometabolic dysregulation in BMI-defined normal weight subjects

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Introduction

The global prevalence of obesity has increased substantially in the past three decades and is now estimated to affect over 400 million (1). Obesity is closely associated with a number of comorbidities such as T2D, hypertension, coronary artery disease, and many types of cancer (124). Recently, recognition of different subtypes of obesity has been reported in the literature including MHO (elevated body fat but normal metabolic profiles) and MONW (26). Although lean, MONW individuals present with clustering of metabolic and cardiovascular risk factors similar to what is often associated with being overweight or obese including elevated fasting glucose, insulin resistance, increased TG and decreased HDL cholesterol levels, and systemic inflammation (27, 154, 168). In addition, MONW women demonstrate an increased risk for cardiovascular mortality (169). The prevalence of this phenotype is substantial; it has been estimated that approximately 25% of normal weight individuals have abnormal metabolic profiles and are at increased risk of developing obesity-associated diseases (27, 168).

Although obesity is characterized by an excessive amount of body fat, it is commonly measured using BMI which is unable to differentiate between elevated body fat content and preserved or increased lean mass (20, 140, 157). This is especially true in normal weight individuals (BMI ≤ 24.9 kg·m^{-2}) (139, 157). Specifically, we recently demonstrated that a wide range in %BF measured using DXA exists for BMI-defined normal weight men (5.6 – 31.2%) and women (4.6 – 51.1 %) (157). Similarly, a recent study involving a large multiethnic sample from the US population found significant inter-subject variability in %BF for individuals with a BMI of 25.0 kg·m^{-2} (20). Taken

together, these data suggest that some individuals with a normal BMI are in fact obese and as such, may display the MONW phenotype. Indeed, it was recently demonstrated that individuals with a normal BMI and high body fat content had a higher prevalence of cardiometabolic dysregulation, metabolic syndrome, and cardiovascular disease risk factors (169-170). In these studies, however, body fat was estimated using BIA, a less accurate method of estimating %BF. We have shown that BIA tends to overestimate %BF in lean adults and underestimate %BF in obese adults compared to DXA, a more accurate reference method (171). At the present time, there is no data available regarding the prevalence of cardiometabolic disease in a large cohort of BMI-defined normal weight individuals with high %BF determined using more reliable DXA measurements. Therefore, the objectives of the current study were as follows: 1) to determine if %BF measured using DXA is associated with a number of cardiometabolic abnormalities in a large NL cohort of BMI-defined normal weight individuals; 2) to determine the prevalence of MONW individuals (defined using BMI) according to gender-specific %BF tertiles; 3) to estimate the risk of cardiometabolic disease associated with higher %BF if normal weight.

Methods

Study Population

A total of 977 subjects (192 men, 785 women) from the Canadian province of NL were recruited from an ongoing large scale nutrigenomics study (CODING Study) to take part in the current study (134-136, 157, 159, 168). Inclusion criteria were as follows: 1)

subjects with a normal BMI (18.5 – 24.9 kg m^{-2}); 2) between the ages of 20 and 79 years old; 3) at least third generation Newfoundlander; 4) healthy, without any serious metabolic, cardiovascular or endocrine diseases; and 5) not pregnant at the time of the study. The Human Investigation Committee of the Faculty of Medicine, Memorial University of Newfoundland approved the study and all subjects provided written and informed consent.

Anthropometric and body composition measurements

Subjects were weighed to the nearest 0.1 kg in standardized light clothes on a platform manual scale balance as previously described (Health O Meter Inc., Bridgeview, IL, USA) (134-136, 157, 168). Specifically, subjects were instructed to remove all clothing, including shoes and any jewelry, and change into a provided hospital gown. Height was measured using a fixed stadiometer to the nearest 0.1 cm. BMI was calculated as a person's weight in kilograms divided by their height in meters squared. Waist circumference was measured as the horizontal distance around the abdomen at the level of the umbilicus while the participant was in a standing position to the nearest 0.1 cm using a flexible metric measuring tape. Whole body composition measurements including fat mass and lean body mass were measured using DXA Lunar Prodigy (GE Medical Systems, Madison, WI, USA). Measurements were performed on subjects while lying in a supine position as previously described (134-136, 157, 168). Percent body fat (%BF) was determined using the manufacturer's software (Version 4.0) as a ratio of fat

mass over total body mass (including bone mineral densities). All measurements were performed following a 12 hour fasting period.

Measurement of cardiometabolic biomarkers

Blood samples were collected in the morning following a 12-hour fasting period; serum was isolated and stored at -80 °C for subsequent analyses. Serum concentrations of glucose, TG, and HDL cholesterol were performed on an Lx20 analyzer (Beckman Coulter Inc., CA, USA) using Synchron reagents. Serum insulin was measured on an Immulite immunoassay analyzer (DPC, CA, USA) and the homeostasis model assessment was used to measure insulin resistance (HOMA-IR; ref 160). hsCRP was measured by nephelometry according to the manufacturer's protocol (Beckman Coulter Inc). Blood pressure was measured using an automatic blood pressure monitor (Omron Healthcare, Burlington, Ont, Canada). Up to two readings were taken while the subject was in a seated position and averaged.

Definition of MONW and cardiometabolic risk factors

Normal weight obesity was defined as those subjects with a normal BMI (18.5 – 24.9 kg m^{-2}) and high %BF content (highest sex-specific tertile of %BF; $\geq$ 20.8% for men, $\geq$35.0 % for women). In addition, six cardiometabolic abnormalities were considered (elevated TG and glucose levels, decreased HDL cholesterol levels, insulin resistance, hypertension, and elevated hsCRP). Because there are no standard criteria to categorize metabolically abnormal individuals, we performed our analyses using the

definition recommended by Wildman et al (ref 27; Table 2.4) as it gives a more comprehensive representation of an individual's metabolic health by incorporating components of the metabolic syndrome, insulin resistance, as well as systemic inflammation. Subjects were classified as metabolically abnormal if they fulfilled ≥ 2 cardiometabolic abnormalities listed in Table 2.4 and metabolically healthy if they did not fulfill the above criteria (0 or 1 cardiometabolic abnormality).

Statistical Analyses

Anthropometric measurements and cardiometabolic variables are presented as mean (SD). Normal weight subjects (BMI 18.5 – 24.9 kg m^{-2}) were divided into sex-specific %BF tertiles as follows: low ($\leq$ 15.2% men, $\leq$ 29.7% women), medium (15.3 – 20.7%% men, 29.8 – 34.9%% women) and high ($\geq$ 20.8% men, $\geq$ 35.0% women) %BF groups. Subjects' physical characteristics and metabolic profiles were calculated according to the %BF tertiles. Differences in these characteristics among the three groups of subjects (low, medium, and high %BF) were analyzed using ANCOVA analyses with age and gender included as model covariates. To investigate the association between %BF as a continuous variable and cardiometabolic abnormalities among normal weight subjects, Partial correlation analyses were performed, controlling for age and gender. We also wanted to assess the effects of central adiposity on cardiovascular disease risk factors, therefore Partial correlation analyses were repeated using waist circumference, controlling again for age and gender. In addition, the prevalence of the metabolically abnormal phenotype was calculated as the mean

percentage within each %BF tertile. As waist circumference is a more appropriate method for field and clinical settings, we also sought to investigate the prevalence of the metabolically abnormal phenotype among normal weight subjects according to waist circumference tertiles (Low $\leq$ 82.0 cm men, $\leq$ 76.5 cm women; Medium 82.1 – 88.0 cm men, 76.6 – 83.2 cm women; High $\geq$ 88.1 cm men, $\geq$ 83.3 cm women). The Cochran-Armitage test for trend was used to explore the overall association between %BF and waist circumference with the prevalence of the metabolically abnormal phenotype. Chi-square analyses were then performed to assess differences between specific tertiles (ie. low vs medium, low vs high, medium vs high). The adjusted odds ratio of being metabolically abnormal according to %BF tertiles was calculated using logistic regression with age, gender and waist circumference included as model covariates (with the lowest tertile as the reference) to assess the risk associated with higher %BF given a normal BMI. PASW Statistics version 18.0 (SPSS Inc., Chicago, IL) was used for all analyses. Statistical analyses were two-sided and a p value < 0.05 was considered to be statistically significant.

Results

Physical characteristics and cardiometabolic biomarkers in subjects

Physical and biochemical characteristics of all subjects according to gender-specific %BF tertiles are shown in Table 2.7. As those in the low %BF group were

Table 2.7 Physical and biochemical characteristics of subjects according to %BF tertiles (n = 977).[1]

Variables	Low (n = 324)	Medium (n = 324)	High (n = 329)
Age (y)	34.5 (13.1)	40.2 (13.0)[2]	44.2 (13.6)[2,3]
Height (cm)	167.0 (8.2)	165.3 (8.2)[2]	165.0 (7.8)[2]
Weight (kg)	60.0 (7.7)	62.0 (7.6)[2]	64.1 (7.2)[2,3]
BMI ($kg\,m^{-2}$)	21.5 (1.5)	22.6 (1.4)[2]	23.5 (1.2)[2,3]
Waist circumference (cm)	77.0 (6.3)	80.6 (6.8)[2]	85.2 (8.0)[2,3]
Body Fat (%)	22.8 (6.8)	29.6 (6.1)[2]	35.8 (6.0)[2,3]
SBP (mm Hg)	116.7 (13.7)	119.9 (16.0)	121.5 (15.4)[2]
DBP (mm Hg)	75.5 (10.3)	77.6 (10.2)	79.3 (10.1)[2]
Total cholesterol (mmol/L)	4.61 (0.97)	5.05 (0.90)[2]	5.23 (1.12)[2]
TG (mmol/L)	0.85 (0.40)	0.97 (0.55)[2]	1.06 (0.58)[2,3]
HDL-C (mmol/L)	1.58 (0.42)	1.64 (0.50)	1.56 (0.38)
LDL-C (mmol/L)	2.67 (0.82)	2.97 (0.77)[2]	3.18 (0.95)[2,3]
Glucose (mmol/L)	4.7 (0.5)	4.8 (0.5)	5.0 (0.6)[2,3]
Insulin (pmol/L)	43.7 (29.5)	45.0 (22.1)	54.9 (35.5)[2,3]
HOMA-IR	1.34 (0.91)	1.41 (0.73)	1.78 (1.40)[2,3]
hsCRP (mg/L)	1.70 (3.30)	2.14 (4.22)[2]	2.42 (3.00)[2]

[1]Data are presented as mean (SD). Differences in variables (aside from age) among %BF tertiles determined using ANCOVA analyses with age and gender included as model covariates. Low ≤ 15.2% for men, ≤29.7 for women; Medium 15.3 – 20.7% for men, 29.8 – 34.9 for women; High ≥ 20.8% for men, ≥35.0 for women. SBP, systolic blood

pressure; DBP, diastolic blood pressure; TG, triacylglycerol; HDL-C, HDL cholesterol; HOMA-IR, homeostasis model for insulin resistance; hsCRP, high-sensitivity C-reactive protein.

[2] $p < 0.05$ versus low

[3] $p < 0.05$ versus medium

significantly younger, analyses of all variables were adjusted for age as well as gender. As expected, weight, waist circumference, BMI, and %BF were significantly higher in the medium and high %BF groups compared to the low group. In terms of cardiometabolic biomarkers, both medium and high %BF subjects had abnormal metabolic profiles compared to low %BF subjects. Specifically, blood pressure (systolic and diastolic) was elevated in the high %BF group compared to the low %BF group although no significant differences were detected between medium and low %BF subjects. Serum TG was higher in both the medium and high %BF groups, however no significant differences in HDL cholesterol levels were evident among the three groups. In addition, both fasting glucose and insulin resistance (HOMA-IR) were elevated in high %BF subjects compared to both medium and low %BF subjects. In terms of systemic inflammation, circulating hsCRP was significantly higher in medium %BF subjects as well as high %BF subjects compared to the low group.

Associations between cardiometabolic abnormalities and %BF as a continuous variable

We also sought to assess the association between %BF as a continuous variable and markers of cardiometabolic disease (Table 2.8). After controlling for gender and age, %BF was positively correlated with both systolic and diastolic blood pressure, TG levels, insulin resistance and circulating hsCRP. Moreover, %BF was negatively associated with HDL cholesterol levels. Interestingly, %BF was not associated with fasting glucose levels. To assess the role of central adiposity on cardiometabolic biomarkers in normal

Table 2.8 Partial correlations between waist circumference and%BF with cardiometabolic abnormalities among normal weight subjects controlling for age and gender (n = 977).[1]

	Waist Circumference		%BF	
Variables	***r***	***p* value**	***R***	***p* value**
SBP (mm Hg)	0.056	ns	0.093	0.007
DBP (mm Hg)	0.039	ns	0.112	0.001
TG (mmol/L)	0.168	<0.001	0.154	<0.001
HDL-C (mmol/L)	-0.206	<0.001	-0.081	0.019
Glucose (mmol/L)	0.003	ns	0.054	ns
HOMA-IR	0.158	<0.001	0.186	<0.001
hsCRP (mg/L)	0.098	0.005	0.132	<0.001

[1]SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triacylglycerol; HDL-C, HDL cholesterol; HOMA-IR, homeostasis model for insulin resistance; hsCRP, high-sensitivity C-reactive protein.

weight subjects, we investigated associations between waist circumference and the variables measured. Similar to %BF, waist circumference was positively associated with TG, insulin resistance and hsCRP, and negatively correlated with HDL cholesterol. No significant associations were evident with systolic or diastolic blood pressure as well as glucose concentrations.

Prevalence of metabolically abnormal phenotype according to waist circumference and %BF tertiles

Figure 2.5A demonstrates the prevalence (%) of the metabolically abnormal phenotype according to gender-specific %BF tertiles. According to the Cochran-Armitage test, the prevalence of cardiometabolic disease increased as %BF increased ($p < 0.001$). Specifically, the prevalence was lowest in the low %BF group at 7.4%. Both the medium and high %BF groups had a significantly greater proportion of subjects displaying the metabolically abnormal phenotype (12.0% and 19.5% respectively, $p < 0.05$). Again, we wanted to assess the influence of central adiposity on the prevalence of this phenotype therefore data were reanalyzed according to gender-specific waist circumference tertiles (Figure 2.5A). Similar trends were evident using this index of adiposity (Cochran-Armtiage test, $p < 0.001$). Although no significant difference was found between the low and medium waist circumference tertiles (8.2% versus 10.7%, respectively), the high waist circumference group had a significantly greater proportion of metabolically abnormal subjects compared to the low group (20.5%, $p < 0.05$). In addition, we wanted to investigate the influence of gender on presentation of

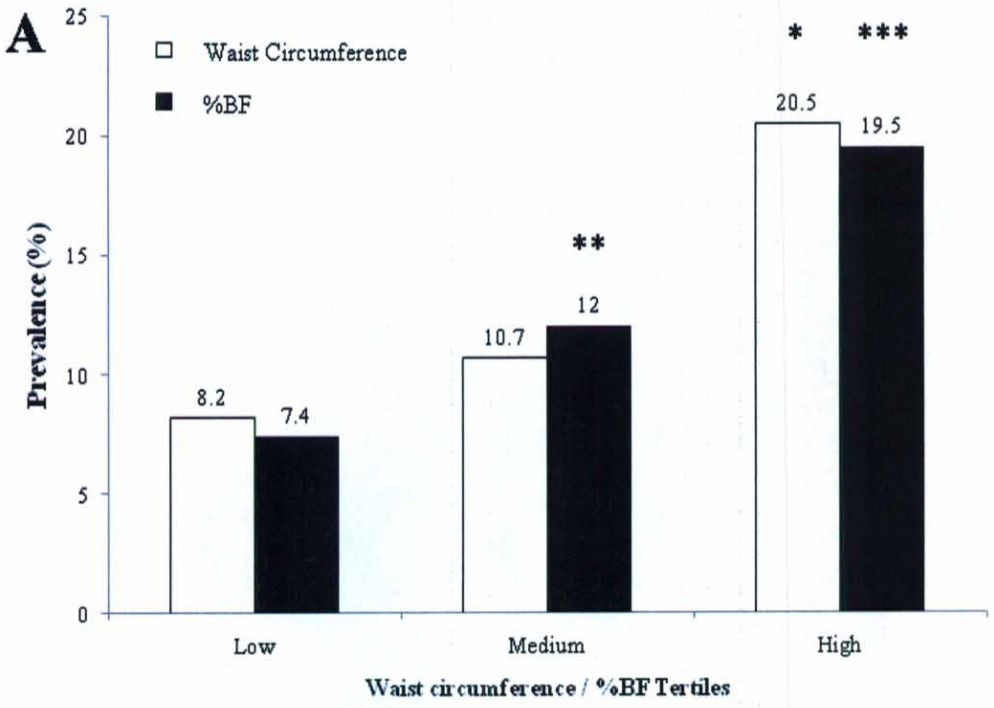

Figure 2.5. Prevalence of metabolically abnormal phenotype among normal weight subjects (n = 977) A. Prevalence according to waist circumference and %BF tertiles. * $p < 0.05$ compared to low and medium waist circumference tertiles; ** $p < 0.05$ compared to low %BF tertile; ***$p < 0.05$ compared to low and medium %BF tertiles. B. Prevalence according to gender (n = 192 men, n = 785 women). * $p < 0.05$ compared to low %BF tertile for men; ** $p < 0.05$ compared to low %BF tertile for women.

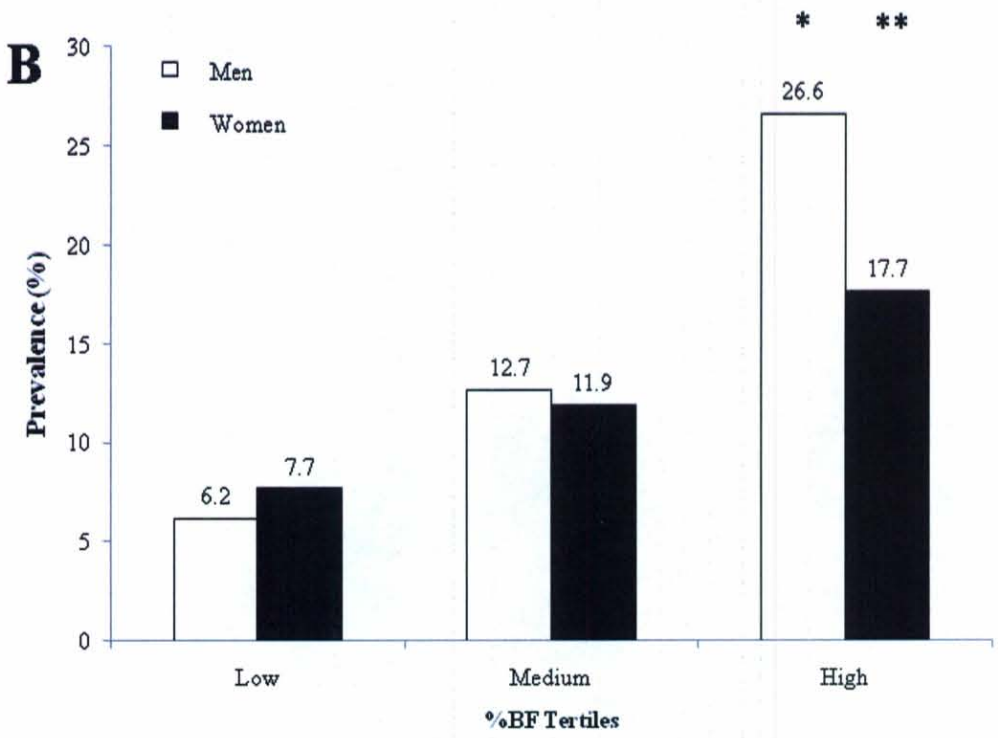

Figure 2.5 continued…

cardiometabolic disease (Figure 2.5B). Although similar trends were seen when subjects were dichotomized according to sex, significance was only reached in the high %BF group compared to the low %BF group for both sexes. In men, the prevalence of being metabolically abnormal was 6.2% among low %BF subjects compared to 26.6% in high %BF subjects ($p < 0.05$). Among women, the prevalence in the low group was 7.7% compared to 17.7% in the high %BF group ($p > 0.05$). There were no significant differences in the prevalence of cardiometabolic abnormalities between men and women for any of the %BF groups.

Risk associated with MONW

Lastly, we explored the risk associated with being MONW compared to those normal weight subjects with low %BF. Figure 2.6 shows the adjusted odds ratio associated with the metabolically abnormal phenotype given medium or high %BF compared to low %BF subjects. After adjusting for age and gender, %BF was significantly associated with greater risk of having an abnormal metabolic profile. In particular, the odds ratio for the medium %BF group was 1.61 (95% CI 0.94 – 2.77), while the odds nearly tripled for high %BF subjects (OR 2.73, 95% CI 1.63 – 4.86). To assess whether the risk associated with high %BF was partly attributed to these subjects having a higher waist circumference, we reanalyzed our data after further adjustment for waist circumference. The odds ratio for the medium %BF group was 1.37 (95% CI 0.79 – 2.38), while the odds of being metabolically abnormal among high %BF subjects was almost doubled compared to the low %BF group (OR 1.87, 95% CI 1.07 – 3.26).

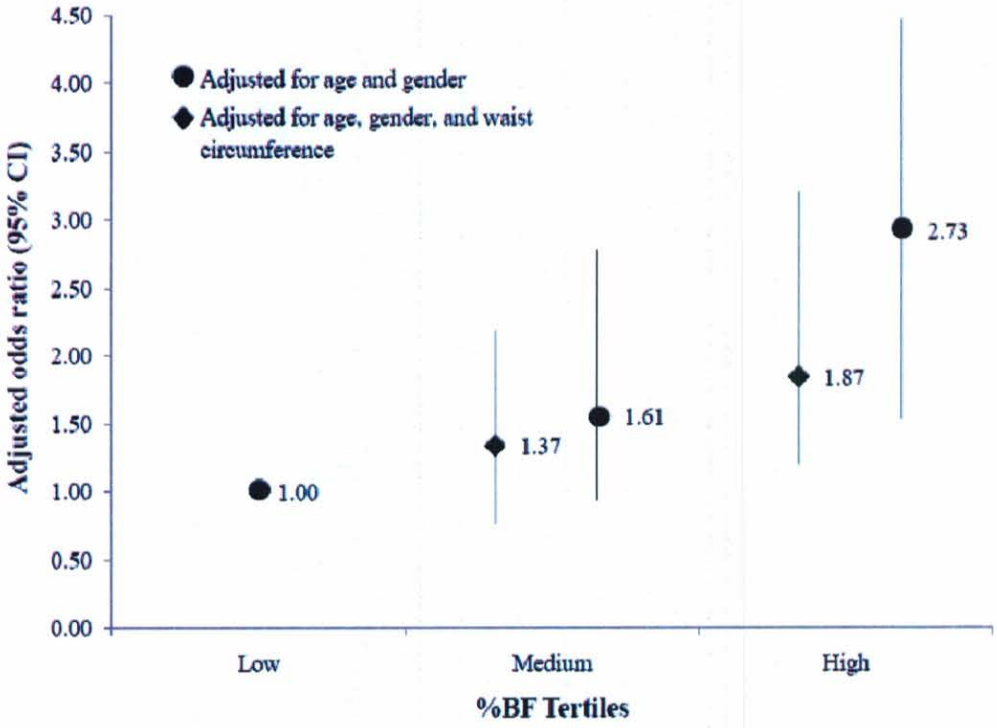

Figure 2.6 Risk of metabolically abnormal phenotype according to %BF tertiles (lowest tertile as reference) in subjects with a normal BMI (n = 977). Adjusted odds ratios (95% CI) were calculated using logistic regression with age, gender, and waist circumference included as model covariates.

Discussion

Recent evidence suggests that some individuals cannot be classified as obese based solely on their BMI. Indeed, a substantial proportion of BMI-defined normal weight subjects display clustering of cardiometabolic abnormalities that puts them at an increased risk for future development of cardiovascular and other obesity-related disease. Although these people tend to have higher %BF, work to date has measured adiposity using less accurate methods. In the present study, we investigated the prevalence of MONW individuals according to DXA-defined %BF tertiles, a more accurate index of obesity. In agreement with previous studies, we found that the prevalence of cardiometabolic abnormalities was highest among high %BF individuals compared to those with low %BF. This was also true when subjects were stratified according to waist circumference tertiles and gender. Furthermore, the risk associated with the metabolically abnormal phenotype was significantly higher among subjects with elevated %BF.

It has been well documented that an excessive amount of body fat is associated with numerous comorbidities including hypertension, insulin resistance, dyslipidemia and systemic inflammation however this has typically been studied in individuals with a BMI $> 25.0\ kg\ m^{-2}$ (172-173). As we, and others, have demonstrated that a wide range in %BF exists for BMI-defined normal weight individuals (139, 157), it is reasonable to expect that these same associations seen in overweight and obese individuals are also true in normal weight subjects with high %BF. Although this has been shown in other Caucasian populations, these studies have subdivided subjects according to BMI

increments (174) or relied on less accurate measures of adiposity, namely BIA (169-170, 175). Furthermore, although De Lorenzo et al established similar relationships between DXA-determined %BF in normal weight Italian women, their sample size was quite small (160). To the best of our knowledge, we are the first to show that cardiovascular risk factors including hypertension, serum lipids, insulin resistance and hsCRP are associated with %BF measured using DXA in a large population of BMI-defined normal weight individuals. Furthermore, individuals in the high %BF group had an almost 3-fold increased risk of being metabolically abnormal. Consequently, our results indicate that a normal BMI does not necessarily imply protection from cardiometabolic dysregulation and obesity-associated disease. When considered along the entire spectrum of BMI and the already well-characterized concept of metabolically healthy but obese subjects, our data provide further evidence of the need for more comprehensive awareness of obesity subtypes among clinicians to increase detection of these individuals.

To further assess the influence of central adiposity, we analyzed our data according to gender-specific waist circumference tertiles. Similar to our results using %BF, the prevalence of the metabolically abnormal phenotype was higher among those with an increased waist circumference compared to those in the lowest tertile. This has significant implications as DXA measurements are not readily available in clinical settings whereas waist circumference can be easily measured by physicians. It is important to note however, that unlike %BF, there was no significant difference between the medium and low waist circumference groups indicating that waist circumference measurements are not as sensitive at predicting cardiometabolic dysregulation among

those in the lower ranges compared to more accurate reference methods such as DXA. Moreover, the risk associated with elevated %BF remained significant after further adjustment for waist circumference suggesting that central adiposity does not fully account for the increased risk of cardiometabolic disease. This is an important finding as it demonstrates the need for development of more accurate algorithms based on %BF measurements to screen for obesity subtypes in field settings and clinical practice.

As there are known differences in the prevalence of cardiometabolic abnormalities between men and women, we examined the influence of gender among our cohort of normal weight subjects. Contrary to previous findings, we observed no significant differences in the prevalence of the metabolically abnormal phenotype between men and women. Earlier studies have demonstrated substantially lower proportions of metabolic syndrome in men with high %BF compared to women (169, 174). The reason for the discrepancy between the current study and others is unclear however it may be due to the fact that the previous studies both used data from the Third National Health and Nutrition Examination Survery, which includes a number of different ethnicities. Furthermore, each of these studies used NCEP ATP III criteria for metabolic syndrome to characterize the metabolically abnormal phenotype. Our use of the more comprehensive definition by Wildman et al (27) may also contribute to the observed differences.

Our study is not without limitations. Although %BF measurements determined using DXA are considered one of the more accurate methods to measure adiposity, certain assumptions are made including uniform hydration of lean tissue (147). In an

attempt to limit the impact of this, all subjects fasted for 12 hours prior to DXA measurements. Furthermore, we used arbitrary cut-off points based on %BF tertiles to define our low, medium and high %BF groups. At the current time, the WHO has not defined a normal range for %BF therefore we believe our use of tertiles is the most valid method. When subjects were divided into %BF groupings according to criteria recommended by Bray (Table 2.1; ref 137) that is based on BMI cut points, similar trends were found (data not shown). Other limitations include the low number of male participants and our uniquely homogeneous study cohort. These two considerations limit application to other populations and therefore further studies are necessary.

In summary, we examined the association between elevated %BF and cardiometabolic abnormalities among 977 BMI-defined normal weight subjects (192 men, 785 women) from the Canadian province of NL. We found a greater prevalence of cardiometabolic disease among individuals in the highest tertile of %BF compared to those with low %BF, regardless of gender. Similar trends were evident when subjects were classified according to waist circumferences tertiles. The odds of being metabolically abnormal were nearly tripled for the high %BF group; this held true after further adjustment for waist circumference. Screening for cardiometabolic risk factors among normal weight individuals will become an important contribution to the prevention of cardiovascular disease and other obesity-related disorders.

3

Common genetic variants are associated with obesity-related traits: A candidate gene approach

3.1

No association between visfatin (*NAMPT*) gene variants and metabolic traits in the Newfoundland population

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Introduction

Visfatin was first reported as a novel adipokine predominantly secreted from visceral adipose tissue however evidence now supports a pro-inflammatory role. Specifically, visfatin is expressed by macrophages infiltrating adipose tissue and is produced in response to inflammatory signals (176-177). Although it was initially suggested that visfatin had insulin-mimetic properties that increased insulin sensitivity (81), this study was later retracted (82) as a subsequent report could not confirm the insulin-mimetic action of this adipocytokine (83). Despite this, it has been demonstrated that circulating visfatin is positively correlated with T2D (84) and obesity (86, 178). Furthermore, we recently demonstrated that visfatin is positively associated with serum TGs and down-regulated by a short-term positive energy balance (179).

Visfatin is a 473 amino acid protein with a molecular mass of 52kD that is encoded by the gene *NAMPT* (Entrez ID: 10135), previously known as pre-B cell colony-enhancing factor gene (80). *NAMPT* is mapped to 7q22.3 and includes 11 exons encompassing 34.7 kb (180). This region of the genome has shown linkage to phenotypes related to the metabolic syndrome including BMI, HDL cholesterol and TG levels (181-183). In addition, recent studies have shown that SNPs within the promoter region of the visfatin gene are associated with increased risk of T2D (184) and its related parameters (185-186), total apolipoprotein B and the apolipoprotein B component of LDL cholesterol (185), higher HDL cholesterol (187-188), total cholesterol (185), systemic inflammation (184) and reduced risk of cardiovascular disease (189). Moreover, a very rare, noncoding SNP has been associated with severe obesity in both

adults and children (190). Contrary to these findings, others have found no association between genetic variation in the visfatin gene and T2D using case-control analyses (186, 188, 191), measures of obesity (184, 186-188, 192) or serum lipids (192). Sample sizes were small in the majority of these studies and therefore, were likely underpowered to detect a true association. Furthermore, the study by Korner et al was not corrected for multiple testing, and consequently their results should be interpreted with caution. As such, the influence of genetic variation in *NAMPT* on the aforementioned variables remains unclear. In the current study, we sought to clarify the influence of genetic variation in the visfatin gene on obesity, parameters of glucose and lipid metabolism, as well as systemic inflammation. We assessed the effect of 10 SNPs in this gene on measurements of body composition, insulin resistance, serum lipids, and hsCRP in a large, healthy NL population.

Research Design and Methods

Subjects

Subjects (n = 1838; 413 men, 1425 women) were recruited from an ongoing, large-scale nutrigenomics study (CODING Study; refs 134, 157, 159). All participants were from the genetically homogenous population of NL, Canada. Inclusion criteria were as follows: 1) at least third generation Newfoundlander; 2) healthy, without any serious metabolic, cardiovascular or endocrine diseases; and 3) not pregnant at the time of the study. The Human Investigation Committee of the Faculty of Medicine, Memorial

University of Newfoundland approved the study and all subjects provided written and informed consent.

Measurements of body composition

All measurements were performed following a 12 hour fasting period. Subjects were weighed to the nearest 0.1 kg in standardized light clothes and without shoes on a platform manual scale balance as previously described by us (Health O Meter Inc., Bridgeview, IL; refs 134, 157, 159). Height was measured using a fixed stadiometer to the nearest 0.1 cm. BMI was calculated as a person's weight in kilograms divided by their height in meters squared. Waist and hip circumference were measured while the participant was in a standing position to the nearest 0.1 cm using a flexible metric measuring tape. Waist circumference was measured as the horizontal distance around the abdomen at the level of the umbilicus; hip circumference was measured as the largest circumference between the waist and thighs and waist-to-hip ratio was calculated. In addition to anthropometric measurements, whole body composition measurements including %BF and percentage trunk fat (%TF) were measured using DXA Lunar Prodigy (GE Medical Systems, Madison, WI, USA). Measurements were performed on subjects following the removal of all metal accessories, while lying in a supine position as previously described (134, 157, 159).

Serum Measurements

Blood samples were taken from all subjects in the morning following a 12-hour fasting period. Serum was isolated and stored at -80 °C for subsequent analyses. Insulin levels were measured on an Immulite immunoassay analyzer (DPC, CA, USA). HOMA-IR was used as a measure of insulin resistance (HOMA-IR = insulin (μU/ml) × glucose (mmol/L)/22.5)) and β-cell function (HOMAβ = 20 × insulin (μU/ml) / (glucose-3.5)) (160). Serum concentrations of glucose, TG, total cholesterol, and HDL cholesterol were measured using Synchron reagents and performed on an Lx20 analyzer (Beckman Coulter Inc., CA, USA). LDL cholesterol was calculated using the following formula: (Chol) – (HDL) – (TG/2.2) which is reliable in the absence of severe hyperlipidemia. hsCRP was measured by nephelometry according to the manufacturer's protocol (Beckman Coulter Inc).

Genomic DNA Isolation, Genotyping and Selection of SNPs

Genomic DNA was isolated from approximately 5ml of whole blood using the Wizard Genomic DNA Purification kit (Promega, WI, USA) according to the manufacturer's protocol as previously described by us (134, 159). To assess the reproducibility of genotyping, 5% of samples were randomly selected and re-genotyped; all genotypes matched their initial called genotype. The ten SNPs investigated in the visfatin gene were rs7789066 (A>G 5' flanking region), rs3801266 (A>G intron), rs6963243 (G>C intron), rs2058539 (A>C intron), rs6947766 (C>T intron), rs4730153 (G>A intron), rs10808150 (G>A intron), rs2098291 (C>T intron), rs10953502 (T>C

intron), and rs10953501 (A>G 3' UTR). Tagging SNPs were selected using a pairwise r^2 approach with an $r^2 \geq 0.9$ and minor allele frequency (MAF) ≥ 0.05 (SNPbrowser Version 3.5; based on HapMap data for CEU population). A total of seven tagging SNPs were genotyped (rs3801266, rs6963243, rs2058539, rs6947766, rs10808150, rs2098291, and rs10953502) as well as two additional SNPs to include the flanking regions (rs7789066 and rs10953501). In addition, rs4730153 was chosen to confirm findings from previous studies (186-187, 190).

Statistical Analyses

All statistical analyses were performed using the statistical software R or SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Hardy-Weinberg equilibrium was tested using X^2 analysis with one degree of freedom. Multiple regression analyses using an additive model were used to assess the association between *NAMPT* variants and body composition, markers of insulin resistance, serum lipids, and hsCRP with sex and age included as covariates. Hypotheses regarding the effect of *NAMPT* variants on parameters measured were two-sided and a p-value of 0.05 was taken as the threshold of statistical significance.

Results

Physical and biochemical characteristics of all subjects are shown in Table 3.1. A description of the ten SNPs genotyped, including location, type of variant, and minor

Table 3.1. Physical and biochemical characteristics of subjects.[1]

Variables	Men N = 413	Women N = 1425	Overall N = 1838
Age (yrs)	41.0 ± 14.0	44.5 ± 11.0	43.7 ± 11.8
BMI (kg/m^2)	27.6 ± 4.5	26.6 ± 5.2	26.8 ± 5.1
Waist Circumference (cm)	97.9 ± 12.0	90.9 ± 14.3	92.4 ± 14.1
Waist-to-hip Ratio	0.97 ± 0.06	0.89 ± 0.07	0.90 ± 0.08
Body fat (%)	25.3 ± 7.5	37.8 ± 7.4	35.0 ± 9.1
Trunk fat (%)	30.1 ± 8.8	39.0 ± 8.7	37.0 ± 9.5
Glucose (mmol/L)	5.3 ± 1.0	5.1 ± 0.9	5.1 ± 0.9
Insulin (pmol/L)	77.8 ± 58.0	68.4 ± 67.8	69.7 ± 65.9
HOMA-IR	2.67 ± 3.55	2.32 ± 2.96	2.40 ± 3.10
HOMAβ	130.1 ± 149.7	135.9 ± 251.4	134.6 ± 232.6
Cholesterol (mmol/L)	5.06 ± 1.10	5.21 ± 1.03	5.17 ± 1.05
HDL cholesterol (mmol/L)	1.24 ± 0.27	1.56 ± 0.38	1.49 ± 0.38
LDL cholesterol (mmol/L)	3.12 ± 0.95	3.12 ± 0.88	3.11 ± 0.90
Triacylglycerol (mmol/L)	1.50 ± 0.99	1.15 ± 0.71	1.23 ± 0.79
hsCRP (mg/L)	2.12 ± 2.73	3.43 ± 4.59	3.14 ± 4.28

[1]Values are expressed as mean ± standard deviation. BMI, body mass index; HOMA-IR, homeostasis model assessment for insulin resistance; HOMAβ homeostasis model assessment for β cell function; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein; hsCRP, high-sensitivity C-reactive protein.

Table 3.2. Summary of single nucleotide polymorphisms, allele frequencies, and Hardy-Weinberg equilibrium.[1]

Position	RS Number	Variant	Location	MAF	HWE (*p*-value)
-2423	rs7789066	A/G	5' flanking	0.07	0.514
+1079	rs3801266[2]	A/G	Intron 1	0.18	0.211
+1382	rs6963243[2]	G/C	Intron 1	0.24	0.890
+8692	rs2058539[2]	A/C	Intron 2	0.41	0.798
+14663	rs6947766[2]	C/T	Intron 4	0.25	0.415
+21179	rs4730153	G/A	Intron 6	0.43	0.703
+24037	rs10808150[2]	G/A	Intron 8	0.43	1.00
+24666	rs2098291[2]	C/T	Intron 8	0.31	0.815
+32898	rs10953502[2]	T/C	Intron 10	0.42	0.717
+37697	rs10953501	A/G	3' UTR	0.43	0.962

[1]Hardy-Weinberg was estimated using X^2 analysis with one degree of freedom. MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium.

[2]Tagging SNPs (SNPbrowser Version 3.5, based on HapMap CEU population).

allele frequencies (MAF), is shown in Table 3.2. All SNPs were fairly common variants (MAF 0.18 – 0.43) aside from rs7789066 (MAF 0.07) which is located in the promoter region of *NAMPT* and therefore is likely highly conserved. Genotype distributions were in Hardy-Weinberg equilibrium at all 10 loci (Table 3.2). Furthermore, linkage disequilibrium was estimated among the variants and is shown in Table 3.3.

Table 3.4 shows the genotype effect of the 10 SNPs investigated in this study on measurements of body composition (BMI, waist circumference, waist-to-hip ratio, %BF, and %TF), markers of insulin resistance (fasting glucose, insulin, HOMA-IR, and HOMAβ), serum lipids (total cholesterol, HDL cholesterol, LDL cholesterol, and TG), as well as systemic inflammation (measured as circulating hsCRP). Multiple regression analyses were performed to test for association between the 10 SNPs in *NAMPT* and the above parameters with gender and age included as model covariates. Under an additive model, we did not find any significant associations between variation in *NAMPT* and any variables investigated. As can be seen, the p values are from significant thresholds, despite not correcting for multiple testing. This was also true when both dominant and recessive models were applied (data not shown).

Power calculations were carried out for a quantitative trait genetic association study. Figure 3.1 illustrates the power profiles as a function of varying coefficients of determination for a range of heritability estimates ($h^2 = 0.05 – 0.15$); type 1 error was fixed to 10^{-6} for these calculations. As demonstrated by Figure 3.1, we had sufficient power ($\beta \geq 0.80$) at $R^2 > 0.4$ to detect a positive association, given our sample size.

Table 3.3 Measures of linkage disequilibrium (D' and r^2) among ten SNPs in *NAMPT*.[1]

r^2/D'	rs7789066	rs3801266	rs6963243	rs2058539	rs6947766	rs4730153	rs10808150	rs2098291	rs10953502	rs10953501
						p values				
rs7789066		0.350	0.477	0.389	0.995	0.999	0.986	0.996	0.367	0.998
rs3801266	0.038		0.964	0.083	0.939	0.988	0.999	0.988	0.708	0.968
rs6963243	0.049	0.066		0.995	0.999	0.997	0.994	0.992	0.997	0.994
rs2058539	0.015	0.190	0.458		0.971	0.973	0.977	0.989	0.974	0.970
rs6947766	0.023	0.068	0.108	0.221		0.987	0.983	0.953	0.987	0.974
rs4730153	0.093	0.298	0.428	0.880	0.246		0.996	0.997	0.903	0.983
rs10808150	0.090	0.292	0.421	0.878	0.247	0.982		0.994	0.909	0.989
rs2098291	0.031	0.098	0.140	0.301	0.139	0.329	0.330		0.862	0.981
rs10953502	0.013	0.157	0.440	0.905	0.239	0.792	0.795	0.239		0.908
rs10953501	0.997	0.284	0.423	0.870	0.241	0.960	0.972	0.320	0.797	

[1]D' is given in upper right half and r^2 is given in lower left half of table.

Table 3.4 Genotype effect of ten SNPs within *NAMPT* on body composition, markers of insulin resistance, serum lipids, and systemic inflammation.[1,2]

Variables	rs7789066	rs3801266	rs6963243	rs2058539	rs6947766	rs4730153	rs10808150	rs2098291	rs10953502	rs10953501
						p values[3]				
BMI (kg/m^2)	1.00	1.00	0.84	1.00	0.96	1.00	0.99	1.00	1.00	1.00
Waist circumference (cm)	1.00	1.00	1.00	1.00	0.91	1.00	1.00	1.00	1.00	1.00
Waist-to-hip ratio	1.00	1.00	0.99	1.00	1.00	0.95	1.00	1.00	1.00	0.95
Body fat (%)	1.00	1.00	1.00	0.99	1.00	1.00	0.99	1.00	1.00	1.00
Trunk fat (%)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Glucose (mmol/L)	1.00	0.97	0.95	0.99	1.00	1.00	1.00	1.00	1.00	1.00
Insulin (pmol/L)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
HOMA-IR	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
HOMAβ	1.00	0.95	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol (mmol/L)	1.00	1.00	1.00	0.88	1.00	1.00	0.98	1.00	0.99	0.89
HDL-c (mmol/L)	0.85	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.99	1.00
LDL-c (mmol/L)	1.00	0.98	1.00	0.63	1.00	0.71	0.77	1.00	0.81	0.55
Triacylglycerol (mmol/L)	0.95	0.99	1.00	1.00	0.95	1.00	1.00	1.00	0.99	1.00
hsCRP (mg/L)	0.81	1.00	1.00	1.00	1.00	1.00	0.98	1.00	1.00	1.00

[1]BMI, body mass index; HOMA-IR, homeostasis model assessment for insulin resistance; HOMAβ homeostasis model assessment for β cell function; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein; hsCRP, high-sensitivity C-reactive protein.

[2]Multiple regression using an additive model was used for quantitative trait analyses to assess the association between *NAMPT* variants and measurements of body composition, markers of insulin resistance, serum lipids, and systemic inflammation. Sex and age were included as model covariates.

[3]No significant genotype effects when dominant or recessive models were applied.

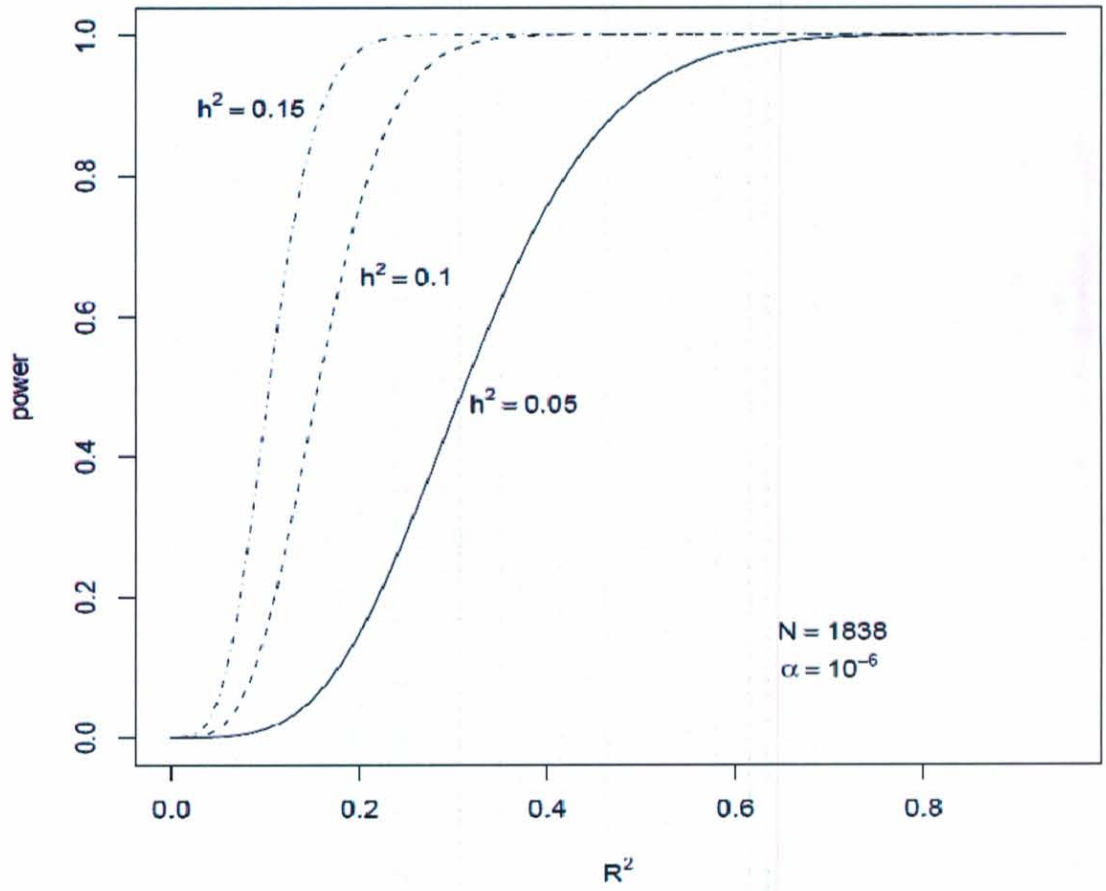

Figure 3.1 Power profiles as a function of varying coefficients of determination for a range of heritability estimates.

Discussion

The physiological function of visfatin seems to be diverse. Initially, attention was focused on its relationship with T2D however recent studies have demonstrated that visfatin may also be involved in the etiology of obesity (86, 186), dyslipidemia (193), systemic inflammation (194) and increased cardiovascular disease risk (194) although these associations remain controversial. Furthermore, a number of genetic association studies have been performed investigating the effect of variants in *NAMPT* on these parameters with contradictory results (184-188). The main findings from our work do not support a significant role for genetic variation in the visfatin gene on differences in body composition, markers of insulin resistance, serum lipids, and systemic inflammation in the NL population.

The current status of genetic association studies regarding visfatin is questionable at best. Although Zhang et al report a significant association with *NAMPT* variants between T2D cases and healthy controls, after adjustment for the number of SNPs investigated by permutation testing, the association no longer remained significant (184). A number of other studies have also found no significant differences in allele, genotype, or haplotype frequencies between T2D patients and healthy controls (186, 188, 191). Interestingly, the study by Bottcher et al did observe a marginally significant association between variation in *NAMPT* and fasting plasma insulin levels as well as 2-h plasma glucose in 626 Caucasian subjects without T2D in quantitative trait analyses (186). This was in contrast to the study by Zhang et al who did not find any association with fasting glucose or insulin levels in 630 healthy individuals (184). In our much larger cohort, we

did not observe any significant associations with any variant in *NAMPT* and parameters of glucose metabolism, including fasting glucose, insulin, HOMA-IR and HOMAβ supporting the notion that genetic variation in the visfatin gene has little, if any, affect on modulating insulin sensitivity.

We also sought to investigate the influence of variants within *NAMPT* on additional metabolic traits, namely obesity and serum lipids. A rare SNP (rs1047818; MAF < 0.01) was recently identified that conferred a protective effect against obesity in both children and adults (190). Interestingly, this SNP was not associated with BMI or waist-to-hip ratio in 5212 healthy controls from this same study. In our cohort, we also failed to identify any significant associations between BMI nor waist-to-hip ratio and variation in *NAMPT*. Furthermore, we also examined the influence of *NAMPT* variants on more accurate markers of obesity, specifically %BF and %TF measured using DXA, and failed to detect any significant associations. In addition, we did not find any significant relationship with any parameter of lipid metabolism. Taken together, our results suggest that genetic variation in the visfatin gene does not influence metabolic health in the NL population.

Recent studies have indicated that visfatin may also be involved in low grade inflammation, as evidenced by associations with circulating markers of inflammation, including monocyte chemoattractant protein 1 and interleukin-6 (194). Recently, Zhang et al found that a SNP in the promoter region of the visfatin gene (-948G>T) was significantly associated with higher plasma levels of fibrinogen and C-reactive protein in a group of 630 non-diabetic individuals (184). In addition, carriers of the minor allele of

-1535C>T had lower hsCRP and interleukin-6 levels in a group of patients presenting with either stable or unstable angina pectoris (195). Contrary to these findings, we found no association between the 10 SNPs genotyped in our cohort and serum hsCRP levels. Interestingly, Wang et al. found no association between -1535C>T and circulating levels of hsCRP, IL-6, and tumor necrosis factor α in patients with acute myocardial infarction (195). At present, it appears that the role of genetic variation within the visfatin gene on regulating circulating factors related to systemic inflammation is unclear. Further work is required to address this issue.

In summary, no significant association was observed between 10 SNPs in *NAMPT* and parameters of body composition, markers of insulin resistance, serum lipids, and systemic inflammation, after accounting for gender and age. Consequently, the results of this study do not support a significant role for genetic variations in the visfatin gene with the above mentioned variables in the NL population.

3.2

Association of *RBP4* gene variants and serum HDL cholesterol levels in the Newfoundland population

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Introduction

RBP4 is a novel adipokine that contributes to systemic insulin resistance in mice (90). Specifically, serum RBP4 levels are elevated in adipocyte-specific glucose transporter 4 knockout mice and are normalized by an insulin sensitizing drug (90). In humans, the role of RBP4 as a mediator of insulin resistance is less clear. Although initial studies demonstrated strong associations between serum RBP4 and insulin resistant states such as impaired glucose intolerance, T2D, and obesity (90-91), others have failed to confirm these findings (93, 196-197).

RBP4 maps to chromosome 10q23-q24, a region that has been linked to elevated fasting blood glucose in European Caucasians (198) as well as an increased risk of T2D in Mexican Americans (199). Recent studies have shown that noncoding SNPs in *RBP4* may increase diabetes susceptibility in Caucasians (200-201), Mongolians (202), and Chinese (203-204), however this finding could not be replicated in an African American population (200). When insulin resistance has been analyzed as a quantitative trait in healthy subjects, results have been more conflicting. Although significant associations have been reported between variants in *RBP4* and insulin levels (201) as well as insulin sensitivity (200), others have found no association with insulin resistance (203). Sample sizes were quite small in these studies and were therefore likely under-powered to detect a true association. Additionally, Kovacs et al failed to correct for multiple testing, therefore their results should be interpreted with caution (201). At the current time,

critical data is lacking regarding the influence of genetic variants in *RBP4* on insulin resistance in a large, healthy population.

Aside from its possible involvement in the development of insulin resistance, growing evidence suggests that RBP4 may also play a role in lipid metabolism. Significant associations have been reported between RBP4 and serum lipids including TG levels, HDL cholesterol and LDL cholesterol (91, 197, 205). In a previous study in our lab, serum RBP4 was positively correlated with LDL cholesterol levels in a group of young men (196). Wu et al. recently reported a significant association between a noncoding SNP in the 5' flanking region of *RBP4* and lower risk of hypertriglyceridemia however, no significant association was evident with any other parameter of lipid metabolism (206). In the current study, we sought to clarify the relationship between genetic variation in *RBP4* with insulin resistance and serum lipids. We assessed the effect of five SNPs within the *RBP4* gene on clinical parameters related to glucose and lipid metabolism in the NL population.

Methods and Procedures

Subjects

A total of 1836 subjects (414 men, 1422 women) were recruited from an ongoing nutrigenomics study (CODING Study; 134, 157) to participate in the current study. All subjects were from the genetically homogeneous population of NL, Canada. Inclusion

criteria were as follows: 1. at least third generation Newfoundlander; 2. healthy, without any serious metabolic, cardiovascular or endocrine disease; 3. not pregnant at the time of study. All subjects provided written and informed consent, and the Human Investigation Committee of the Faculty of Medicine, Memorial University of Newfoundland approved the study.

Serum Measurements

Blood samples were taken from all subjects in the morning following a 12-hour fasting period. Serum was isolated and stored at -80 °C for subsequent analyses. Serum concentrations of glucose, TG, total cholesterol, and HDL cholesterol were measured using Synchron reagents and performed on an Lx20 analyzer (Beckman Coulter Inc., CA, USA). LDL cholesterol was calculated using the following formula: (Chol) – (HDL) – (TG/2.2) which is reliable in the absence of severe hyperlipidemia. Insulin levels were measured on an Immulite immunoassay analyzer (DPC, CA, USA). HOMA-IR was used as a measure of insulin resistance (HOMA-IR=insulin (μU/ml) × glucose (mmol/L)/22.5)) and β-cell function (HOMAβ=20 × insulin (μU/ml) / (glucose-3.5)) (160).

Genomic DNA Isolation, Genotyping and Selection of SNPs

Genomic DNA was isolated from approximately 5ml of whole blood using the Wizard Genomic DNA Purification kit (Promega, WI, USA) according to the manufacturer's protocol. Genotyping was performed using Taqman validated or functionally tested SNP Genotyping Assays (Applied Biosystems, CA, USA) according to the manufacturer's protocol on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). To assess the reproducibility of genotyping, 5% of samples were randomly selected and re-genotyped; all genotypes matched their initial called genotype.

The five SNPs investigated in *RBP4* were rs3758539 (G/A: 5' flanking region), rs61461737 (A/G: intron), rs10882280 (C/A: intron), rs11187545 (A/G: intron), and rs12265684 (C/G: intron). Tagging SNPs were selected using a pairwise r^2 approach with an $r^2 \geq 0.9$ and MAF ≥ 0.05 (SNPbrowser Version 3.5; based on HapMap data for CEU population). This yielded three tagging SNPs that capture all common variants in the *RBP4* gene (rs11187545, rs10882280, and rs17484721). Of these three SNPs, one SNP assay did not work (rs17484721) therefore only two tagging SNPs were genotyped in the entire cohort. An additional four SNPs (rs3758539, rs12265684, rs61461737 and +559 G>A) were genotyped based on recent publications and to increase coverage of the gene. The MAF of +559 G>A (missense mutation) was too low to be informative therefore this SNP was not included in further analyses.

Statistical Analyses

All statistical analyses were performed using the statistical software R or SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Hardy-Weinberg equilibrium was tested using X^2 analysis with one degree of freedom. Multiple regression analyses using an allele dosage or additive model (ie. having two copies of the minor allele doubles the protective effect compared to having just one copy) were used to assess the association between *RBP4* variants and markers of insulin resistance and serum lipids with sex and age included as covariates. Prior to performing any statistical analysis, TG, insulin, HOMA-IR and HOMAβ were log-transformed to reach a normal distribution. Hypotheses regarding the effect of *RBP4* variants on parameters measured were two-sided and significance after correcting for multiple testing was assessed through 10,000 permutations; a corrected p-value of 0.05 was taken as the threshold of statistical significance.

Results

Physical and biochemical characteristics of all subjects are shown in Table 3.5. A summary of the five SNPs genotyped in the entire cohort, including allele frequencies, can be found in Table 3.6. The five SNPs included in final analyses in this study cover approximately 7.8 kb of the *RBP4* gene and includes the 5' flanking region. The observed MAF for all SNPs was ≥ 0.05 (aside from +559 G>A which was excluded from analysis) and all SNPs were in Hardy-Weinberg equilibrium (Table 3.6). Linkage

Table 3.5 Physical and biochemical characteristics of subjects[1].

Variables	Men N = 414	Women N = 1422	Overall N = 1836
Age (yrs)	41.2 ± 14.0	44.5 ± 11.1	43.8 ± 8.9
Height (cm)	175.5 ± 6.4	162.1 ± 5.9	165.1 ± 8.2
Weight (kg)	84.9 ± 14.2	69.8 ± 14.3	73.2 ± 15.6
BMI (kg/m^2)	27.6 ± 4.5	26.6 ± 5.3	26.8 ± 5.1
Waist Circumference (cm)	97.9 ± 12.0	91.0 ± 14.4	95.2 ± 14.2
Body fat (%)	25.4 ± 7.5	37.7 ± 7.5	35.0 ± 9.1
Glucose (mmol/L)	5.3 ± 1.0	5.1 ± 0.9	5.1 ± 0.9
Insulin (pmol/L)	77.8 ± 58.0	70.1 ± 68.3	71.8 ± 66.2
HOMA-IR	2.78 ± 3.60	2.37 ± 2.99	2.46 ± 3.13
HOMAβ	138.8 ± 141.5	144.3 ± 194.2	143.1 ± 183.9
Cholesterol (mmol/L)	5.06 ± 1.11	5.21 ± 1.03	5.17 ± 1.05
HDL cholesterol (mmol/L)	1.24 ± 0.27	1.56 ± 0.38	1.49 ± 0.39
LDL cholesterol (mmol/L)	3.12 ± 0.95	3.12 ± 0.89	3.12 ± 0.90
Triacylglycerol (mmol/L)	1.50 ± 0.99	1.15 ± 0.71	1.23 ± 0.79
Cholesterol/HDL	4.23 ± 1.16	3.48 ± 0.94	3.64 ± 1.04

[1]Values are expressed as mean ± standard deviation. BMI, body mass index; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; HOMAβ homeostasis model assessment for β cell function.

Table 3.6 Summary of single nucleotide polymorphisms, allele frequencies, and Hardy-Weinberg equilibrium[1].

Position	RS Number	Variant	Location	Frequency	HWE (*p*-value)
-803	rs3758539	G/A	5' Flanking	0.84 / 0.16	0.263
+406	n/a[2]	T/C	Intron 3	0.91 / 0.09	0.936
+1473	rs10882280[3]	T/G	Intron 4	0.92 / 0.08	0.651
+3681	rs11187545	T/C	Intron 4	0.91 / 0.09	0.775
+6969	rs12265684[3]	G/C	Intron 4	0.83 / 0.17	0.652

[1]Hardy-Weingberg was estimated using using X^2 analysis.

[2]n/a, not applicable

[3]Tagging SNPs (SNPbrowser Version 3.5, based on HapMap CEU population)

LD was also estimated among the variants and is shown in Table 3.7.

The genotype effect of the five SNPs within *RBP4* on markers of insulin resistance and serum lipids are shown in Table 3.8. We tested for associations between the five SNPs within *RBP4* and parameters of glucose and lipid metabolism using multiple regression analyses with gender and age as model covariates. The *p* values for the studied SNPs for glucose, insulin, HOMA-IR, HOMAβ, cholesterol, HDL cholesterol, LDL cholesterol, TG, and risk factor, after adjusting for gender and age, are presented in Table 3.8. We did not find any significant associations between variation in *RBP4* with markers of insulin resistance (fasting glucose, insulin, HOMA-IR or HOMAβ). Analyses were repeated after excluding volunteers with self-reported T2D or fasting glucose $\geq$ 7.0 mmol/L (n = 72) and again, no SNP was significantly associated with any marker of insulin resistance (data not shown).

Two noncoding SNPs (rs10882280 and rs11187545) were significantly associated with serum HDL choelsterol (p = 0.043 and 0.042, respectively) in our cohort (Table 3.8). Carriers of the minor allele of rs10882280 and rs11187545 had significantly higher fasting levels of HDL cholesterol compared to homozygotes for the major allele (Table 3.9). Although serum HDL cholesterol was even greater in homozygotes for the minor allele, this did not reach statistical significance, likely due to the small number of subjects with this genotype. A suggestive association was also detected between rs61461737 and

Table 3.7 Estimated pairwise linkage disequilibrium (right upper) and sample size (left lower).

N/D'	rs3758539	rs61461737	rs10882280	rs11187545	rs12265684
rs3758539		0.819	0.846	0.693	0.930
rs12265684	1836		0.940	0.965	0.768
rs61461737	1836	1835		0.954	0.778
rs11187545	1836	1835	1836		0.630
rs10882280	1836	1835	1835	1835	

Table 3.8 Genotype effect of five SNPs within *RBP4* on markers of insulin resistance and serum lipids.[1,2]

Variables	rs3758539	rs61461737	rs10882280	rs11187545	rs12265684
			p values[3]		
Glucose (mmol/L)	0.839	0.831	0.851	1.000	1.000
Insulin (pmol/L)	1.000	1.000	0.915	0.901	1.000
HOMA-IR	0.907	1.000	0.957	0.957	1.000
HOMAβ	1.000	1.000	0.777	1.000	0.606
Cholesterol (mmol/L)	0.237	1.000	0.449	1.000	0.383
HDL (mmol/L)	1.000	1.000	**0.043**[4]	**0.042**[4]	1.000
LDL (mmol/L)	0.210	0.771	0.815	0.746	0.298
Triacylglycerol (mmol/L)	1.000	0.107	0.417	0.379	1.000
Cholesterol/HDL	1.000	1.000	0.861	0.802	1.000

[1]HOMA-IR, homeostasis model assessment for insulin resistance; HOMAβ homeostasis model assessment for β cell function; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol.

[2]Multiple regression using an allele dosage or additive model were used for quantitative trait analyses to assess the association between *RBP4* variants and markers of insulin resistance and serum lipids. Sex and age were included as model covariates.

[3]*p* values based on 10,000 permutations.

[4]Using a dominant model, p values were 0.044 and 0.70 for rs10882280 and rs11187545, respectively.

Table 3.9 Serum HDL cholesterol levels according to *RBP4* genotype.[1]

Genotype	n	HDL Cholesterol (mmol/L)
rs10882280		
CC	1535	1.48 ± 0.37
CA	290	1.55 ± 0.39[2]
AA	11	1.55 ± 0.47
rs11187545		
AA	1520	1.48 ± 0.37
AG	303	1.54 ± 0.39[2]
GG	13	1.60 ± 0.45

[1]Serum HDL cholesterol is presented as mean ± SD. Differences in serum HDL between genotypes determined using ANOVA analyses, adjusted for age and gender.

[2]Significantly higher compared to homozygotes for the major allele ($p < 0.05$).

serum TGs ($p = 0.032$; corrected $p = 0.107$) (Table 3.8). We did not observe any significant associations with any other parameter related to lipid metabolism.

As we found a significant association between variants within *RBP4* and serum HDL cholesterol, we sought to further explore this relationship using a case-control study design. Using a minimum-Chi-squared method to determine cut-off values for cases (HDL < 1.23 mmol/L, n = 476) and controls (HDL > 1.56 mmol/L, n = 671), we found a significant difference in genotype frequencies between cases and controls for rs10882280 ($p = 0.009$) and rs11187545, ($p = 0.014$), as would be expected given our previous findings. We also found a significant difference in genotype frequencies for rs61461737 ($p = 0.029$).

Power calculations were carried out for a quantitative trait genetic association study and are shown in Figure 3.1. The causal locus was reflected indirectly by rs11187545 and type 1 error was fixed to 10^{-6}. Figure 3.1 shows the power profiles as a function of varying coefficients of determination for a range of heritability estimates ($h^2 = 0.05 - 0.15$). As demonstrated by this figure, we had sufficient power ($\beta \geq 0.80$) at $R^2 > 0.4$, given our sample size, to detect a positive association.

Discussion

RBP4 has come under scrutiny for its possible involvement in insulin resistance and more recently, lipid metabolism. Previously known solely as a transporter of retinol

(vitamin A), RBP4 was identified as a marker of insulin resistance in both mice and humans (90-91). Further studies have suggested that RBP4 may play a more important role in lipid metabolism, as evidenced by numerous associations between RBP4 levels and serum lipids (197, 205). In the present study, we investigated the relationship between variation in the *RBP4* gene with parameters of glucose and lipid metabolism in a large NL population. We found a significant association between two noncoding SNPs (rs10882280 and rs11187545) and serum HDL cholesterol however no association was evident with any other parameter of lipid metabolism. Similarly, we did not observe any significant associations between any variant sites and markers of insulin resistance.

The current status of genetic association studies on *RBP4* has indicated that -803G/A (rs3758539) influences transcription efficiency in a hepatocarcinoma cell line as well as binding efficiency of hepatocyte nuclear factor 1 alpha to its motif (202). The A-allele of this SNP has also been associated with an increased risk of T2D in Mongolians (202) as well as Caucasians (207). We failed to confirm an association between this variant, or any other variant, and insulin resistance in the present study. Although the reason for this discrepancy is unclear, it may be due to smaller sample sizes in previous studies resulting in spurious associations. Differences in effect size of the risk allele and/or exposure to environmental factors may also play a role. Our results are consistent with two recent studies in Caucasians where no significant association was evident between -803 G/A and diabetes risk (200-201). Although both of these studies demonstrated an association between a haplotype containing this SNP and increased diabetes risk, both risk haplotypes contained the -803 G allele, not the A allele as would

be expected if this allele does confer diabetes risk. Interestingly, SNPs within *RBP4* are in a tight LD block that continues until the 3' end of the downstream gene, *GPR120* (202), a receptor for unsaturated long-chain fatty acids and another candidate gene for T2D. It is possible that the risk haplotypes identified in previous studies are a result of a signal from this gene.

Aside from investigating the effect of genetic variants in RBP4 on insulin resistance, we were also interested in examining their effect on parameters of lipid metabolism. Perhaps, the most interesting finding in our study was that noncoding SNPs within RBP4 may offer a protective effect against low serum levels of HDL cholesterol. To the best of our knowledge, this is the first study to demonstrate that genetic variation in RBP4 is associated with serum HDL cholesterol. Specifically, we found that individuals carrying the minor allele of rs10882280 or rs11187545 had higher levels of serum HDL cholesterol in quantitative trait analyses. This was also true when a case-control design was used. For these SNPs, it appears that harboring one or two copies of the minor allele offers a protective effect against low levels of HDL cholesterol. It should be noted that mean HDL cholesterol levels in homozygotes for the minor allele were not significantly different from levels in heterozygotes for both polymorphisms, likely due to the small number of homozygotes for the minor allele. Our findings, therefore, should be replicated in another population with a greater sample size to confirm the results. Interestingly, recent evidence has suggested that RBP4 is a marker of metabolic abnormalities, as serum levels of this adipokine correlate with a number of components of the metabolic syndrome (205, 208), including HDL cholesterol (91). Our

findings support a role for RBP4 in the differences in serum HDL cholesterol, however, the exact mechanism through which these variants result in higher serum HDL cholesterol remains to be elucidated.

Recently, a SNP in the promoter region of *RBP4* (rs3758538, C allele) was significantly associated with both reduced hypertriglyceridemia risk and lower TG levels in Chinese Hans (206). Although we did not type this variant in our cohort, we did observe an association between the minor allele of rs64161737 and lower TG levels. After accounting for the number of tests performed, however, the association no longer remained significant. Although this appears contradictory to recent reports indicating significant correlations between serum RBP4 and TG levels (205, 209-210), our findings are in agreement with previous studies that found no association between variants in *RBP4* and serum TG (202-203; 207). Further studies are required to clarify the role of variants within *RBP4* and lipid metabolism.

In summary, we have identified two noncoding SNPs (rs10882280 and rs11187545) that are significantly associated with higher levels of serum HDL cholesterol in the NL population after controlling for age and gender. We did not find any significant associations between genetic variation in *RBP4* and markers of insulin resistance. Due to high LD between the variants typed in this study, we believe we have likely captured the majority of genetic variation in and around this gene. Consequently, the results of our study support a role for genetic variations in *RBP4* with differences in serum HDL cholesterol, but not with insulin resistance, in the NL population.

4

Examining the genetic and endocrine determinates of obesity through an intervention approach: Response to a positive energy balance

4.1

Serum retinol-binding protein 4 concentrations in response to short term overfeeding in normal weight, overweight and obese men

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Introduction

It has been well documented that an increase in adipose tissue is linked to both insulin resistance and T2D (211). One of the major causes of these two conditions is impaired insulin action in adipose tissue, skeletal muscle and liver. In fact, adipose tissue secretes many adipokines that influence insulin action in other tissues including leptin, adiponectin and tumor necrosis factor α (212-214). It has recently been discovered that RBP4, previously known solely as a transporter of retinol (vitamin A), is also secreted from adipose tissue where it induces insulin resistance in the liver and skeletal muscle of mice (90). Following this finding, a small number of studies have demonstrated a correlation between RBP4 and insulin resistant states in humans (90-92, 215-216). However, the role that adiposity status plays in this is controversial. Although some studies have observed higher concentrations of RBP4 in obese subjects (90-92) others have found no association between RBP4 and adiposity status (93). Yet others have found significant correlations between elevated RBP4 and specific fat depots, including liver fat (215) and trunk fat percentage (216).

With limited data available regarding this adipokine, the mechanism through which RBP4 acts in the development of insulin resistance and T2D in humans is still unclear. There is also no information available regarding the nutritional regulation of RBP4. Studies from our lab and others have shown that changes in nutritional status such as overfeeding can have major impacts on adipose tissue metabolism (217) as well as influence circulating concentrations of adipokines (179) and consequently, may

influence RBP4. The subsequent responses to changes in nutritional status can provide insight regarding the role of RBP4 in the development of insulin resistance and T2D. Furthermore, past studies have shown that the response of leptin to a high fat meal differs between lean and obese men (218) and it is therefore possible that the response of RBP4 to short term overfeeding may also be dependent on adiposity status. The objectives of this study were to further understand the role RBP4 plays in the development of insulin resistance by investigating 1. the correlations of RBP4 with phenotypes of insulin resistance, glucose and lipid metabolism and other adipokines (interleukin-6 and visfatin); 2. the response of RBP4 to short-term overfeeding in young men without diabetes; and 3. the role of adiposity status on the effects of RBP4.

Methods

Subjects

Subjects were recruited from an ongoing overfeeding study investigating the effects of a positive energy balance on endocrine factors as well as glucose and lipid metabolism (179). A total of sixty-five young males were recruited from the city of St. John's and surrounding area in the Canadian province of NL to participate in this study. Younger males are often targeted in overfeeding studies because they tend to tolerate overfeeding better than older subjects. Inclusion criteria were: 1. male; 2. 19-29 years of age; 3. at least third generation Newfoundlander; 4. healthy, without any serious metabolic, cardiovascular, or endocrine disease; 5. not on medication for lipid

metabolism; 6. reported having a stable weight (± 2.5 kg) in the previous six months. This study was approved by the Ethical Committee of the Faculty of Medicine, Memorial University, St. John's, NL and all subjects provided written consent.

Serum Measurements

Blood samples were taken from all subjects before and after completion of the overfeeding study, following a 12-hour fasting period. Serum was stored at -80 °C for subsequent analyses. Serum RBP4 concentrations were measured in duplicate by radioimmunoassay kits purchased from Phoenix Pharmaceuticals, Inc. (Belmont, CA, USA). Serum insulin levels were measured on an Immulite immunoassay analyzer (DPC, CA, USA). The homeostasis model assessment (HOMA) was used as a measure of insulin resistance (HOMA-IR=insulin (μU/ml) × glucose (mmol/L)/22.5)) and β-cell function (HOMAβ=20*insulin (μU/ml) / (glucose-3.5)) (160). Serum concentrations of glucose, TG, total cholesterol, and HDL cholesterol were measured using Synchron reagents and performed on an Lx20 analyzer (Beckman Coulter Inc., CA, USA). LDL cholesterol (LDL-c) was calculated using the following formula: (Cholesterol) – (HDL cholesterol) – (TG/2.2) which is reliable in the absence of severe hyperlipidemia. Serum interleukin-6 levels (IL-6) were measured in duplicate using the Access® IL-6 kits (Beckman Coulter Inc., CA, USA) performed on a Unicel DxI 800 Access Immunoassay system (Beckman Coulter Inc., CA, USA). Serum visfatin concentrations were measured in duplicate with a human visfatin (COOH-terminal) enzyme immunometric assay

(Phoenix Pharmaceuticals, Belmont, CA) performed on an Alisei Quality System (SEAC Radim Group, Pomezia, Italy).

Measurement of body composition

%BF was determined using DXA Lunar Prodigy (GE Medical Systems, Madison, WI, USA). Measurements were performed on subjects following the removal of all metal accessories, while lying in a supine position as previously described (136). Software version 4.0 was used for analysis. All measurements were completed prior to and one day following overfeeding.

Overfeeding protocol

Although both short and long term overfeeding strategies have been used to investigate biochemical and metabolic responses to a hypercaloric diet (60, 219-220), the majority of studies have been short term, ranging from 12 hours to 22 days. A 7 day overfeeding protocol was chosen for this study to ensure that the intervention would induce metabolic changes. Subjects consumed 70% more calories than their normal energy requirements and this consisted of 15% protein, 35% fat, and 50% carbohydrates to mimic the common daily diet in North America.

Full details of the overfeeding protocol have been previously described by us (179). Briefly, subjects consumed 70% more calories than their normal energy requirements and this consisted of 15% protein, 35% fat, and 50% carbohydrates to

mimic the common daily diet in North America. Baseline energy intake assessments were completed for each subject prior to commencing the overfeeding protocol. Baseline energy requirements were estimated for each subject using three 24-h food recalls and completion of a 30-d dietary inventory. Subjects were then started on a 40% hypercaloric diet for 7 days. Subjects were offered three meals per day and energy values and macronutrient content of the food was measured using the Food Processor SQL, version 9.5.0.0 (ESHA Research, Salem, Oregon). Subjects were asked to maintain their usual pattern of physical activity. Total energy expenditure was estimated by an Actical physical activity level monitor (Mini Mitter Co, Inc, Bend, OR) for 7 days prior to starting the study and during the overfeeding period. Any differences in physical activity levels between baseline and the overfeeding period were controlled below 15%. The average baseline calorie intake and calorie intake during overfeeding was 2969 kcal and 5471 kcal, respectively. On average, subjects gained 2.2±0.18 kg body weight of which 28% (0.615kg±0.131) was body fat.

Statistical Analysis

Data are presented as mean ± SD. Prior to performing any statistical analyses, subjects were grouped according to adiposity status. Subjects were classified using BMI as normal weight (<24.9 $kg \cdot m^{-2}$) or overweight/obese (>25.0 $kg \cdot m^{-2}$) (143). Overweight and obese subjects were grouped together due to the small number of subjects in each group. Statistical analyses were also performed according to %BF according to criteria recommended by Bray (137). Differences in RBP4 levels between the two groups as

well as changes in RBP4 in response to overfeeding were analyzed using two-factor ANOVA with interaction analysis using SAS PROC GLM. Pearson correlation analyses were performed to screen for potential factors related to fasting RBP4 levels followed by partial correlation analyses controlling for age and BMI. Parameters of the metabolic syndrome, including HDL cholesterol, fasting TG and glucose, decline with age (221) and we therefore controlled for age within each group. Bonferroni testing was applied to correct for multiple comparisons. The correlation analyses were performed at the following three levels:

1. RBP4 at baseline vs. all variables at baseline
2. RBP4 at baseline vs. changes in all variables in response to overfeeding to investigate whether baseline RBP4 could predict the changes in related markers
3. Change in RBP4 vs. changes in all variables in response to overfeeding.

SPSS version 14.0 (SPSS Inc., Chicago, IL) was used for all analyses unless otherwise stated. Statistical analyses were two-sided and a p value < 0.05 was considered to be statistically significant.

Results

Comparison of characteristics at baseline and in response to short term overfeeding

Physical and biochemical characteristics of subjects at baseline are shown in Table 4.1. There were no significant differences in age and height between the two groups, however the differences in BMI, body weight and %BF were significant. There was no significant difference in fasting glucose between both groups. Overweight/obese subjects had higher fasting serum insulin levels as well as increased HOMA-IR and elevated HOMAβ compared to normal weight subjects. Although all lipid profile markers (cholesterol, LDL cholesterol, and TG) were higher in overweight/obese subjects compared to lean subjects, this did not reach statistical significance. HDL cholesterol was significantly lower in overweight/obese individuals compared to lean. There were no significant differences in fasting IL-6 or visfatin concentrations between groups. The average fasting serum RBP4 concentrations at baseline were 29.53±6.02 and 29.14±5.30 μg/ml for the normal weight and overweight/obese subjects respectively, with no significant differences between the groups. RBP4 concentrations were also analyzed according to %BF criteria, and again, no significant differences were found (data not shown). As well, subjects were divided into three groups according to HOMA-IR, controlling for BMI. There were also no significant differences in fasting serum RBP4 among the low, medium or high HOMA-IR groups (data not shown).

Changes in body composition as well as phenotypes of glucose metabolism and lipids in response to the 7-day overfeeding period are described in Table 4.1. Briefly, there was a significant increase in body weight in both groups following overfeeding. A significant increase in %BF was evident in both the normal weight and overweight/obese groups as well. Total cholesterol, HDL-c and TG were significantly increased in both normal weight and overweight/obese subjects. Insulin levels and HOMAβ were also significantly increased. There were no significant differences in fasting serum RBP4 in response to overfeeding within each group.

Correlations of RBP4 with phenotypes of glucose and lipid metabolism

Pearson correlation analysis was used as an initial screening tool between RBP4 and phenotypes of glucose and lipid metabolism followed by partial correlation analyses, controlling for BMI and age. At baseline, RBP4 was positively correlated with baseline LDL cholesterol in normal weight subjects and with baseline HOMAβ in overweight/obese subjects. When all subjects were combined, only the positive correlation with LDL cholesterol was evident. However, after multiple comparison testing was applied, no significant results remained (Table 4.2). Correlations between baseline RBP4 and the changes in parameters were also assessed. Significant negative correlations were evident between baseline RBP4 and changes in both insulin and HOMA-IR in normal weight subjects (Table 4.3). The significant correlation between RBP4 and the change in HOMA-IR remained significant even after Bonferroni

Table 4.1 Physical and biochemical characteristics of subjects at baseline and in response to 7 days of overfeeding[1]

	Lean (n=37-40)		Overweight/Obese (n=24-28)		ANOVA for repeated measurements[3]	
Variables	Pre-overfeeding	Post-overfeeding	Pre-overfeeding	Post-overfeeding	Change from baseline (p value)	Group comparison (p value)
Age (yrs)	23.2±3.6	n/a	22.0±0.5	n/a	n/a	n/a
Height (cm)	180.3±6.6	n/a	181.1±1.3	n/a	n/a	n/a
Weight (kg)[2,4]	72.58±8.85	75.00±8.83	92.26±15.51	94.37±15.83	<0.001	<0.001
Body fat (%)[2,4]	17.95±6.64	18.51±6.36	28.80±7.64	28.45±7.16	0.09	<0.001
BMI ($kg \cdot m^{-2}$)[4]	22.31±2.16	23.09±2.16	29.18±4.54	29.88±4.77	<0.001	<0.001
Glucose (mmol/L)	5.01±0.39	4.96±0.44	5.22±0.67	5.12±0.45	0.67	0.12
Insulin (pmol/L)[2]	55.07±46.60	71.35±55.68	93.72±93.09	111.22±71.62	0.04	0.02
Cholesterol (mmol/L)	4.47±0.91	4.64±0.97	4.65±0.79	4.80±0.57	0.031	0.52
Triacylglycerol (mmol/L)	0.99±0.45	1.16±0.78	1.20±0.66	1.82±2.10	0.031	0.08
HDL-c (mmol/L)[2]	1.40±0.29	1.47±0.28	1.22±0.21	1.36±0.24	<0.001	0.04
LDL-c (mmol/L)	2.62±0.74	2.73±0.73	2.89±0.67	2.61±1.16	0.47	0.94

Variables	Lean (n=37-40)		Overweight/Obese (n=24-28)		ANOVA for repeated measurements[3]	
	Pre-overfeeding	Post-overfeeding	Pre-overfeeding	Post-overfeeding	Change from baseline (*p* value)	Group comparison (*p* value)
HOMA-IR[2]	1.76±1.71	2.22±1.85	3.26±3.75	3.60±2.51	0.18	0.02
HOMAβ[2]	97.86±55.39	143.36±103.12	142.52±90.07	193.54±104.68	<0.001	0.03
IL-6 (pg/ml)	1.62±3.86	1.29±3.48	1.32±1.24	1.32±1.77	0.26	0.83
Visfatin (ng/ml)[4]	31.01±20.98	28.33±23.25	27.85±19.62	18.15±19.44	<0.001	0.21
RBP4 (μg/ml)	29.53±6.02	30.20±6.89	29.14±5.30	31.57±7.18	0.10	0.71

[1] All values are mean ± SD. RBP4, retinol-binding protein 4; IL-6, interleukin 6; HOMA-IR, homeostasis model assessment for insulin resistance; HOMAβ, homeostasis model assessment for β cell function; n/a, not available.

[2] Significant differences between lean and overweight/obese subjects at baseline were analyzed using Student's *t* test. Weight, %BF, BMI, HDL-c, insulin, HOMA-IR, and HOMA-β were significantly different between groups ($p < 0.05$).

[3] Adiposity status and responses to overfeeding were analyzed using two-factor ANOVA with interaction analysis using SAS PROC GLM.

[4] Significant between-subject interactions ($p < 0.05$).

Table 4.2 Partial correlations of baseline variables related to baseline fasting serum RBP4 (μg/ml), controlling for BMI and age.[1]

Variables	Normal weight (n = 37-40)		Overweight/Obese (n =24-28)		All Subjects (n = 65)	
	r	*p*	*r*	*p*	*r*	*P*
Glucose (mmol/L)	0.108	ns	-0.018	ns	0.021	ns
Insulin (pmol/L)	-0.139	ns	0.314	ns	0.036	ns
Cholesterol (mmol/L)	0.365	ns	0.154	ns	0.278	ns
Triacylglycerol (mmol/L)	0.286	ns	0.251	ns	0.237	ns
HDL-c (mmol/L)	-0.131	ns	-0.139	ns	-0.128	ns
LDL-c (mmol/L)	0.380	0.042[2]	0.093	ns	0.290	0.035[2]
HOMA-IR	-0.114	ns	0.275	ns	0.036	ns
HOMAβ	-0.210	ns	0.424	0.049[2]	0.041	ns
IL-6 (pg/ml)	-0.093	ns	-0.098	ns	-0.076	ns
Visfatin (ng/ml)	0.028	ns	0.221	ns	0.065	ns

[1]RBP4, retinol-binding protein 4; IL-6, interleukin 6; HOMA-IR, homeostasis model assessment for insulin resistance; HOMAβ, homeostasis model assessment for β cell function. Partial correlation analysis controlling for BMI and age was used to screen for potential factors related to fasting RBP4.

[2]Not significant after Bonferroni correction.

Table 4.3 Partial correlations of changes in variables related to baseline fasting serum RBP4 (μg/ml), controlling for BMI and age.[1]

Variables	Normal weight (n = 37-40)		Overweight/Obese (n = 24-28)		All subjects (n = 65)	
	r	*p*	*r*	*p*	*r*	*P*
Glucose (mmol/L)	-0.368	ns	-0.101	ns	-0.202	ns
Insulin (pmol/L)	-0.467	0.014[2]	-0.084	ns	-0.078	ns
Cholesterol (mmol/L)	-0.067	ns	0.002	ns	-0.038	ns
Triacylglycerol (mmol/L)	-0.115	ns	0.410	ns	0.130	ns
HDL-c (mmol/L)	0.067	ns	0.048	ns	0.059	ns
LDL-c (mmol/L)	-0.056	ns	-0.122	ns	-0.081	ns
HOMA-IR	**-0.614**	**0.001**[3]	-0.094	ns	-0.083	ns
HOMAβ	0.050	ns	-0.061	ns	-0.019	ns
IL-6 (pg/ml)	0.265	ns	0.293	ns	-0.076	ns
Visfatin (ng/ml)	-0.089	ns	0.030	ns	-0.043	ns

[1]RBP4, retinol-binding protein 4; IL-6, interleukin 6; HOMA-IR, homeostasis model assessment for insulin resistance; HOMAβ, homeostasis model assessment for β cell function. Partial correlation analysis controlling for BMI and age was used to screen for potential factors related to fasting RBP4.

[2]Not significant after Bonferroni correction.

[3]$p = 0.01$ after Bonferroni correction.

correction. Lastly, we investigated correlations between the changes in RBP4 and the changes in parameters measured. Although a number of significant correlations were detected between the change in RBP4 and the changes in insulin and HOMA-IR in both normal weight and overweight/obese subjects none of these survived Bonferroni correction in any of the groups or in the entire study cohort (Table 4.4).

Discussion

T2D is one of the fastest growing diseases in North America as well as some developing countries and is closely associated with both insulin resistance and obesity. Although the discovery of various adipokines has shed light on the etiology of this disease, the molecular link between obesity, insulin resistance, and T2D in humans is still largely unknown. It appears that RBP4 is a factor that acts to induce insulin resistance in the liver and skeletal muscle of rodents (90) however the mechanism through which this adipokine acts and the role that adiposity status plays in humans is still unclear.

Although RBP4 is secreted primarily by the liver (221), studies in rats have shown that adipose tissue has the second highest expression level (222) where it is expressed almost exclusively by adipocytes (223). Janke et al. were able to demonstrate that RBP4 is also highly expressed in mature human adipocytes and is secreted by differentiating human adipocytes (93). If adipose tissue is an important site contributing to circulating RBP4, it is reasonable to expect significant differences in serum RBP4 between normal weight and obese humans due to variations in the amount of adipose

Table 4.4 Partial correlations of changes in variables related to changes in fasting serum RBP4 (μg/ml), controlling for BMI and age.[1]

Variables	Normal weight (n = 37-40)		Overweight/Obese (n = 24-28)		All subjects (n = 65)	
	r	*p*	*r*	*p*	*r*	*P*
Glucose (mmol/L)	0.024	ns	-0.328	ns	-0.123	ns
Insulin (pmol/L)	0.413	0.032[2]	-0.513	0.021[2]	-0.194	ns
Cholesterol (mmol/L)	0.018	ns	0.015	ns	0.012	ns
Triacylglycerol (mmol/L)	0.169	ns	0.077	ns	0.098	ns
HDL-c (mmol/L)	0.000	ns	0.085	ns	0.026	ns
LDL-c (mmol/L)	-0.009	ns	0.081	ns	0.015	ns
HOMA-IR	0.445	0.020[2]	-0.515	0.020[2]	-0.202	ns
HOMAβ	0.113	ns	-0.505	0.023[2]	-0.200	ns
IL-6 (pg/ml)	-0.062	ns	-0.350	ns	-0.136	ns
Visfatin (ng/ml)	-0.040	ns	0.376	ns	0.075	ns

[1]RBP4, retinol-binding protein 4; IL-6, interleukin 6; HOMA-IR, homeostasis model assessment for insulin resistance; HOMAβ, homeostasis model assessment for β cell function. Partial correlation analysis controlling for BMI and age was used to screen for potential factors related to fasting RBP4.

[2]Not significant after Bonferroni correction.

tissue. Graham et al. demonstrated an increase in serum RBP4 in both obese humans and obese humans with T2D compared to lean controls (91). Cho et al. found a significant difference in plasma RBP4 levels between subjects with normal glucose tolerance and those with T2D (92). Conversely, Janke et al. found no significant difference in circulating levels of RBP4 amongst normal weight, overweight, and obese women (93). Stefan et al. found a positive correlation between circulating RBP4 and liver fat but not with total body, visceral or subcutaneous fat (215). In the current study we found no significant difference in fasting serum RBP4 levels between normal weight and overweight/obese men. This was also true when subjects were classified according to %BF. Our data does not support the hypothesis that total body fat, indexed by BMI or %BF, determines circulating RBP4 levels in young, healthy men without diabetes.

We also sought to understand the nutritional regulation of RBP4. To the best of our knowledge, this is the first study of its kind to explore the response of RBP4 to a short-term positive energy challenge. Overfeeding studies provide a means in which the biochemical changes that would be evident with extended overeating in both adipokines and hormones can be investigated. A positive energy balance is one of the major causes of obesity (224) and triggers many hormonal responses including both an increase and/or decrease in the secretion of various adipokines (225-226). In the present study there were no significant differences in RBP4 concentrations before and after overfeeding suggesting that RBP4 is not significantly regulated by a short-term positive energy challenge at physiological conditions in healthy, young men. Interestingly, Janke et al found no significant change in circulating RBP4 in women following a five percent

weight loss (93) indicating that a change in overall energy balance, whether positive or negative, appears to have no significant effect on circulating RBP4 concentrations.

Recent studies have suggested that RBP4 is a key player in the etiology of insulin resistance in both healthy subjects (215-216) and subjects with T2D (90-92). In the current study, however, we did not find any significant associations between RBP4 and phenotypes of insulin resistance at baseline without overfeeding. However, we found that in normal weight men, changes in HOMA-IR were inversely correlated with baseline serum RBP4 suggesting that RBP4 may serve as a predictor of insulin resistance when exposed to a positive energy challenge such as in the development of obesity. To further understand the relationship between RBP4 and insulin resistance, we classified subjects according to HOMA-IR status as either low, medium or high to determine if subjects with a greater degree of insulin resistance had elevated levels of circulating RBP4. We found no significant differences in serum RBP4 between these three groups suggesting that at physiological conditions in healthy, young men, RBP4 is not a significant factor in determining the difference in insulin resistance between normal weight and obese subjects. However, it can't exclude the possibility that such a relationship might be detected if a more sensitive method to measure insulin resistance was used such as euglycemic clamp test as well as a larger sample size.

We also investigated correlations between RBP4 and phenotypes of lipid metabolism. It has been suggested that RBP4 may be linked to the metabolic syndrome. Specifically, elevated serum RBP4 was associated with higher concentrations of serum

TGs and decreased HDL cholesterol (91) as well as waist circumference (92). Although we found a weak positive correlation between baseline RBP4 and LDL cholesterol, it did not survive bonferroni correction. Further studies are warranted to investigate associations between RBP4 and lipid metabolism.

It has been suggested that adipokines may interact with one another in the regulation of energy balance. For example, it has been hypothesized that the stimulatory effects of ghrelin and inhibitory effects of leptin assimilate in the regulation of energy intake and expenditure (227). Previous studies have investigated the relationship between RBP4 and both leptin and adiponectin (92, 213) however no significant correlations were detected in either study. In the present study we investigated the relationship between RBP4 and both IL-6 and visfatin. We did not find any association between RBP4 and these two adipokines.

One limitation of our study is the homogeneous study group. By targeting males 19-29 years of age, we are limiting the population to which these findings can be applied. Further studies are warranted investigating these issues in other age groups as well as in females. Another potential limitation is the short overfeeding period. Although this is the first study of its kind to explore the nutritional regulation of RBP4, future studies examining the effects of prolonged overeating are necessary.

In summary, we measured serum RBP4 in 65 men before and after a 7-day overfeeding protocol. Circulating RBP4 was similar amongst normal weight and overweight/obese young men. Likewise, the changes in RBP4 in response to overfeeding

were not significant within the two adiposity groups. RBP4 was not associated with phenotypes of insulin resistance or lipid metabolism in either normal weight or overweight/obese subjects at baseline. RBP4 was inversely associated with the change in insulin resistance in normal weight men suggesting that it may be a predictor of the changes in insulin resistance in response to a short term positive energy challenge.

4.2

Changes in the transcriptome of abdominal subcutaneous adipose tissue in response to short term overfeeding in lean and obese men

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Introduction

Obesity can be defined as the excessive accumulation of adipose tissue caused by a chronic energy imbalance between energy intake and energy expenditure (228). Obesity rates amongst developed countries have increased substantially in the past three decades and are now affecting billions globally. The consequences of excess body weight are numerous and include type II diabetes, hypertension, coronary artery disease, and many types of cancer (124). Increases in caloric intake combined with decreased physical activity levels and an underlying genetic predisposition, all contribute to the obesity epidemic. Current research indicates that there are many genes that play a role in the development of obesity. As of October 2005, more than 600 genes, markers, and chromosomal regions have been associated with obesity phenotypes (39). Estimates of the heritability of BMI, a marker of obesity, are between 30-70% (35, 229-230). However, the underlying molecular and genetic basis surrounding this phenomenon is still unclear.

Aside from its role in storage, adipose tissue actively communicates with cells, tissues, and the central nervous system through a network of endocrine, paracrine and autocrine signals. The discovery of numerous adipocyte-derived hormones has demonstrated an active role of this tissue in the development of obesity and related metabolic disorders (90, 231-232). With the recent advent of microarrays, researchers have been able to examine the global gene expression profiles of adipose tissue to investigate its role in obesity. Microarray profiling of adipose tissue in numerous

populations has led to the discovery of a number of processes that are now thought to be involved in the pathogenesis of this disease including lipolysis (233), inflammation/immune response (234-235), apoptosis (236-237), adipogenesis (238), as well as extracellular matrix constituents (239). Moreover, the use of microarray technology has led to the discovery of adipokines that have been implicated in the pathogenesis of obesity (90, 240). However, the majority of these studies have been cross-sectional in nature with analysis performed on lean vs. obese individuals or subcutaneous vs. visceral adipose tissue.

Energy homeostasis is a key factor in the regulation of body weight and subsequently obesity, and because of this, studying the effects of changes in energy balance may provide further insight into the underlying genetic and molecular mechanisms responsible for obesity. A number of studies have identified genes modulated by a negative energy balance, induced by exercise (241-243) or caloric restriction (244-245). Surprisingly, critical data are lacking regarding changes in gene expression under conditions of a positive energy balance, which is the fundamental cause of the rising prevalence of human obesity. In the current study, we investigated changes in global gene expression profiles in abdominal subcutaneous adipose tissue in response to a positive energy balance induced by overfeeding. Overfeeding studies provide a means to investigate genetic and biochemical changes as well as individual differences that would be evident with extended overeating. Previous studies have shown that changes in nutritional status such as overfeeding can have major impacts on adipose tissue metabolism (217), gene expression (246), and adipocytokine regulation (179). The

objectives of the current study were as follows: 1) Define the mRNA expression profiles of abdominal subcutaneous adipose tissue in lean and obese males at baseline and identify any differences between the two; 2) Identify genes that are induced and/or suppressed in response to a positive energy challenge to provide novel obesity candidate genes for the study of human obesity.

Methods

Subjects

Subjects were recruited from an ongoing overfeeding study investigating the effects of a positive energy balance on endocrine factors as well as glucose and lipid metabolism (179, 196). A total of 65 subjects participated in the previous study. A subset of these subjects agreed to receive an adipose tissue biopsy for the current microarray study. Twenty-six subjects participated in the baseline study (13 lean and 13 obese) and of these, 16 (eight lean and eight obese) agreed to take part in the overfeeding intervention. All subjects were from the city of St. John's and surrounding area in the Canadian province of NL. Inclusion criteria were as follows: 1) male; 2) 19-29 years of age; 3) at least third generation Newfoundlander; 4) healthy, without any serious metabolic, cardiovascular, or endocrine disease; 5) not on medication for lipid metabolism; 6) reported having a stable weight (± 2.5 kg) in the previous six months. All subjects provided written and informed consent, and the Research Ethics Board of the Faculty of Medicine, Memorial University of Newfoundland approved the study.

Study Design

This study employed a longitudinal design. All measurements, blood samplings, and adipose tissue samplings were performed twice: prior to commencing the one week overfeeding protocol and the day following the week of overfeeding (8^{th} day).

Overfeeding protocol

Full details of the overfeeding protocol have previously been described by us (179, 196). Briefly, individual energy requirements were estimated for each subject prior to commencing a 40% hypercaloric diet for seven days. Subjects were offered three meals per day and energy values and macronutrient content of the food were measured using the Food Processor SQL, version 9.5.0.0 (ESHA Research, Salem, Oregon).

Measurement of body composition

%BF and %TF were measured using DXA Lunar Prodigy (GE Medical Systems, Madison, WI, USA). Measurements were performed on subjects following the removal of all metal accessories, while lying in a supine position as previously described (136). Software version 4.0 was used for analysis. All measurements were completed prior to and one day following the overfeeding protocol.

Serum Measurements

Blood samples were taken from all subjects before and after completion of the overfeeding period, following 12 hours of fasting. Serum was stored at -80 °C for subsequent analyses. Serum insulin levels were measured on an Immulite immunoassay analyzer (DPC, CA, USA). HOMA-IR was used as a measure of insulin resistance (HOMA-IR=insulin (μU/ml) × glucose (mmol/L)/22.5)) and β-cell function (HOMAβ=20*insulin (μU/ml) / (glucose-3.5)) (160). Serum concentrations of glucose, total cholesterol, HDL cholesterol, and TG were measured using Synchron reagents and performed on an Lx20 analyzer (Beckman Coulter Inc., CA, USA). LDL cholesterol was calculated using the following formula: (total cholesterol) – (HDL cholesterol) – (TG/2.2) which is reliable in the absence of severe hyperlipidemia. Detailed description of all serum measurements can be found in our previous papers (135-136, 179, 196).

Adipose tissue biopsy and RNA isolation

Subcutaneous adipose tissue samples were obtained following a 12 hour fast, before and one day following overfeeding. Adipose tissue was removed from the sub-umbilical region with use of a local anesthetic composed of 10 cc of lidocaine in dilute bupivacaine (40cc of 0.25% bupivacaine in 250 cc of normal saline). Approximately 1-2 g of subcutaneous adipose tissue was removed and immediately flash frozen in liquid nitrogen, and subsequently stored in liquid nitrogen until further analysis. Total RNA was isolated from approximately 500 mg of adipose tissue using RNeasy lipid tissue midi

kit (Qiagen, CA., U.S.A). RNA concentration and purity were determined spectrophotometrically (Eppendorf, Hamburg Germany), and integrity was assessed on a 2100 bioanalyzer by electrophoresis on an agarose gel (Agilent Technologies, CA., USA). All samples showed an approximate 2:1 ratio of 28S to 18S RNA.

Microarrays

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 (247). 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. Samples were then purified using RNeasy mini elute kit (Qiagen). Hybridization of amplified, labeled RNA samples was accomplished by use of Agilent's *in situ* hybridization kit. 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. Reverse hybridization (dye swap) was performed to account for differences in signal strength between Cy3 and Cy5. Arrays were hybridized at 60^0C for 17.5 hours. All arrays were scanned with the ScanArray Express (Perkin Elmer, MA., U.S.A) and then quantified using Imagene software, version 5.6 (Biodiscovery Inc., CA., U.S.A.).

RT-PCR Validation

We chose to validate expression of six genes from our microarray data (transferrin (*TF*), stearoyl-CoA desaturase (*SCD*), transaldolase 1 (*TALDO1*), cathepsin C (*CTSC*), insulin receptor substrate 2 (*IRS2*), and pyruvate dehydrogenase kinase, isozyme 4 (*PDK4*)). These genes were identified as being significantly differentially expressed between lean and obese subjects in response to the overfeeding intervention. Total RNA was extracted from adipose tissue samples as described above. Reverse transcription was performed with 300 ng of total RNA from each sample and 100ng of cDNA was used as a template for RT-PCR as recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA). Beta-2-microglobulin (B2M) was used as an endogenous control to normalize gene expression (Applied Biosystems). PCR was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays (Applied Biosystems) which contain a mixture of forward and reverse primers as well as a specific TaqMan probe. Each probe was labeled at the 5' end with the reporter dye, FAM, and at the 3' end with the quencher, 6-minor groove binder. Each reaction contained 100 ng cDNA, PCR Master Mix, 900 nmol L^{-1} of each primer and 250 nmol L^{-1} of TaqMan probe in a final volume of 20 µl. Thermal cycling conditions were as follows: 50 °C for 2 minutes, 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute.

All samples were measured in triplicate and a negative control was included. The Comparative C_T method was used for calculation of mean fold change. The comparative

C_T method is similar to the standard curve method except it uses the formula $2^{-\Delta\Delta Ct}$ to achieve the same result for relative quantification. The C_T method also eliminates the need for a standard curve, thereby giving higher throughput and also reducing the adverse effect of any dilution errors made when creating the standard curve. In order for the comparative C_T method to be valid, there must be no major difference in amplification efficiencies of the target and endogenous control. We tested the amplification efficiency for each of the probes and endogenous control and found them to be approximately equal.

Data Analysis

Physical characteristics of subjects are presented as mean ± SE. Prior to performing statistical analyses, subjects were grouped according to adiposity status. Subjects were classified using %BF criteria as lean (<20%) or obese (>26%) according to criteria recommended by Bray (137). Differences in physical and biochemical parameters between the two groups at baseline (prior to overfeeding) and in response to overfeeding were assessed using GLM for Repeated Measures. SPSS version 15.0 (SPSS Inc., Chicago, IL) was used for all analyses. Statistical analyses were two-sided and a p value < 0.05 was considered to be statistically significant.

The raw microarray data obtained was analyzed using GeneSifter (http://www.genesifter.net/web/), which has been used in the analyses of microarray data (248-250). Analysis was performed on two levels:

1. Identification of genes that were significantly up- or down-regulated between lean and obese subjects at baseline (prior to the overfeeding intervention) was performed using Student's *t*-test (n = 26).
2. Identification of genes that were significantly differentially expressed due to the overfeeding intervention, adiposity status (lean vs obese) or genes that were significant due to an interaction effect between the two was done using two-factor ANOVA analyses (n = 16).

GeneSifter establishes biological significance based on both Gene Ontology (GO) Consortium and Kyoto Encyclopedia of Genes and Genomes (KEGG) public pathway. A z-score report was used to analyze the biological process ontologies and KEGG pathway terms associated with the differentially expressed genes. The z-score was derived by dividing the difference between the observed number of genes meeting a specific GO term and the expected number of genes, based on the total number of genes in the array. A positive z-score indicates that more genes than expected fulfilled the criteria in a certain group or pathway, therefore that group or pathway is likely to be affected by the treatment. The parameters used in the analyses were: threshold = 1.5 and log transformation. A threshold set at 1.5 indicates the minimum fold change required to be deemed significantly differentially expressed (in comparison to the selected control sample, in our case, lean, pre-overfeeding). Although the chosen threshold has no statistical significance, it was chosen with the assumption that a larger fold change increases the likelihood of that gene having a significant biological effect. Data were transformed to a logarithmic scale to ensure a normal distribution of data on all arrays.

Benjamini and Hochberg false discovery rate (FDR) method was used for the correction of multiple testing (251-252). This method controls the expected proportion of falsely rejected hypotheses and is the recommended FDR procedure for microarray data.

Results

Analyses of physical and biochemical parameters at baseline and in response to a 7-day overfeeding protocol

Physical and biochemical characteristics of subjects at baseline are shown in Table 4.5. There were no significant differences in age and height between the two groups however, weight, BMI, %BF, and %TF were all significantly higher in obese subjects. Total body fat (kg) and total trunk fat (kg) was also significantly higher in obese subjects. Obese subjects had higher fasting serum insulin concentrations as well as higher insulin resistance and ß-cell function. Changes in physical and biochemical characteristics in response to overfeeding are also shown in Table 4.5. Following the overfeeding intervention weight, BMI, and total body fat (kg) were significantly higher in subjects. TG were also significantly increased in subjects in response to the hypercaloric diet. There were no significant between-subject interactions.

Table 4.5 Physical and biochemical characteristics of subjects at baseline and in response to 7 days of overfeeding.[1]

	Lean (n=8)		Obese (n=8)		GLM Repeated Measures[2,3]	
Variables	Pre-overfeeding	Post-overfeeding	Pre-overfeeding	Post-overfeeding	Change from baseline (p value)	Group comparison (p value)
Age (yrs)	22.9±2.3	n/a	24.3±2.0	n/a	n/a	n/a
Height (cm)	176.5±3.5	n/a	176.9±6.5	n/a	n/a	n/a
Weight (kg)	74.7±10.3	78.0±10.9	105.4±23.9	108.0±25.4	<0.001	0.006
BMI ($kg \cdot m^{-2}$)	23.9±3.1	25.1±3.2	33.5±6.0	34.2±6.2	<0.001	0.002
Body fat (%)	14.8±5.0	15.6±5.0	33.9±4.6	33.7±4.0	ns	<0.001
Trunk fat (%)	16.6±5.9	17.7±5.6	38.4±4.4	38.4±4.1	ns	<0.001
Total Body Fat (kg)	11.3±4.8	12.5±5.0	36.2±12.0	36.9±12.4	0.030	<0.001
Total Trunk Fat (kg)	5.9±2.3	6.7±2.8	20.3±6.1	20.2±5.2	ns	<0.001
Cholesterol (mmol/L)	3.47±0.64	5.32±0.96	4.24±1.13	5.03±0.88	ns	ns
LDL-c (mmol/L)	2.82±0.75	3.08±0.82	3.18±0.76	2.95±0.78	ns	ns
HDL-c (mmol/L)	1.33±0.20	1.54±0.17	1.16±0.33	1.26±0.33	ns	ns
Triacylglycerol (mmol/L)	0.89±0.20	1.56±0.58	1.50±0.39	1.83±0.90	0.007	ns
Glucose (mmol/L)	5.08±0.32	5.56±0.62	5.88±0.87	5.56±0.62	ns	ns

Table 4.5 continued…

Variables	Lean (n=8)		Obese (n=8)		GLM Repeated Measures[2,3]	
	Pre-overfeeding	Post-overfeeding	Pre-overfeeding	Post-overfeeding	Change from baseline (p value)	Group comparison (p value)
HOMA-IR	1.48±0.75	2.41±0.94	6.94±5.61	6.33±4.10	ns	0.004
HOMAβ	82.3±36.0	101.9±45.3	206.6±134.5	240.3±159.1	ns	0.005

[1]All values are mean ± SD. HOMA-IR, homeostasis model assessment for insulin resistance; HOMAβ, homeostasis model assessment for β cell function; n/a, not available; ns, not significant.

[2]Adiposity status and responses to overfeeding were analyzed using the GLM Repeated Measures procedure.

[3]There were no significant between-subject interactions ($p > 0.05$)

Identification of differentially expressed genes between lean and obese subjects at baseline (n =26)

Using Student's t-test with a threshold of 1.5, log transformation and $p < 0.05$ (corrected by Benjamini and Hochberg method), 385 genes were found to be differentially expressed in adipose tissue of lean and obese individuals (Table 4.6). Of these, 158 were up-regulated and 227 were down-regulated in adipose tissue of obese subjects compared to lean.

Identification of differentially expressed genes due to overfeeding (n = 16)

Using a threshold of 1.5 and a two-way ANOVA where $p < 0.05$ (corrected by Benjamini and Hochberg method), a total of 45 genes were significantly differentially expressed due to the overfeeding intervention (Table 4.7) while 398 were significant due to adiposity status. Numerous studies have investigated differences in global gene expression between lean and obese individuals, therefore we decided to focus on the 45 genes significantly affected by the overfeeding intervention. The majority of genes affected by the dietary intervention were up-regulated in response to overfeeding in both lean and obese subjects, however seven genes were down-regulated in response to the hypercaloric diet. These genes were zinc finger, HIT type 3 (*ZNHIT3*), CD44 molecule (Indian blood group) (*CD44*), met proto-oncogene (hepatocyte growth factor receptor) (*MET*), cyclin-dependent kinase inhibitor 1C (*CDKN1C*), solute carrier family 19

Table 4.6 Differentially expressed genes in subcutaneous adipose tissue between lean and obese males at baseline.[1]

Gene ID	Gene Symbol	Gene Name	GO Biological Process	*p* value
Up-regulated				
NM_000582	SPP1	Secreted phosphoprotein 1	Ossification	<0.001
NM_004181	UCHL1	Ubiquitin carboxyl-terminal esterase L1	Ubiquitin-dependent protein catabolic process	<0.001
NM_000211	ITGB2	Integrin, beta 2	Apoptosis	<0.001
NM_005807	PRG4	Proteoglycan 4	Cell proliferation	0.017
NM_001611	ACP5	Acid phosphatase 5	Acid phosphatase activity	0.002
NM_024021	MS4A4A	Membrane-spanning 4-domains, subfamily A, member 4	Signal transduction	<0.001
NM_015507	EGFL6	EGF-like-domain, multiple 6	Cell cycle	0.010
NM_004355	CD74	CD74 molecule, major histocompatibility complex, class II invariant chain	Prostaglandin biosynthetic process	0.003
NM_002872	RAC2	Ras-related C3 botulinum toxin substrate 2	Chemotaxis	0.010
NM_004994	MMP9	Matrix metallopeptidase 9	Peptidoglycan metabolic process	0.001
NM_002298	LCP1	Lymphocyte cytosolic protein 1	Actin filament bundle formation	<0.001
NM_024563	C5orf23	Chromosome 5 open reading frame 23	Protein complex	0.004
NM_001803	CD52	CD52 molecule	GPI anchor binding	0.001
NM_015147	CEP68	Centrosomal protein 68kDa	n/a	0.022
NM_016206	VGLL3	Vestigial like 3 (Drosophila)	Regulation of transcription	0.009
NM_020792	AADACL1	Arylacetamide deacetylase-like 1	Metabolic process	0.010
NM_144569	SPOCD1	SPOC domain containing 1	Transcription	0.004
NM_144966	FREM1	FRAS1 related extracellular matrix 1	Cell adhesion	0.011
NM_032828	ZNF587	Zinc finger protein 587	Transcription	0.044
NM_014224	PGA5	Pepsinogen 5, group I (pepsinogen A)	Proteolysis	0.001
AK130614	IGHG1	Immunoglobulin heavy constant gamma 1	Immune response	0.017
BC012027	CYP2U1	Cytochrome P450, family 2, subfamily U,	Electron transport	0.017

		polypeptide 1		
NM_006332	IFI30	Interferon, gamma-inducible protein 30	Immune response	0.002
NM_022351	EFCBP1	EF-hand calcium binding protein 1	Antibiotic biosynthetic process	0.037
NM_000397	CYBB	Cytochrome b-245, beta polypeptide	Electron transport	0.003
NM_004694	SLC16A6	Solute carrier family 16, member 6	Transport	0.023
NM_014931	SAPS1	SAPS domain family, member 1	Regulation of phosphoprotein phosphatase activity	0.047
NM_007350	PHLDA1	Pleckstrin homology-like domain, family A, member 1	Apoptosis	0.044
THC2339142	-	THC2339142	n/a	0.045
NM_138715	MSR1	Macrophage scavenger receptor 1	Phosphate transport	0.034
NM_003579	RAD54L	RAD54-like (S. cerevisiae)	DNA repair	0.033
NM_001465	FYB	FYN binding protein	Protein amino acid phosphorylation	0.017
NM_004847	-	Allograft inflammatory factor 1	Response to stress	<0.001
NM_000399	EGR2	Early growth response 2	Regulation of transcription, DNA-dependent	0.046
THC2400010	-	THC2400010	n/a	0.025
NM_000480	AMPD3	Adenosine monophosphate deaminase	AMP catabolic process	0.005
NM_006851	GLIPR1	GLI pathogenesis-related 1	Extracellular region	0.036
NM_001010919	FAM26F	Family with sequence similarity 26, member F	Membrane	0.044
NM_001295	CCR1	Chemokine (C-C motif) receptor 1	Chemotaxis	0.047
NM_001014436	DBNL	Drebrin-like	Endocytosis	0.047
AK024680	-	CDNA: FLJ21027 fis, clone CAE07110	n/a	0.046
NM_013345	GPR132	G protein-coupled receptor 132	G1/S transition of mitotic cell cycle	0.003
BC032910	-	CDNA clone IMAGE:5264904	n/a	0.045
NM_207511	C9orf139	Chromosome 9 open reading frame 139	n/a	0.041
NM_000730	CCKAR	Cholecystokinin A receptor	Neuron migration	0.014
NM_002838	PTPRC	Protein tyrosine phosphatase, receptor type, C	Negative regulation of T cell mediated cytotoxicity	0.045
THC2437474	-	THC2437474	n/a	0.022

NM_006700	TRAFD1	TRAF-type zinc finger domain containing 1	Zinc ion binding	0.049
NM_000887	ITGAX	Integrin, alpha X	Cell adhesion	0.028
X91103	-	Hr44 antigen	Biological process	0.006
NM_148897	SDR-O	Orphan short-chain dehydrogenase / reductase	Metabolic process	0.045
NM_006573	TNFSF13B	Tumor necrosis factor (ligand) superfamily, member 13b	B cell homeostasis	0.047
	-	ENST00000357132	n/a	0.036
NM_001017402	LAMB3	Laminin, beta 3	Cell adhesion	<0.001
NM_004693	KRT5	Keratin 5	Epidermis development	0.034
NM_001677	ATP1B1	ATPase, Na+/K+ transporting, beta 1 polypeptide	Ion transport	0.039
AK022893	-	Homo sapiens, clone IMAGE:4214313	n/a	0.037
NM_006399	BATF	Basic leucine zipper transcription factor, ATF-like	Transcription	0.031
NM_000146	FTL	Ferritin, light polypeptide	Iron ion transport	<0.001
NM_002155	HSPA6	Heat shock 70kDa protein 6	Response to unfolded protein	0.038
NM_012284	KCNH3	Potassium voltage-gated channel, subfamily H, member 3	Two-component signal transduction system (phosphorelay)	0.020
NM_020919	ALS2	Amyotrophic lateral sclerosis 2	Behavioral fear response	0.046
NM_002418	MLN	Motilin	G-protein coupled receptor protein signaling pathway	0.025
BC019824	PTPRJ	Protein tyrosine phosphatase, receptor type, J	Vasculogenesis	0.024
NM_002133	HMOX1	Heme oxygenase 1	Heme oxidation	<0.001
NM_198594	C1QTNF1	C1q and tumor necrosis factor related protein 1	Phosphate transport	0.042
NM_021149	COTL1	Coactosin-like 1	Biological_process	<0.001
NM_177478	FTMT	Ferritin mitochondrial	Iron ion transport	0.042
NM_016212	TP53TG3	TP53TG3 protein	n/a	0.046

BQ028381	-	Transcribed locus	n/a	0.046
NM_001336	CTSZ	Cathepsin Z	Proteolysis	0.013
NM_005060	RORC	RAR-related orphan receptor C	Transcription	0.048
NM_003761	VAMP8	Vesicle-associated membrane protein 8	Protein complex assembly	<0.001
	-	ENST00000313481	n/a	<0.001
NM_024012	HTR5A	5-hydroxytryptamine receptor 5A	Signal transduction	0.020
NM_003290	TPM4	Tropomyosin 4	Cell motility	0.022
NM_000063	C2	Complement component 2	Proteolysis	0.017
NM_021226	ARHGAP22	Rho GTPase activating protein 22	Angiogenesis	0.032
AL133090	-	MRNA; cDNA DKFZp434E0528 (from clone DKFZp434E0528)	n/a	0.038
NM_001014999	SULT1A3	Sulfotransferase family, cytosolic, 1A, phenol- preferring, member 3	Catecholamine metabolic process	0.048
NM_024556	FAM118B	Family with sequence similarity 118, member B	n/a	0.043
NM_147780	CTSB	Cathepsin B	Proteolysis	<0.001
NM_138612	HAS3	Hyaluronan synthase 3	Carbohydrate metabolic process	0.047
	-	ENST00000282163	n/a	0.046
AK021777	GALNT10	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 10	Polypeptide N-acetylgalactosaminyltransferase activity	0.024
NM_006498	LGALS2	Lectin, galactoside-binding, soluble, 2	Sugar binding	0.047
NM_006123	IDS	Iduronate 2-sulfatase	Metabolic process	0.047
U14391	MYO1E	Myosin IE	Actin filament-based movement	0.039
NM_000903	NQO1	NAD(P)H dehydrogenase, quinone 1	Electron transport	0.037
NM_005103	FEZ1	Fasciculation and elongation protein zeta 1	Cell adhesion	0.006
NM_001901	CTGF	Connective tissue growth factor	Cartilage condensation	0.045
BC021189	-	CDNA clone IMAGE:4829245	n/a	0.032
NM_000259	MYO5A	Myosin VA	Transport	0.022
NM_017549	EPDR1	Ependymin related protein 1 (zebrafish)	Cell-matrix adhesion	0.031
NM_021102	SPINT2	Serine peptidase inhibitor, Kunitz type, 2	Cell motility	0.024

NM_001644	APOBEC1	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1	mRNA processing	0.041
NM_002341	LTB	Lymphotoxin beta	Immune response	0.033
NM_024770	METTL8	Methyltransferase like 8	n/a	0.016
NM_002317	LOX	Lysyl oxidase	Blood vessel development	0.036
AF218008	C19orf28	Chromosome 19 open reading frame 28	Transport	0.002
AB050854	C1orf38	Chromosome 1 open reading frame 38	Cell adhesion	0.012
	-	A_24_P281683	n/a	<0.001
NM_052871	MGC4677	Hypothetical protein MGC4677	n/a	0.001
NM_002961	S100A4	S100 calcium binding protein A4	Epithelial to mesenchymal transition	0.004
NM_001005339	RGS10	Regulator of G-protein signalling 10	Negative regulation of signal transduction	0.018
NM_022044	SDF2L1	Stromal cell-derived factor 2-like 1	Hydrolase activity	0.008
NM_006597	HSPA8	Heat shock 70kDa protein 8	Protein folding	<0.001
NM_006810	PDIA5	Protein disulfide isomerase family A, member 5	Electron transport	0.043
	-	ENST00000343149	n/a	<0.001
NM_006303	JTV1	JTV1 gene	Translation	0.034
NM_005720	ARPC1B	Actin related protein 2/3 complex, subunit 1B	Cell motility	0.023
NM_177528	SULT1A2	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 2	Catecholamine metabolic process	0.001
T68867	RPS26	Ribosomal protein S26	Translation	0.025
NM_014624	S100A6	S100 calcium binding protein A6	Regulation of progression through cell cycle	0.008
NM_006597	HSPA8	Heat shock 70kDa protein 8	Protein folding	<0.001
NM_016113	TRPV2	Transient receptor potential cation channel, subfamily V, member 2	Ion transport	<0.001
NM_001311	CRIP1	Cysteine-rich protein 1 (intestinal)	Cell proliferation	0.022
NM_001002033	HN1	Hematological and neurological expressed 1	n/a	0.011
NM_002032	FTH1	Ferritin, heavy polypeptide 1	Iron ion transport	0.001

NM_000636	SOD2	Superoxide dismutase 2, mitochondrial	Response to superoxide	<0.001
NM_006748	SLA	Src-like-adaptor	Intracellular signaling cascade	0.039
NM_080386	TUBA3D	Tubulin, alpha 3d	n/a	0.023
NM_003897	IER3	Immediate early response 3	Apoptosis	0.040
THC2266610	-	THC2266610	n/a	0.003
NM_000404	GLB1	Galactosidase, beta 1	Carbohydrate metabolic process	0.003
NM_152692	C1GALT1C1	C1GALT1-specific chaperone 1	Protein folding	0.011
NM_004636	SEMA3B	Sema domain, immunoglobulin domain, short basic domain, secreted, 3B	Cell-cell signaling	0.017
NM_004838	HOMER3	Homer homolog 3 (Drosophila)	Protein targeting	0.011
	-	A_24_P349756	n/a	0.030
NM_182924	MICALL2	MICAL-like 2	Endocytosis	0.027
NM_001069	TUBB2A	Tubulin, beta 2A	Microtubule-based movement	0.014
NM_199484	-	Chromosome 20 open reading frame 24	Biological_process	<0.001
NM_002807	PSMD1	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 1	Regulation of progression through cell cycle	0.001
NM_003329	TXN	Thioredoxin	Electron transport	0.001
U60266	MAN2B1	Mannosidase, alpha, class 2B, member 1	Carbohydrate metabolic process	0.001
XM_374010	LOC389033	Hypothetical LOC389033	n/a	0.039
NM_000447	PSEN2	Presenilin 2 (Alzheimer disease 4)	Cell fate specification	0.047
NM_003329	TXN	Thioredoxin	Electron transport	0.001
NM_001628	AKR1B1	Aldo-keto reductase family 1, member B1	Carbohydrate metabolic process	0.050
NM_021103	TMSB10	Thymosin, beta 10	Cytoskeleton organization and biogenesis	0.005
NM_003746	DYNLL1	Dynein, light chain, LC8-type 1	Microtubule-based process	0.040
NM_017725	-	G patch domain containing 4	Nucleic acid binding	0.009
NM_002119	HLA-DOA	Major histocompatibility complex, class II, DO alpha	Antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	0.025
NM_001124	ADM	Adrenomedullin	cAMP biosynthetic process	0.013
NM_001666	ARHGAP4	Rho GTPase activating protein 4	Cytoskeleton organization and	0.038

			biogenesis	
L48692	PNO1	Partner of NOB1 homolog (S. cerevisiae)	RNA binding	0.037
NM_006082	TUBA1B	Tubulin, alpha 1b	Microtubule-based movement	0.004
NM_080738	EDARADD	EDAR-associated death domain	Signal transduction	0.049
NM_031894	FTHL17	Ferritin, heavy polypeptide-like 17	Iron ion transport	0.022
NM_022736	MFSD1	Major facilitator superfamily domain containing 1	Transport	0.025
NM_005717	ARPC5	Actin related protein 2/3 complex, subunit 5	Cell motility	0.005
NM_005347	HSPA5	Heat shock 70kDa protein 5	Nucleotide binding	0.019
NM_006058	TNIP1	TNFAIP3 interacting protein 1	Translation	0.032
NM_024101	MLPH	Melanophilin	Protein targeting	0.032
NM_005335	HCLS1	Hematopoietic cell-specific Lyn substrate 1	Regulation of transcription, DNA-dependent	0.026
NM_015476	C18orf10	Chromosome 18 open reading frame 10	Cytoplasm	0.042
NM_001540	HSPB1	Heat shock 27kDa protein 1	Regulation of translational initiation	0.002
Down-regulated				
NM_005181	CA3	Carbonic anhydrase III, muscle specific	One-carbon compound metabolic process	0.025
AL713792	LOC131873	Hypothetical protein LOC131873	Phosphate transport	0.001
NM_025135	FHOD3	Formin homology 2 domain containing 3	Cellular component organization and biogenesis	<0.001
NM_032717	MAG1	Lung cancer metastasis-associated protein	Metabolic process	0.001
XM_930891	-	XM_930891	n/a	0.025
BC041636	LOC401320	Hypothetical LOC401320	n/a	0.019
NM_020207	C9orf102	Chromosome 9 open reading frame 102	n/a	0.040
	-	A_32_P51313	n/a	0.004
AK023047	ZNF702	Zinc finger protein 702	Transcription	0.032
NM_001185	AZGP1	Alpha-2-glycoprotein 1, zinc-binding	Immune response	<0.001

NM_145244	DDIT4L	DNA-damage-inducible transcript 4-like	n/a	0.032
NM_003273	TM7SF2	Transmembrane 7 superfamily member 2	Cholesterol biosynthetic process	0.006
	-	A_32_P331700	n/a	0.010
	-	ENST00000256969	n/a	0.047
NM_015678	NBEA	Neurobeachin	Cytoplasm	0.042
NM_014959	CARD8	Caspase recruitment domain family, member 8	Regulation of apoptosis	0.028
NM_002065	GLUL	Glutamate-ammonia ligase (glutamine synthetase)	Regulation of neurotransmitter levels	0.017
THC2404058	-	THC2404058	n/a	0.022
NM_003471	KCNAB1	Potassium voltage-gated channel, shaker-related subfamily, beta member 1	Ion transport	0.009
NM_018530	GSDML	Gasdermin-like	n/a	<0.001
NM_182568	FLJ36492	Hypothetical protein FLJ36492	n/a	0.027
AK125162	-	CDNA FLJ43172 fis, clone FCBBF3007242	n/a	0.049
NM_000165	GJA1	Gap junction protein, alpha 1, 43kDa	Transport	0.012
AK023572	-	CDNA FLJ13510 fis, clone PLACE1005146	n/a	0.045
NM_019849	SLC7A10	Solute carrier family 7, member 10	Transport	0.042
NM_002126	HLF	Hepatic leukemia factor	Transcription	<0.001
NM_020682	AS3MT	Arsenic methyltransferase	Metabolic process	0.046
NM_022746	MOSC1	MOCO sulphurase C-terminal domain containing 1	Oxidoreductase activity	0.023
NM_130436	DYRK1A	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A	Nervous system development	0.029
NM_015423	AASDHPPT	Aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase	Macromolecule biosynthetic process	0.012
NM_016542	RP6-213H19.1	Serine/threonine protein kinase MST4	Protein amino acid phosphorylation	0.005
NM_024867	FLJ23577	KPL2 protein	Protein dimerization activity	0.047
	-	A_24_P16361	n/a	0.042
AK001975	-	Methyl-CpG binding domain protein 5	DNA binding	0.049

BX640978	PCNXL3	Pecanex-like 3 (Drosophila)	Integral to membrane	0.028
BC025324	-	Homo sapiens, clone IMAGE:3344449	n/a	0.039
CR749800	IKZF5	IKAROS family zinc finger 5 (Pegasus)	Nucleic acid binding	0.034
THC2314599	-	THC2314599	n/a	0.045
NM_001902	CTH	Cystathionase	Amino acid biosynthetic process	0.005
NM_015323	KIAA0776	KIAA0776	Protein binding	0.042
R60067	RTN4RL1	Reticulon 4 receptor-like 1	Axon regeneration	0.004
NM_001551	IGBP1	Immunoglobulin (CD79A) binding protein 1	Response to biotic stimulus	0.009
NM_001039361	PRAMEF10	PRAME family member 10	n/a	0.039
NM_003970	MYOM2	Myomesin 2	Striated muscle contraction	0.009
NM_006996	SLC19A2	Solute carrier family 19 (thiamine transporter), member 2	Transport	<0.001
AK025816	RGNEF	Rho-guanine nucleotide exchange factor	Intracellular signaling cascade	0.001
NM_018374	TMEM106B	Transmembrane protein 106B	Membrane	0.026
NM_001004356	FGFRL1	Fibroblast growth factor receptor-like 1	Regulation of cell growth	0.043
	-	A_24_P212997	n/a	0.025
NM_178549	ZNF678	Zinc finger protein 678	Transcription	0.001
NM_004071	CLK1	CDC-like kinase 1	Regulation of progression through cell cycle	0.018
NM_022058	SLC4A10	Solute carrier family 4, sodium bicarbonate transporter-like, member 10	Sodium ion transport	0.044
	-	A_24_P167059	n/a	0.046
NM_024581	C6orf60	Chromosome 6 open reading frame 60	n/a	0.042
NM_152686	DNAJC18	DnaJ (Hsp40) homolog, subfamily C, member 18	Protein folding	0.043
	-	A_24_P298143	n/a	0.049
NM_001012651	-	Natural killer-tumor recognition sequence	Protein folding	0.045
BX647070	RORB	RAR-related orphan receptor B	Transcription	0.008
NM_002591	PCK1	Phosphoenolpyruvate carboxykinase 1	Gluconeogenesis	0.038
NM_018974	UNC93A	Unc-93 homolog A (C. elegans)	Biological_process	0.047
AF333762	-	AF333762	n/a	0.046

AI694800	-	Transcribed locus, strongly similar to XP_001096828.1 similar to RP42 homolog	n/a	0.048
AW994037	-	CDNA clone IMAGE:6025865	n/a	0.018
CR749233	ZNF626	Zinc finger protein 626	Transcription	0.002
THC2373083	-	THC2373083	n/a	0.040
NM_001063	TF	Transferrin	Ion transport	0.049
BX648831	LOC132430	Similar to poly(A) binding protein, cytoplasmic 4 (inducible form)	n/a	0.046
NM_032523	OSBPL6	Oxysterol binding protein-like 6	Lipid transport	0.026
NM_012076	-	Crumbs homolog 1 (Drosophila)	Establishment and/or maintenance of cell polarity	0.042
NM_001025366	VEGFA	Vascular endothelial growth factor A	Regulation of progression through cell cycle	0.017
NM_002612	PDK4	Pyruvate dehydrogenase kinase, isozyme 4	Carbohydrate metabolic process	0.036
NM_006007	ZFAND5	Zinc finger, AN1-type domain 5	Biological_process	0.006
NM_002736	PRKAR2B	Protein kinase, cAMP-dependent, regulatory, type II, beta	Protein amino acid phosphorylation	0.045
NM_033410	ZNF764	Zinc finger protein 764	Transcription	0.024
NM_022471	-	Germ cell-less homolog 1 (Drosophila)-like	Multicellular organismal development	0.012
NM_024786	ZDHHC11	Zinc finger, DHHC-type containing 11	Zinc ion binding	0.043
NM_016027	LACTB2	Lactamase, beta 2	Hydrolase activity	0.032
NM_022910	NDRG4	NDRG family member 4	Response to stress	0.018
	-	A_24_P75888	n/a	0.022
NM_032947	MST150	MSTP150	Membrane	0.027
NM_014326	DAPK2	Death-associated protein kinase 2	Protein amino acid phosphorylation	0.045
NM_005831	CALCOCO2	Calcium binding and coiled-coil domain 2	Viral reproduction	0.046
NM_024336	IRX3	Iroquois homeobox protein 3	Regulation of transcription, DNA-dependent	0.037
NM_001919	DCI	Dodecenoyl-Coenzyme A delta isomerase	Lipid metabolic process	0.042
NM_138732	NRXN2	Neurexin 2	Cell adhesion	0.048
NM_152271	LONRF1	LON peptidase N-terminal domain and ring	ATP-dependent proteolysis	0.006

		finger 1		
NM_002611	PDK2	Pyruvate dehydrogenase kinase, isozyme 2	Carbohydrate metabolic process	0.004
NM_001896	CSNK2A2	Casein kinase 2, alpha prime polypeptide	Protein amino acid phosphorylation	0.027
	-	A_23_P113453	n/a	0.043
	-	A_24_P938006	n/a	0.039
NM_001698	AUH	AU RNA binding protein/enoyl-Coenzyme A hydratase	mRNA catabolic process	0.019
NM_000016	ACADM	Acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain	Electron transport	0.001
THC2404169	-	THC2404169	n/a	0.047
NM_148170	CTSC	Cathepsin C	Proteolysis	0.001
NM_002971	SATB1	SATB homeobox 1	Establishment and/or maintenance of chromatin architecture	0.02
NM_032901	C12orf62	Chromosome 12 open reading frame 62	Membrane	0.001
NM_138779	C13orf27	Chromosome 13 open reading frame 27	n/a	0.025
THC2437430	-	THC2437430	n/a	0.047
NM_001185	AZGP1	Alpha-2-glycoprotein 1, zinc-binding	Immune response	0.011
NM_032329	ING5	Inhibitor of growth family, member 5	Protein amino acid acetylation	0.027
NM_018424	EPB41L4B	Erythrocyte membrane protein band 4.1 like 4B	Structural constituent of cytoskeleton	0.042
NM_003798	CTNNAL1	Catenin, alpha-like 1	Apoptosis	0.024
	-	ENST00000355095	n/a	0.031
NM_005839	SRRM1	Serine/arginine repetitive matrix 1	mRNA processing	0.049
NM_024790	CSPP1	Centrosome and spindle pole associated protein 1	Microtubule	0.015
NM_018196	TMLHE	Trimethyllysine hydroxylase, epsilon	Electron transport	0.017
NM_024685	BBS10	Bardet-Biedl syndrome 10	Protein folding	0.025
NM_004726	REPS2	RALBP1 associated Eps domain containing 2	Protein complex assembly	0.047
NM_015570	AUTS2	Autism susceptibility candidate 2	Biological_process	0.008
	-	ENST00000244221	n/a	0.042

NM_006271	S100A1	S100 calcium binding protein A1	Intracellular signaling cascade	0.042
NM_006348	COG5	Component of oligomeric golgi complex 5	Intra-Golgi vesicle-mediated transport	0.036
NM_000466	PEX1	Peroxisome biogenesis factor 1	Peroxisome organization and biogenesis	0.018
NM_024051	C7orf24	Chromosome 7 open reading frame 24	n/a	0.014
NM_001417	-	Eukaryotic translation initiation factor 4B	Translation	0.010
NM_145345	UBXD5	UBX domain containing 5	Cytoplasm	0.047
NM_015595	SGEF	Src homology 3 domain-containing guanine nucleotide exchange factor	Regulation of Rho protein signal transduction	0.042
NM_016272	TOB2	Transducer of ERBB2, 2	Regulation of progression through cell cycle	0.015
NM_175910	ZNF493	Zinc finger protein 493	Transcription	0.036
NM_001206	KLF9	Kruppel-like factor 9	Regulation of transcription from RNA polymerase II promoter	0.007
NM_032902	PPP1R16A	Protein phosphatase 1, regulatory subunit 16A	Protein binding	<0.001
AK128731	ATF2	Activating transcription factor 2	Transcription	0.046
NM_000474	TWIST1	Twist homolog 1 (Drosophila)	Negative regulation of transcription from RNA polymerase II promoter	0.027
NM_005327	HADH	Hydroxyacyl-Coenzyme A dehydrogenase	Lipid metabolic process	<0.001
NM_138333	FAM122A	Family with sequence similarity 122A	n/a	0.030
NM_032515	BOK	BCL2-related ovarian killer	Induction of apoptosis	0.040
NM_006859	LIAS	Lipoic acid synthetase	Metabolic process	0.043
NM_178335	CCDC50	Coiled-coil domain containing 50	n/a	<0.001
NM_021818	SAV1	Salvador homolog 1 (Drosophila)	Signal transduction	0.040
	-	ENST00000296015	n/a	0.047
NM_018660	ZNF395	Zinc finger protein 395	Transcription	0.032
AK126842	FLJ44894	Similar to zinc finger protein 91	n/a	0.026
NM_004428	EFNA1	Ephrin-A1	Cell-cell signaling	0.020
NM_033107	GTPBP10	GTP-binding protein 10	GTP binding	0.030
NM_006586	TNRC5	Trinucleotide repeat containing 5	n/a	0.047

NM_003273	TM7SF2	Transmembrane 7 superfamily member 2	Cholesterol biosynthetic process	0.001
	-	A_23_P96017	n/a	0.033
	-	ENST00000308603	n/a	0.032
NM_133443	GPT2	Glutamic pyruvate transaminase 2	Biosynthetic process	0.005
NM_133474	ABCA11	ATP-binding cassette, sub-family A, member 11 (pseudogene)	n/a	0.039
NM_033017	TRIM4	Tripartite motif-containing 4	Protein binding	0.011
NM_001257	CDH13	Cadherin 13, H-cadherin (heart)	Cell adhesion	0.022
AK098569	DKFZp667G2110	Hypothetical protein DKFZp667G2110	n/a	0.014
NM_006243	PPP2R5A	Protein phosphatase 2, regulatory subunit B, alpha isoform	Signal transduction	0.032
NM_001006641	SLC25A25	Solute carrier family 25	Transport	0.045
NM_015141	GPD1L	Glycerol-3-phosphate dehydrogenase 1-like	Carbohydrate metabolic process	0.008
	-	A_24_P897062	n/a	0.040
NM_021643	TRIB2	Tribbles homolog 2 (Drosophila)	Protein amino acid phosphorylation	0.010
NM_018357	LARP6	La ribonucleoprotein domain family, member 6	RNA processing	0.025
NM_002676	PMM1	Phosphomannomutase 1	Metabolic process	0.007
NM_145753	PHLDB2	Pleckstrin homology-like domain, family B, member 2	Cytoplasm	0.026
NM_006007	ZFAND5	Zinc finger, AN1-type domain 5	Biological_process	0.009
NM_012241	SIRT5	Sirtuin 5 (S. cerevisiae)	Chromatin silencing	0.038
NM_014802	KIAA0528	KIAA0528	Transport	0.040
	-	A_24_P928489	n/a	0.032
NM_058183	-	SON DNA binding protein	Anti-apoptosis	0.049
NM_005760	CEBPZ	CCAAT/enhancer binding protein zeta	Transcription	0.027
NM_001011724	RP11-78J21.1	Heterogeneous nuclear ribonucleoprotein A1-like	Nucleotide binding	0.041
NM_016205	PDGFC	Platelet derived growth factor C	Regulation of progression through cell cycle	0.017
NM_006667	PGRMC1	Progesterone receptor membrane	Receptor activity	0.042

		component 1		
NM_003932	ST13	Suppression of tumorigenicity 13	Protein folding	0.021
NM_003576	STK24	Serine/threonine kinase 24 (STE20 homolog, yeast)	Protein amino acid phosphorylation	0.001
NM_006918	SC5DL	Sterol-C5-desaturase (ERG3 delta-5-desaturase homolog, S. cerevisiae)-like	Lipid metabolic process	0.048
	-	ENST00000316369	n/a	0.022
NM_138330	ZNF675	Zinc finger protein 675	Regulation of transcription, DNA-dependent	0.003
NM_016824	ADD3	Adducin 3 (gamma)	Structural constituent of cytoskeleton	0.037
NM_005836	HRSP12	Heat-responsive protein 12	Regulation of translational termination	0.031
NM_001025356	TMEM16F	Transmembrane protein 16F	Membrane	0.010
NM_002291	LAMB1	Laminin, beta 1	Cell adhesion	0.041
NM_021922	FANCE	Fanconi anemia, complementation group E	DNA repair	0.012
NM_012081	ELL2	Elongation factor, RNA polymerase II, 2	Transcription	0.039
NM_001823	CKB	Creatine kinase, brain	Nucleotide binding	0.036
NM_017572	MKNK2	MAP kinase interacting serine/threonine kinase 2	Regulation of translation	<0.001
NM_005611	RBL2	Retinoblastoma-like 2 (p130)	Transcription	0.042
NM_181708	BCDIN3D	BCDIN3 domain containing	n/a	0.032
NM_002413	MGST2	Microsomal glutathione S-transferase 2	Signal transduction	0.028
NM_152350	C17orf45	Chromosome 17 open reading frame 45	Mitochondrion	0.022
	-	A_24_P118391	n/a	0.047
NM_003605	-	O-linked N-acetylglucosamine (GlcNAc) transferase	Protein amino acid O-linked glycosylation	<0.001
NM_152783	D2HGDH	D-2-hydroxyglutarate dehydrogenase	Electron transport	0.040
NM_080632	UPF3B	UPF3 regulator of nonsense transcripts homolog B (yeast)	mRNA catabolic process, nonsense-mediated decay	0.049
NM_004453	ETFDH	Electron-transferring-flavoprotein dehydrogenase	Electron transport	0.042
	-	A_24_P814872	n/a	0.029

NM_005433	YES1	V-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	Protein modification process	0.036
NM_033535	FBXL5	F-box and leucine-rich repeat protein 5	Protein ubiquitination	0.036
NM_017801	CMTM6	CKLF-like MARVEL transmembrane domain containing 6	Chemotaxis	0.020
NM_004096	EIF4EBP2	Eukaryotic translation initiation factor 4E binding protein 2	Insulin receptor signaling pathway	0.030
THC2280003	-	THC2280003	n/a	0.044
NM_001967	EIF4A2	Eukaryotic translation initiation factor 4A, isoform 2	Translation	0.032
NM_003430	ZNF91	Zinc finger protein 91	Transcription	0.028
NM_000245	MET	Met proto-oncogene	Activation of MAPK activity	0.012
NM_203282	ZNF254	Zinc finger protein 254	Negative regulation of transcription from RNA polymerase II promoter	0.001
NM_014452	TNFRSF21	Tumor necrosis factor receptor superfamily, member 21	Apoptosis	0.008
NM_001277	CHKA	Choline kinase alpha	Lipid metabolic process	0.005
NM_000189	HK2	Hexokinase 2	Regulation of progression through cell cycle	0.025
NM_003576	STK24	Serine/threonine kinase 24 (STE20 homolog, yeast)	Protein amino acid phosphorylation	0.022
NM_003489	NRIP1	Nuclear receptor interacting protein 1	Negative regulation of transcription from RNA polymerase II promoter	0.045
NM_152858	WTAP	Wilms tumor 1 associated protein	Nucleus	0.043
NM_019058	DDIT4	DNA-damage-inducible transcript 4	n/a	0.007
NM_012215	MGEA5	Meningioma expressed antigen 5	Glycoprotein catabolic process	0.006
NM_004331	BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like	Apoptosis	0.020
NM_006806	BTG3	BTG family, member 3	Regulation of progression through cell cycle	0.034
NM_000076	CDKN1C	Cyclin-dependent kinase inhibitor 1C	Regulation of cyclin-dependent protein	0.026

			kinase activity	
NM_002129	HMGB2	High-mobility group box 2	DNA replication	0.047
NM_033281	MRPS36	Mitochondrial ribosomal protein S36	Translation	0.032
THC2436072	-	THC2436072	n/a	0.049
NM_024573	C6orf211	Chromosome 6 open reading frame 211	Protein binding	0.032
NM_001568	EIF3S6	Eukaryotic translation initiation factor 3, subunit 6	Translation	0.042
NM_007236	CHP	Calcium binding protein P22	Potassium ion transport	0.013
NM_022157	RRAGC	Ras-related GTP binding C	Transcription	0.021
NM_003429	ZNF85	Zinc finger protein 85	Transcription	0.049
NM_007080	LSM6	LSM6 homolog, U6 small nuclear RNA associated (S. cerevisiae)	mRNA processing	0.003
NM_001967	EIF4A2	Eukaryotic translation initiation factor 4A, isoform 2	Translation	0.001
NM_016304	C15orf15	Chromosome 15 open reading frame 15	Translation	0.025
NM_006265	RAD21	RAD21 homolog (S. pombe)	Double-strand break repair	0.008
NM_032303	HSDL2	Hydroxysteroid dehydrogenase like 2	Metabolic process	0.041
NM_019095	CRLS1	Cardiolipin synthase 1	Phospholipid biosynthetic process	0.015
NM_000255	MUT	Methylmalonyl Coenzyme A mutase	Metabolic process	0.047
	-	ENST00000361227	n/a	0.037
NM_138286	ZNF681	Zinc finger protein 681	Nucleic acid binding	0.022
	-	ENST00000331425	n/a	0.019
NM_173531	ZNF100	Zinc finger protein 100	Transcription	0.003
NM_005871	SMNDC1	Survival motor neuron domain containing 1	Spliceosome assembly	0.043
	-	ENST00000361789	n/a	0.001
	-	ENST00000361845	n/a	0.017
NM_031372	HNRPDL	Heterogeneous nuclear ribonucleoprotein D-like	Transcription	0.025
NM_178558	ZNF680	Zinc finger protein 680	Transcription	0.017
NM_022818	MAP1LC3B	Microtubule-associated protein 1 light chain 3 beta	Ubiquitin cycle	0.040

[1]Significant differentially expressed genes due to overfeeding were identified by Student's *t*-test using GeneSifter®

n/a - not applicable (no assigned GO Biological Process)

Table 4.7 Differentially expressed genes in subcutaneous adipose tissue in lean (n = 8) and obese (n = 8) subjects due to a 40% hypercaloric diet.[1]

Gene ID	Gene Symbol	Gene Title	GO Biological Process	Fold Change[2]	*p* value
Up-regulated					
NM_080744	SRCRB4D	Scavenger receptor cysteine rich domain containing, group B (4 domains)	Receptor activity	2.17	<0.001
A_24_P212314	-	A_24_P212314	n/a	1.65	<0.001
NM_001037984	MGC15523	Hypothetical protein MGC15523	n/a	1.82	0.001
NM_004265	FADS2	Fatty acid desaturase 2	Lipid metabolic process	2.06	<0.001
NM_032525	TUBB6	Tubulin, beta 6	Microtubule-based movement	1.55	<0.001
NM_003681	PDXK	Pyridoxal (pyridoxine, vitamin B6) kinase	Pyridoxine biosynthetic process	1.88	<0.001
NM_001017389	SULT1A4	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 4	Synaptic transmission	1.37	0.008
NM_006088	TUBB2C	Tubulin, beta 2C	Cell motility	1.42	<0.001
NM_006755	TALDO1	Transaldolase 1	Carbohydrate metabolic process	1.48	<0.001
NM_014172	PHPT1	Phosphohistidine phosphatase 1	Dephosphorylation	1.42	<0.001
NM_004343	CALR	Calreticulin	Regulation of transcription, DNA-dependent	1.23	0.001
NM_004146	NDUFB7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7, 18kDa	Mitochondrial electron transport, NADH to ubiquinone	1.42	<0.001
NM_005063	SCD	Stearoyl-CoA desaturase (delta-9-desaturase)	Lipid metabolic process	3.56	<0.001
AF132203	-	PRO1933	n/a	2.23	<0.001

NM_013402	FADS1	Fatty acid desaturase 1	Lipid metabolic process	2.21	<0.001
NM_153837	GPR114	G protein-coupled receptor 114	Signal transduction	2.07	<0.001
NM_018479	ECHDC1	Enoyl Coenzyme A hydratase domain containing 1	Metabolic process	1.95	<0.001
NM_015415	BRP44	Brain protein 44	Mitochondrion	1.73	<0.001
NM_001063	TF	Transferrin	Ion transport	2.06	<0.001
NM_004823	KCNK6	Potassium channel, subfamily K, member 6	Ion transport	1.75	<0.001
NM_002300	LDHB	Lactate dehydrogenase B	Tricarboxylic acid cycle intermediate metabolic process	1.52	<0.001
NM_005729	PPIF	Peptidylprolyl isomerase F (cyclophilin F)	Protein folding	1.73	<0.001
A_24_P600622	-	A_24_P600622	n/a	1.62	<0.001
NM_001686	ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	Generation of precursor metabolites and energy	1.34	<0.001
NM_031280	MRPS15	Mitochondrial ribosomal protein S15	Translation	1.45	<0.001
NM_001001937	ATP5A1	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	Ion transport	1.45	<0.001
NM_007361	NID2	Nidogen 2 (osteonidogen)	Cell adhesion	1.47	<0.001
NM_024090	ELOVL6	ELOVL family member 6, elongation of long chain fatty acids	Fatty acid elongation	3.29	0.004
NM_000284	PDHA1	Pyruvate dehydrogenase (lipoamide) alpha 1	Acetyl-CoA metabolic process	1.38	0.011
NM_004092	ECHS1	Enoyl Coenzyme A hydratase, short chain, 1, Mitochondrial	Generation of precursor metabolites and energy	1.64	<0.001
NM_001931	DLAT	Dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex)	Acetyl-CoA biosynthetic process	1.56	0.004

A_24_P213175	-	A_24_P213175	n/a	1.40	0.009
NM_198402	PTPLB	Protein tyrosine phosphatase-like, member b	Protein binding	1.53	0.015
NM_145693	LPIN1	Lipin 1	Lipid metabolic process	1.56	<0.001
NM_001262	CDKN2C	Cyclin-dependent kinase inhibitor	Cell cycle	1.40	0.010
NM_007236	CHP	Calcium binding protein P22	Potassium ion transport	1.25	0.011
NM_002291	LAMB1	Laminin, beta 1	Cell adhesion	1.23	0.049
Down-regulated					
NM_004773	ZNHIT3	Zinc finger, HIT type 3	Regulation of transcription, DNA-dependent	-1.22	0.011
NM_000610	CD44	CD44 molecule (Indian blood group)	Cell adhesion	-1.41	0.029
NM_000245	MET	Met proto-oncogene	Activation of MAPK activity	-1.66	0.001
NM_000076	CDKN1C	Cyclin-dependent kinase inhibitor 1C	Regulation of cyclin-dependent protein kinase activity	-1.63	<0.001
NM_006996	SLC19A2	Solute carrier family 19, member 2	Transport	-1.55	0.011
NM_148170	CTSC	Cathepsin C	Proteolysis	-1.70	0.001
NM_002612	PDK4	Pyruvate dehydrogenase kinase, isozyme 4	Carbohydrate metabolic process	-1.73	0.002

[1]Significant differentially expressed genes due to overfeeding were identified by two-way ANOVA analyses using GeneSifter®.

n/a - not applicable (no assigned GO Biological Process)

[2]Fold change was calculated relative to pre-overfeeding values.

(thiamine transporter), member 2 (*SLC19A2*), cathepsin C (*CTSC*), and pyruvate dehydrogenase kinase, isozyme 4 (*PDK4*).

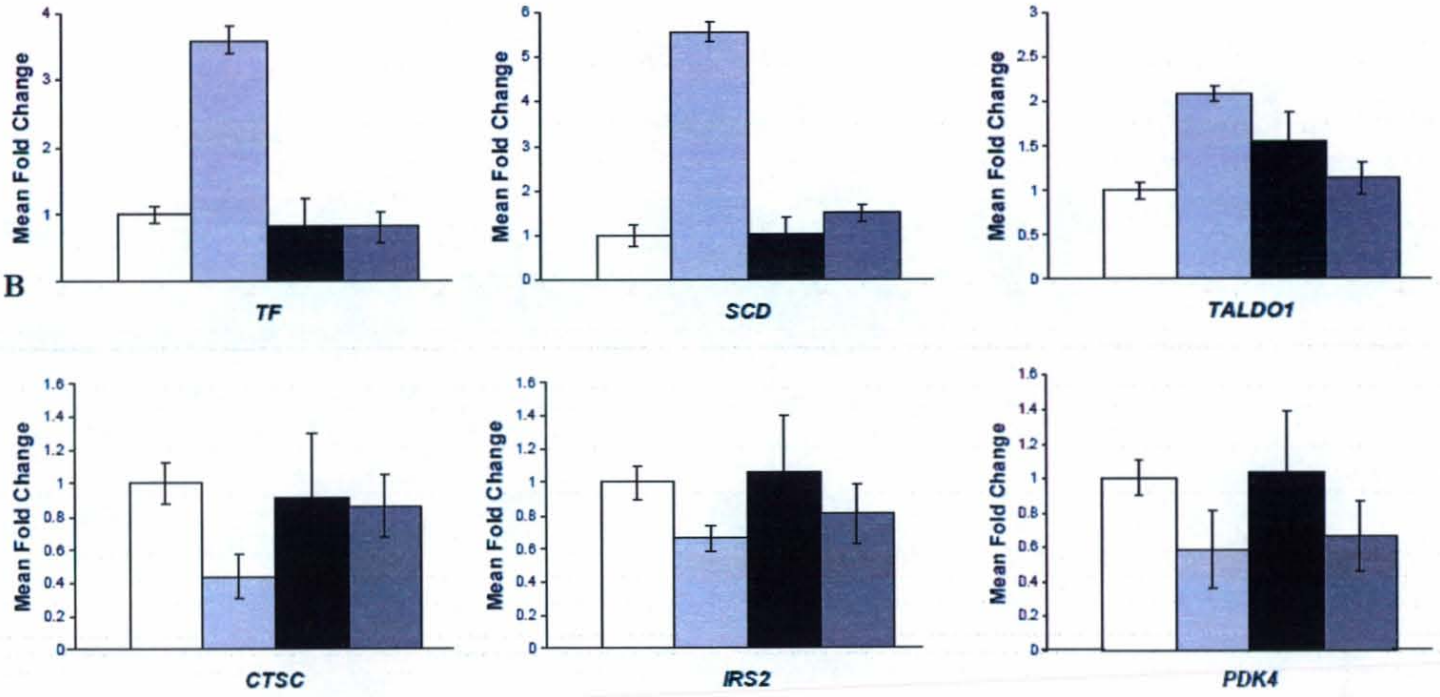
The unique design of our study allowed us to investigate possible genes that were differentially regulated between lean and obese subjects in response to overfeeding (an interaction effect). Six genes demonstrated a significant adiposity status by overfeeding interaction (Table 4.8). These were transferrin (*TF*), stearoyl-CoA desaturase (*SCD*), transaldolase 1 (*TALDO1*), cathepsin C (*CTSC*), insulin receptor substrate 2 (*IRS2*), and pyruvate dehydrogenase kinase, isozyme 4 (*PDK4*). Expression of these six genes was verified using RT PCR and demonstrated similar expression trends compared to the microarray data. Expression of *TF* was significantly increased in lean subjects following overfeeding, however no significant change in expression levels was evident in obese subjects (Figure 4.1). *SCD* was significantly increased in both lean and obese subjects, however the increase in expression level was much more pronounced in lean subjects. *TALDO1* was significantly increased in lean subjects in response to the overfeeding intervention while expression was slightly decreased in obese subjects. Expression of *CTSC* was decreased in lean subjects after overfeeding, while no change was evident in obese subjects. *IRS2* and *PDK4* were significantly decreased in both lean and obese individuals following overfeeding however the change was more substantial in lean individuals.

Table 4.8 Genes displaying a significant adiposity status by overfeeding interaction effect.[1]

Gene ID	Gene Symbol	Gene Title	GO Biological Process	*p* value
NM_006755	TALDO1	Transaldolase 1	Carbohydrate metabolic process	<0.001
NM_005063	SCD	Stearoyl-CoA desaturase (delta-9-desaturase)	Lipid metabolic process	0.043
NM_001063	TF	Transferrin	Ion transport	0.005
NM_148170	CTSC	Cathepsin C	Proteolysis	0.030
NM_002612	PDK4	Pyruvate dehydrogenase kinase, isozyme 4	Carbohydrate metabolic process	0.015
NM_003749	IRS2	Insulin receptor substrate 2	Glucose metabolic process	0.034

[1]Significant differentially expressed genes were identified by two-way ANOVA analyses using GeneSifter®

A


Mean Fold Change
TF
SCD
TALDO1
CTSC
IRS2
PDK4
Lean, Pre-overfeeding
Lean, Post-overfeeding
Obese, Pre-overfeeding
Obese, Post-overfeeding

B

Figure 4.1 Mean fold change in a subset of genes selected for validation of microarray results using real-time PCR (n = 16; 8 lean, 8 obese). Data (mean ± SE) are expressed as mean fold change compared to lean, pre-overfeeding values. A. *TF*, *SCD*, and *TALDO1* were increased in lean subjects in response to the overfeeding intervention while no significant change was evident in obese subjects. B. *CTSC*, *IRS2*, and *PDK4* were decreased in lean subjects in response to the overfeeding intervention while no significant change was evident in obese subjects.

Significance of biological pathways represented in genes differentially regulated by overfeeding

Table 4.9 represents KEGG pathways significantly affected by the overfeeding intervention. A z-score report was generated displaying only those terms with a z-score greater than 2.0. Genes involved in both carbohydrate and lipid metabolism were significantly affected by the overfeeding intervention. Specifically, pyruvate metabolism, glycolysis/gluconeogenesis, propanoate metabolism, and the pentose phosphate pathway all had a significant over-representation of genes following the overfeeding protocol. In terms of lipid metabolism, fatty acid elongation in the mitochondria and linoleic acid metabolism both had a z-score greater than 2.0. Interestingly, oxidative phosphorylation and sulfur metabolism, two pathways involved in energy metabolism, also had significant z-scores. A number of other pathways involved in cell communication (gap junction), cell signaling (ECM-receptor interaction), the endocrine system (PPAR signaling pathway, and metabolism of cofactors and vitamins {vitamin B6 metabolism, thiamine metabolism, and riboflavin metabolism}) were all significantly affected by the hypercaloric diet.

Discussion

Numerous studies have been conducted in recent years that have examined differences in gene expression profiles of adipose tissue between lean and obese individuals. Others have performed site comparisons (subcutaneous vs. visceral adipose tissue), and few have investigated changes in gene expression in response to a negative

Table 4.9 Effect of overfeeding on the expression of KEGG pathways.[1]

KEGG Pathway	List[2]	Array[3]	z-score
Vitamin B6 metabolism	1	5	5.67
Pyruvate metabolism	3	42	5.58
Thiamine metabolism	1	7	4.74
Glycolysis / Gluconeogenesis	3	63	4.36
Alanine and aspartate metabolism	2	32	4.2
Propanoate metabolism	2	32	4.2
Linoleic acid metabolism	2	35	3.98
Fatty acid elongation in mitochondria	1	10	3.89
Prion disease	1	12	3.51
Valine, leucine and isoleucine biosynthesis	1	12	3.51
Butanoate metabolism	2	44	3.45
Pathogenic Escherichia coli infection - EHEC	2	49	3.21
Sulfur metabolism	1	14	3.21
Caprolactam degradation	1	15	3.08
Cysteine metabolism	1	16	2.96
Riboflavin metabolism	1	16	2.96
Oxidative phosphorylation	3	114	2.89
PPAR signaling pathway	2	66	2.61
beta-Alanine metabolism	1	24	2.29
Pentose phosphate pathway	1	24	2.29
Benzoate degradation via CoA ligation	1	25	2.23
Limonene and pinene degradation	1	26	2.18
ECM-receptor interaction	2	84	2.17
Aminosugars metabolism	1	28	2.07
Gap junction	2	91	2.03

[1]Pathway analyses and z-score report were generated using GeneSifter®. The z-score was derived by dividing the difference between the observed number of genes meeting a specific GO term and the expected number of genes, based on the total number of the genes in the array.

[2]"List" refers to the number of genes belonging to that pathway that were significantly differentially expressed due to the overfeeding intervention.

[3]"Array" refers to the total number of genes belonging to that pathway that are represented on the array.

energy balance, created through an increase in energy expenditure (241-243) or through calorie restriction (244-245). However, the major factor leading to obesity is an energy surplus, and there is currently no data available regarding changes in the transcriptome of adipose tissue during a positive energy balance in humans. We have performed, for the first time, a study to comprehensively investigate the expression profiles of abdominal subcutaneous adipose tissue in response to an energy surplus induced through a short-term positive energy challenge using whole human genome microarray technology.

Physical and biochemical parameters were compared between the current study and our previous overfeeding studies (179, 196). The lean subgroup of 8 subjects from the current study was statistically similar to lean subjects (n = 37) from our previous study for all parameters measured. In terms of the obese subgroup, these subjects had a significantly higher BMI, weight, and %TF compared to overweight/obese subjects from the previous study (data not shown). In addition, glucose was elevated in obese subjects as well as insulin and insulin resistance. In the present study, we were interested in investigating differences in gene expression between two extremes of the weight spectrum (lean vs obese) whereas the total overfeeding cohort contains subjects spanning the entire weight spectrum (lean, normal weight, overweight and obese) and therefore these results are not unexpected.

A major finding in our study was the identification of 45 genes that were differentially expressed in response to overfeeding. These genes are involved in a wide variety of biological processes known to be implicated in the development of obesity

including the immune response, lipid metabolism, and energy production. A number of genes regulated by the overfeeding intervention have been identified in previous studies as being differentially expressed in adipose tissue of lean and obese individuals (235), in obese individuals following weight loss (239) and in diet-induced obese rats (253). These include genes involved in lipid metabolism (*SCD*, *FADS1*), glucose metabolism (*IRS2*, *PDK4*), cell adhesion processes (*NID2, CD44, LAMB1*), immune response (*CTSC*), and energy pathway/electron transport (*ECHS1*, *NDUFB7*). We have also identified novel genes not previously examined in the context of obesity that are regulated by a positive energy challenge. Their role in the development of human obesity and in individual differences in the predisposition to weight gain is a valuable issue to investigate.

It is known that there is a genetic basis for the predisposition to weight gain when exposed to a positive energy balance, such as the current situation in Western societies. However, as to how adiposity status may influence the genetic response to an energy surplus in human adipose tissue has not been studied. This is very important because the information obtained will provide insight into the genetic targets responsible for the inter-individual differences in weight gain. An important finding in our study was the discovery of six genes whose expression levels were significantly affected by adiposity status during a positive energy challenge. These genes may represent the most promising targets for future obesity research.

Of the six genes that displayed a significant adiposity status by treatment interaction effect, three were up-regulated in lean subjects while no significant change was evident in obese subjects including *SCD*, *TALDO1*, and *TF*. SCD is an iron-containing enzyme involved in lipid metabolism, where it catalyzes a rate-limiting step in the synthesis of unsaturated fatty acids (254). TALDO1 is involved in both energy and lipid metabolism where it acts as a key enzyme in the nonoxidative pentose phosphate pathway. TF is involved in iron transport into cells by receptor-mediated endocytosis (255) and is also routinely used as a nutritional marker in clinical settings. At present, it is difficult to speculate the role that each of these may play in the development of obesity without further study. The products of SCD are the most abundant fatty acids in triacylglycerols, cholesterol esters, and phospholipids. Aside from being components of lipids, unsaturated fatty acids also serve as mediators of signal transduction, cellular differentiation and apoptosis (256-258) and therefore changes in SCD activity would be expected to have an effect on a variety of metabolic pathways including those involved in obesity (259). Indeed, evidence indicates that high SCD activity favors fat storage and obesity. In a recent study, diet-induced obese rats had an approximate 2-fold increase in *SCD* expression compared to lean animals (253). Similarly, TF has also been identified as a robust marker of weight status. Specifically, iron deficiency is a common characteristic of morbidly obese patients as a result of low transferrin saturation (low serum iron in the presence of high transferrin; 260). Up-regulation of these genes in lean subjects in response to overfeeding seems counter-intuitive given their function. As long term expression may be detrimental to maintaining a normal body weight, it is plausible

that obese individuals have adapted to suppress expression as they were under conditions of a chronic energy surplus prior to taking part in this study. Conversely, it is also reasonable to speculate that up-regulation of these genes could be a self-protective mechanism in lean subjects. Further studies are certainly warranted to address the biological significance of each of these genes before making any concrete statements regarding their involvement in obesity.

The three additional genes displaying a significant interaction effect were *CTSC*, *IRS2*, and *PDK4*. Expression of these was downregulated in lean subjects while no significant change was evident in obese subjects. CTSC is involved in the immune response, where it appears to be a central coordinator for activation of many serine proteinases in immune/inflammatory cells (261). Although little is known regarding the role CTSC plays in the development of obesity, it is widely accepted that obesity represents an inflammatory state resulting from chronic activation of the innate immune system. Interestingly, a protein with similar function, cathepsin S (CTSS) has recently been identified as a novel marker of adiposity, specifically as a link between obesity and atherosclerosis (240). It is possible that CTSC acts in a similar manner. The differential expression of this gene requires further investigation to fully comprehend its role in the pathogenesis of obesity.

IRS2 encodes a cytoplasmic signaling molecule that mediates the effects of insulin and other cytokines (262). Numerous studies have shown a direct correlation between common variants in this gene and severe obesity (263) and T2D (264). In our

study, expression of *IRS2* was decreased in lean subjects following the hypercaloric diet which may have contributed to the increase in insulin resistance evident in these individuals. No change was apparent in *IRS2* expression in obese subjects following overfeeding and similarly, no change in insulin resistance. The mechanism through which *IRS2* acts in adipose tissue of lean subjects to in response to a hypercaloric diet, however, is still unclear.

PDK4 is a member of the pyruvate dehydrogenase kinase family, a group of enzymes that inhibit the pyruvate dehydrogenase complex (PDC) by phospohorylating one of its subunits (265). Activation of PDK4 results in metabolic switching of oxidative fuel use from glucose to fatty acids and occurs during times of starvation (266). Dietary intake of carbohydrate results in an increase in activity of the PDC in white and brown adipose tissues and liver of rats, likely through inhibition of PDK4 activity (267). In this manner, a decrease in *PDK4* expression in lean subjects following overfeeding may facilitate glucose clearance, through activation of the PDC, to combat the excess energy intake. This hypothesis is further supported by the fact that increases in expression of two key enzymes comprising the PDC (dihydrolipoamide S-acetyltransferase and pyruvate dehydrogenase alpha 1) were evident in lean subjects in response to overfeeding but not in obese subjects. This protective molecular mechanism appears to be blunted in obese subjects and necessitates further studies to understand the role of PDK4 in the genetic predisposition to weight gain.

The findings from our study highlight the importance of gene expression profiles obtained from specific tissues or organs essential for the development of obesity. Future studies are warranted to investigate expression profiles of other adipose tissue depots as well as skeletal muscle and liver under overfeeding conditions. Our study also reflects the necessity of investigating changes in the transcriptome under conditions similar to obesity, such as a positive energy balance.

In summary, we have examined the transcriptome of abdominal subcutaneous adipose tissue under conditions of a positive energy balance in human subjects. A total of 45 genes were identified as being significantly differentially expressed in response to overfeeding. We were also able to identify six genes that displayed a differential response to the overfeeding intervention between lean and obese subjects. Differential expression of these genes may represent a defense mechanism at the molecular level to protect the body against an energy surplus. Therefore, these genes are important targets to further investigate for the role they play in the genetic predisposition towards obesity.

5

Conclusions, Limitations, and Future Directions

5.1 Concluding remarks

Obesity is one of the leading causes of preventable death worldwide, with increasing prevalence in both adults and children in recent years. It has been called one of the most serious public health problems of the 21st century and because of this, a surge of research has been initiated to delineate the underlying causes of this in an attempt to further our understanding of the mechanism of action of this disease. This thesis has added to the growing body of literature concerning this by characterizing the obesity phenotype in the NL population, investigating the role genetic variation plays in obesity-related traits, and evaluating the genomic and endocrine response to a positive energy challenge. By using a multi-tiered approach, we provide a more comprehensive overview of the etiology of obesity.

First, we evaluated the precision of BMI compared to a more accurate reference method, DXA, in determining adiposity status. We found a significant discrepancy between these two indices of obesity which has important clinical relevance as BMI is the most widespread method used to estimate obesity and its associated health risks. Adding to these findings, we were then able to show that the discrepancy between BMI and DXA affects the reported prevalence of a common obesity subtype, MHO. Specifically, we found that approximately 50% of obese individuals are in fact, metabolically healthy, which is significantly higher than previous reports of approximately 30%. Moreover, the inconsistencies between BMI and DXA also had serious implications for another subtype of obesity, MONW. Given the large range in %BF evident in BMI-defined normal

weight individuals, we explored the risk associated with having high %BF in these subjects. Perhaps not surprisingly, we found that those in the highest tertile for %BF had an almost 3-fold increased risk of being metabolically unhealthy. Taken together, these three studies demonstrate the need for development of additional obesity measurement tools in field settings, as well as increased clinical awareness of both MHO and MONW individuals.

Aside from characterizing the obesity phenotype, efforts were also made to understand the genetic etiology of this disease. Using a candidate gene approach, genetic association studies were performed on *NAMPT* and *RBP4*, encoding two novel adipokines, visfatin and RBP4, respectively. We tested the association between variants within and/or near these genes and obesity-related traits including serum lipids, markers of glucose metabolism, and systemic inflammation. Although we did not find any significant association between genetic variation in *NAMPT* and these traits, we did observe an association between two SNPs in *RBP4* and HDL cholesterol levels. In particular, carriers of the minor allele had significantly higher serum HDL cholesterol suggesting that these variants may offer a protective effect against the development of dyslipidemia.

Lastly, we moved from our large-scale population-based studies to a smaller scale overfeeding intervention. Perhaps the most novel of all findings in this thesis, we generated 45 obesity candidate genes from global gene expression profiling of subcutaneous adipose tissue of men undergoing the overfeeding challenge. Of these 45,

six are extremely promising targets as they demonstrated a differential response between lean and obese individuals to overfeeding and likely contribute to the inter-individual variation in weight gain. It is difficult to speculate the exact mechanism of action for each of these genes regarding the development of obesity with the given data, however, future studies involving murine models are planned to discern an exact role for each of these candidates. Furthermore, we also found that RBP4 was not regulated by a positive energy balance in young men however it may potentially serve as a predictor of the response of insulin resistance to the intervention. The findings from our nutritional intervention are very valuable as few overfeeding studies have currently been completed. By attempting to mimic the chronic energy surplus evident in the Western world today, we have likely identified novel mechanisms of action for obesity that would otherwise go undetected.

In conclusion, this thesis has provided insight into the prevalence of obesity and its subtypes in the NL population as well as the underlying genetic and endocrine mechanisms involved in the development of this disease. The major strength of the current work lies in the fact that we have accomplished this goal using a number of different techniques allowing for full exploitation of the data available. Ultimately, the objective of all obesity research is to enhance current prevention and treatment strategies, resulting in increased quality of life for these patients. It is our hope that the findings presented here will contribute to this goal.

5.2 Limitations of the present work

Although we provide very sound and conclusive results in each of the studies presented in this thesis, the work is not without its weaknesses. Aside from the limitations discussed in each individual paper, there are a small number of additional concerns that warrant mention. First, different criteria were used for classifying adiposity status between different studies. Initially, we categorized subjects as normal weight, overweight, or obese based on BMI criteria (Chapter 4.1). As the utility of BMI came into question, we then chose to follow criteria recommended by Bray based on %BF that is gender- , age-, and ethnicity-specific (Chapters 2, 3, and 4.2). This classification system was first proposed in 2003 and is based on BMI cut points. Although more reliable than BMI, these criteria have also been questioned as they are not based on association with metabolic risk factors and increased risk of mortality. To circumvent this issue, we then chose to use gender-specific %BF tertiles to define adiposity in the most recent paper presented in this thesis (Chapter 2.3). While it is well accepted that DXA-defined %BF measurements are more accurate than BMI, the best cut points used to define each %BF group are still unknown. At the current time, the WHO has not defined a normal range for %BF therefore we believe our use of tertiles is the most valid method. It will be important to define obesity using %BF cut points based on disease biomarkers and increased health risk. Owing to the high cost of DXA measurements, there is limited data available exploring this in a large cohort however, moving forward, this will become an important question to answer.

Another limitation that deserves mention is the difference in the percentage overfed between nutritional intervention studies (Chapters 4.1 and 4.2). In the first overfeeding study exploring the nutritional response of RBP4, subjects were overfed by 70% more than baseline energy requirements compared to 40% in the global gene expression study. The reason for this discrepancy is a result of the increased invasiveness experienced by subjects in the latter study due to the additional requirement of two adipose tissue biopsies. Due to ethical considerations, we felt it best to minimize the burden experienced by these subjects and therefore reduced their caloric load. Previous positive energy challenge studies have shown that being overfed by as little as 30% above normal energy requirements can induce changes in gene expression (268), therefore we are confident that our intervention was sufficient to induce measurable changes in the transcriptome.

5.3 Future directions

The findings from this thesis open up a number of avenues for which future work could continue. As mentioned above, there is a critical need for large scale studies evaluating the appropriate %BF cut points for classifying individuals as obese based on associations with morbidity and mortality. We plan to explore this in the near future using information from the CODING Study, which to date has collected data on over 2500 subjects. Further characterization of obesity subtypes using other biomarkers, including additional adipokines, gut hormones, and markers of inflammation, is also

underway. Moreover, we plan to investigate the genetic background of both MHO and MONW individuals using a candidate gene association approach with the aim of identifying genetic predictors of these two conditions and eventually allow for population level screening.

In terms of the work done on RBP4, plans are ongoing to measure serum levels in the entire CODING Study cohort. Using the genotype data presented in this thesis, we can then determine if common genetic variants in *RBP4* are associated with serum levels of the protein. Furthermore, given the association we found with HDL cholesterol, we can also determine if this is mediated through differences in serum RBP4.

Lastly, my lab is intending to perform additional functional studies on a number of the novel obesity candidate genes identified from the microarray experiments to further clarify their potential role in the development of obesity. Specifically, a small number of genes have been chosen that are most promising based on either some knowledge of their function or having the most significant difference in expression level between lean and obese subjects. This will be done initially using knock-out mouse models for each chosen gene. Knockout (if viable), heterozygous, and wild-type mice will be fed a high fat diet and normal chow and differences in obesity-related phenotypes compared, including studies of the skeletal muscle and liver that would be difficult to perform on human subjects. These studies will provide further understanding of gene function as it relates to obesity. Based on these results, further studies can be performed

using data from the CODING study, including candidate gene association analyses, serum measurements, etc.

References

1. World Health Organization. Obesity and Overweight Fact Sheet #311. 2006. URL http://www.who.int/mediacentre/factsheets/fs311/en/index.html (Accessed January 11th, 2011).

2. Katzmarzyk, P and Mason C. Prevalence of Class I, Class II and Class III obesity in Canada. *CMAJ* 2006;174(2):156-157.

3. Shields M, Tjepkema M. Regional differences in obesity. *Health Reports* (Statistics Canada, Catalogue 82-003) 2006;17(3):61-67.

4. Colditz GA, Willett WC, Rotnitzky A, Manson JE. Weight gain as a risk factor for clinical diabetes mellitus in women. *Ann Intern Med* 1995;122:481-486.

5. Rimm EB, Stampfer MJ, Giovannucci E, Ascherio A, Spiegelman D, Colditz GA, Willett WC. Body size and fat distribution as predictors of coronary heart disease among middle-aged and older US men. *Am J Epidemiol* 1995;141:1117-1127.

6. Chrostowska M, Szyndler A, Paczwa P, Narkiewicz K. Impact of abdominal obesity on the frequency of hypertension and cardiovascular disease in Poland - Results from the IDEA study (International Day for the Evaluation of Abdominal Obesity). *Blood Pressure* 2010; In press. doi 10.3109/08037051.2010.538996.

7. Rexrode KM, Hennekens CH, Willett WC, Colditz GA, Stampfer MJ, Rich-Edwards JW, Speizer FE, Manson JE. A prospective study of body mass index, weight change, and risk of stroke in women. *JAMA* 1997;277:1539-1545.

8. Rabkin SW, Chen Y, Leiter L, Liu L, Reeder BA. Risk factor correlates of body mass index. Canadian Heart Health Surveys Research Group. *CMAJ* 1997;157(Supplement 1):S26-S31.

9. Sellers TA, Kushi LH, Potter JD, Kaye SA, Nelson CL, McGovern PG, Folsom AR. Effect of family history, body-fat distribution, and reproductive factors on the risk of postmenopausal breast cancer. *N Engl J Med* 1992;326:1323-1329.

10. Fontaine KR, Barofsky I. Obesity and health-related quality of life. *Obes Rev* 2001;2:173-182.

11. Peeters A, Barendregt JJ, Willekens F, Mackenbach JP, Al Mamun A, Bonneux L. Obesity in adulthood and its consequences for life expectancy: a life-table analysis. *Ann Intern Med* 1995;122:481-486.

12. Canadian Institute for Health Information. Comparison of obesity among ESRD patients in Canada and provinces and the general population in 2000/2001. 2004. Ref type: Report.

13. Withrow D, Alter DA. The economic burden of obesity worldwide: a systematic review of the direct costs of obesity. *Obes Rev* 2010; In press. doi 10.1111/j.1467-789X.2009.00712.x.

14. Canadian Community Health Survey Cycle 2.2. Statistics Canada 2004. Map of obesity according to measured body mass index (BMI) in adults in Canada (both males and females). URL http://www.hc-sc.gc.ca/fn-an/surveill/atlas/map-carte/mass_adult_obes_mf-hf-eng.php (Accessed January 11th, 2011).

15. World Health Organization. Obesity: preventing and managing the global epidemic. Geneva, Switzerland: World Health Organization, 1998.

16. Paek KW, Chun KH. Sex difference of type 2 diabetes affected by abdominal obesity versus overall obesity. *Yonsei Med J* 2010;51(6):850-6.

17. Testa G, Cacciatore F, Galizia G, Della-Morte D, Mazzella F, Langellotto A, Russo S, Gargiulo G, De Santis D, Ferrara N, Rengo F, Abete P. *J Am Geriatr Soc* 2010;58(8):1433-40.

18. National Institutes of Health, National Heart, Lung, and Blood Institute. Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults: the evidence report. *Obes Res* 1998;6:S51-210.

19. Wellens RI, Roche AF, Khamis HJ, Jackson AS, Pollock ML, Siervogel RM. Relationships between body mass index and body composition. *Obes Res* 1996;4:35-44.

20. Romero-Corral A, Somers VK, Sierra-Johnson J, et al. Accuracy of body mass index in diagnosing obesity in the adult general population. *Int J Obes (Lond)* 2008;32:959-66.

21. Rankinen T, Kim SY, Perusse L, Despres JP, Bouchard C. The prediction of abdominal visceral fat level from body composition and anthropometry: ROC analysis. *Int J Obes Relat Metab Disord* 1999;23:801-809.

22. Katch FI. Practice curves and error of measurement in estimating underwater weight by hydrostatic weighing. *Med Sci Sports* 1969;1:212-6.

23. Dempster P, Aitkens S. A new air displacement method for the determination of human body composition. *Med Sci Sports Exerc* 1995;27:1692-7.

24. Salamone LM, Fuerst T, Visser M, et al. Measurement of fat mass using DXA: a validation study in elderly adults. *J Appl Physiol* 2000;89:345-52.

25. Prior BM, Cureton KJ, Modlesky CM, et al. In vivo validation of whole body composition estimates from dual energy X-ray absorptiometry. *J Appl Physiol* 1997;83:623-30.

26. Karelis AD, St-Pierre DH, Conus F, Rabasa-Lhoret R, Poehlman ET. Metabolic and body composition factors in subgroups of obesity: what do we know? *J Clin Endocrinol Metab* 2004;89(6):2569-2575.

27. Wildman RP, Muntner P, Reynolds K, et al. The obese without cardiometabolic risk factor clustering and the normal weight with cardiometabolic risk factor clustering. *Arch Intern Med* 2008;168(15):1617-1624.

28. Stefan N, Kantartzis K, Machann J, et al. Identification and characterization of metabolically benign obesity in humans. *Arch Intern Med* 2008;168(15):1609-1616.

29. Lacoballis G, Ribaudo MC, Zappaterreno A, Iannucci CV, Leonetti F. Prevalence of uncomplicated obesity in Italian obese population. *Obes Res* 2005;13(6):1116-1122.

30. Loos, R.J.F. and Rankinen, T. Gene-Diet Interactions on Body Weight Changes. *Supplement to the Journal of the American Dietetic Association* 2005;105(5):S29-S34.

31. Hill JO, Wyatt HR, Reed GW, Peters JC. Obesity and the Environment: Where Do We Go from Here? *Science* 2003;299:853-855.

32. Hill JO. Understanding and Addressing the Epidemic of Obesity: An Energy Balance Perspective. *Endocr Rev* 2006. 27(7):750-761.

33. Tremblay A, Perusse L, Bouchard C. Energy balance and body-weight stability: impact of gene-environment interactions. *Br J Nutr* 2004;92(S1):S63-S66.

34. Nativio, D.G. The Genetics, Diagnosis, and Management of Prader-Willi Syndrome. *J Pediatr Health Care* 2002;16(6):298-303.

35. Bell, C.G., Walley, A.J., Froguel, P. The Genetics of Human Obesity. *Nat Rev Genet* 2005; 6:221-234.

36. Montague CT, Farooqi IS, Whitehead JP, Soos MA, Rau H, Wareham NJ, Sewter CP, Digby JE, Mohammed SN, Hurst JA, Cheetham CH, Earley AR, Barnett AH, Prins JB, O'Rahilly S. Congenital leptin deficiency is associated with severe early-onset obesity in humans. *Nature* 1997;387:903-908.

37. Clément K, Vaisse C, Lahlou N, Cabrol S, Pelloux V, Cassuto D, Gourmelen M, Dina C, Chambaz J, Lacorte JM, Basdevant A, Bougnères P, Lebouc Y, Froguel P, Guy-Grand B. A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature* 1998;392:398-401.

38. Lubrano-Berthelier C, Le Stunff C, Bougneres P, Vaisse C. A homozygous null mutation delineates the role of the melanocortin-4 receptor in humans. *J Clin Endocrinol Metab* 2004;89:2028-2032.

39. Rankinen T, Zuberi A, Chagnon YC, Weisnagel SJ, Argyropoulos G, Walts B, Perusse L, Bouchard C. The Human Obesity Gene Map: The 2005 Update. *Obesity* 2006;14(4):529-644.

40. Loos, R.J.F. and Bouchard, C. Obesity – is it a genetic disorder? *J Intern Med* 2003;254:401-425.

41. Marti A, Moreno-Aliaga MJ, Hebebrand J, Martinez JA. Genes, lifestyles and obesity. *Int J Obes Relat Metab Disord* 2004;28(S3):S29-S36.

42. Boutin P, Dina C, Vasseur F, Dubois S, Corset L, Séron K, Bekris L, Cabellon J, Neve B, Vasseur-Delannoy V, Chikri M, Charles MA, Clement K, Lernmark A, Froguel P *GAD2* on chromosome 10p12 is a candidate gene for human obesity. *PLoS Biol* 2003;1(3):E68.

43. Meyre D, Bouatia-Naji N, Tounian A, Samson C, Lecoeur C, Vatin V, Ghoussaini M, Wachter C, Hercberg S, Charpentier G, Patsch W, Pattou F, Charles MA, Tounian P, Clément K, Jouret B, Weill J, Maddux BA, Goldfine ID, Walley A, Boutin P, Dina C, Froguel P.. Variants of *ENPP1* are associated with childhood and adult obesity and increase the risk of glucose intolerance and type 2 diabetes. *Nature Genet* 2005;37(8):863–867.

44. Suviolahti E, Oksanen LJ, Ohman M, Cantor RM, Ridderstrale M, Tuomi T, Kaprio J, Rissanen A, Mustajoki P, Jousilahti P, Vartiainen E, Silander K, Kilpikari R, Salomaa V, Groop L, Kontula K, Peltonen L, Pajukanta P The

SLC6A14 gene shows evidence of association with obesity. *J Clin. Invest* 2003;112(11):1762–72.

45. Durand E, Boutin P, Meyre D, Charles MA, Clement K, Dina C, Froguel P. Polymorphisms in the amino acid transporter solute carrier family 6 (neurotransmitter transporter) member 14 gene contribute to polygenic obesity in French Caucasians. *Diabetes* 2004;53(9):2483–2486.

46. Saunders CL, Chiodini BD, Sham P, Lewis CM, Abkevich V, Adeyemo AA, et al. Meta-analysis of genome-wide linkage studies in BMI and obesity. *Obesity* 2007;15(9):2263–2275.

47. Frayling TM, Timpson NJ, Weedon MN, Zeggini E, Freathy RM, Lindgren CM, et al. A common variant in the *FTO* gene is associated with body mass index and predisposes to childhood and adult obesity. *Science* 2007;316(5826):889–894.

48. Dina C, Meyre D, Gallina S, Durand E, Körner A, Jacobson P, et al. Variation in *FTO* contributes to childhood obesity and severe adult obesity. *Nature Genet* 2007;39(6):724–726.

49. Sun Y, Sun J, Wang X, You W, Yang M. Variants in the fat mass and obesity associated (FTO) gene are associated with obesity and C-reactive protein levels in Chinese Han populations. *Clin Invest Med* 2010;33(6):E405-412.

50. Wing MR, Ziegler JM, Langefeld CD, Roh BH, Palmer ND, Mayer-Davis EJ, Rewers MJ, Haffner SM, Wagenknecht LE, Bowden DW. Analysis of FTO gene variants with obesity and glucose homeostasis measures in the multiethnic Insulin Resistance Atherosclerosis Study cohort. *Int J Obes* 2010; In press. doi: 10.1038/ijo.2010.244.

51. Mei H, Chen W, Srinivasan SR, Jiang F, Schork N, Murray S, Smith E, So JD, Berenson GS. FTO influences on longitudinal BMI over childhood and adulthood and modulation on relationship between birth weight and longitudinal BMI. *Hum Genet* 2010;128(6):589-96.

52. Loos RJ, Lindgren CM, Li S, Wheeler E, Zhao JH, Prokopenko I, Inouye M, et al. Common variants near *MC4R* are associated with fat mass, weight and risk of obesity. *Nature Genet* 2008;40(6):768–775.

53. Chambers JC, Elliott P, Zabaneh D, Zhang W, Li Y, Froguel P, Balding D, Scott J, Kooner JS. Common genetic variation near *MC4R* is associated with waist circumference and insulin resistance. *Nature Genet* 2008;40(6):716–188.

54. Meyre D, Delplanque J, Chèvre JC, Lecoeur C, Lobbens S, Gallina S, et al. Genome-wide association study for early-onset and morbid adult obesity identifies three new risk loci in European populations. *Nature Genet* 2009;41(2):157–159.

55. The Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature. 2007;447(7145):661-78.

56. Bouchard C. Gene-environment interactions in the etiology of obesity: Defining the fundamentals. *Obesity* 2008;16(Suppl 3):S5-S10.

57. Ravussin E, Valencia ME, Esparza J, Bennett PH, Schulz O. Effects of a traditional lifestyle on obesity in Pima Indians. *Diabetes Care*. 1994;17:1067-1074.

58. Afman L, Muller M. Nutrigenomics: from molecular nutrition to prevention of disease. *J Am Diet Assoc* 2006;106:569-576.

59. Poehlman ET, Despres JP, Marcotte M, Tremblay A, Theriault G, Bouchard C. Genotype dependency of adaptation in adipose tissue metabolism after short-term overfeeding. *Am J Physiol* 1986;250:E480-E485.

60. Bouchard C, Tremblay A, Despres JP, Nadeau A, Lupien PJ, Theriault G, Dussault J, Moorjani S, Pinault S, Fournier G. The response to long-term overfeeding in identical twins. *New Engl J Med* 1990;322:1477-1482.

61. Bouchard C, Tremblay A, Despres JP, Theriault G, Nadeau A, Lupien PJ, Moorjani S, Prud'homme D, Fournier G. The response to exercise with constant energy intake in identical twins. *Obes Res* 1994;2:400-410.

62. Tremblay A, Poehlman ET, Despres JP, Theriault G, Danforth E, Bouchard C. Endurance training with constant energy intake in identical twins: changes over time in energy expenditure and related hormones. *Metabolism* 1997;46:499-503.

63. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994;372(6505):425-32.

64. Lord GM, Matarase G, Howard JK, Baker RJ, Bloom SR, Lechler RI. Leptin modulates the T-cell imunne response and reverses starvation-induced immunosupression. *Nature* 1998;394:897-901.

65. Ge H, Huang L, Pourbahrami T, Li C. Generation of soluble leptin receptor by ectodomain shedding of membrane-spanning receptors in vitro and in vivo. *J Biol Chem* 2002;277:45898-45903.

66. Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. *Nature* 1998;395:763-770.

67. Farooqi IS, Wangensteen T, Collins S, Kimber W, Matarese G, Keogh JM, et al. Clinical and molecular genetic spectrum of congenital deficiency of the leptin receptor. *N Engl J Med* 2007;356:237-247.

68. Flier JS. Obesity wars: molecular progress confronts an expanding epidemic. *Cell* 2004;116:337-350.

69. Chin-Chance C, Polonsky KS, Schoeller DA. Twenty-four-hour leptin levels respond to cumulative short-term energy imbalance and predict subsequent intake. *J Clin Endocrinol Metab* 2000;85(8):2685-91.

70. Strobel A, Issad T, Camoin L, Ozata M, Strosberg AD. A leptin missense mutation associated with hypogonadism and morbid obesity. *Nat Genet* 1998;18:213-215.

71. Blum WF, Englaro P, Hatsch S, et al. Plasma leptin levels in healthy children and adolescents: dependence on BMI, body fat mass, gender, pubertal stage, and testosterone. *Clin Endocrinol Metab* 1997;82:2904-2910.

72. Castracane VD, Kraemer RR, Franken MA, Kraemer GR, Gimpel T. Serum leptin concentration in women: effect of age, obesity, and estrogen administration. *Fertil Steril* 1998;70:472-477.

73. Loffreda S, Yang SQ, Lin HZ, Karp CL, Brengman ML, Wang DJ, et al. Leptin regulates proinflammatory immune responses. *FASEB J* 1998;12:57-65.

74. Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, et al. Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat Med* 2002;8:731-737.

75. Nawrocki AR, Rajala MW, Tomas E, Pajvani UB, Saha AK, Trumbauer ME, et al. Mice lacking adiponectin show decreased hepatic insulin sensitivity and reduced responsiveness to PPARγ-agonists. *J Biol Chem* 2006;281:2654-2660.

76. Kim JY, van de WE, Laplante M, Azzara A, Trujillo ME, Hofmann SM, et al. Obesity-associated improvements in metabolic profile through expansion of adipose tissue. *J Clin Invest* 2007;117:2621-2637.

77. Kern PA, Di Gregorio GB, Lu T, Rassouli N, Ranganathan G. Adiponectin expression from human adipose tissue: relation to obesity, insulin resistance, and tumor necrosis factor-α expression. *Diabetes* 2003;52:1779-1785.

78. Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, Tataranni PA. Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *J Clin Endocrinol Metab* 2001;86:1930-1935.

79. Trujillo ME, Scherer PE. Adiponectin: journey from an adipocyte secretory protein to biomarker of the metabolic syndrome. *J Intern Med* 2005;257:167-175.

80. Samal B, Sun Y, Stearns G, Xie C, Suggs S, McNiece I. Cloning and characterization of the cDNA encoding a novelhuman pre-B-cell colony-enhancing factor. *Molec Cell Biol* 1994;14:1431-1437.

81. Fukuhara A, Matsuda M, Nishizawa M, et al. Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. *Science* 2005;307:426-430.

82. Fukuhara A, Matsuda M, NishizawaM, et al. Retraction. *Science* 2007;318(5850):565.

83. Revollo JR, Korner A, Mills KF, et al. Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. *Cell Metab* 2007;67:796-800.

84. Chen MP, Chung FM, Chang DM et al. Elevated plasma level of visfatin/pre-B cell colony-enhancing factor in patients with type 2 diabetes mellitus. *J Clin Endocrinol Metab* 2006;91:295-299.

85. de Luis DA, Sagrado MG, Aller R, Conde R, Izaola O. Circulating visfatin in obese non-diabetic patients in relation to cardiovascular risk factors, insulin resistance, and adipocytokines: a contradictory piece of the puzzle. *Nutrition* 2010;26(11-12):1130-3.

86. Haider DG, Holzer G, Schaller G et al. The adipokine visfatin is markedly elevated in obese children. *J Pediatr Gastroenterol Nutr* 2006;43:548-549.

87. Ersoy C, Sadikoglu G, Orhan H, Guclu M, Sarandol E, Akgun MD, Ozcakir A, Imamoglu S. Body fat distribution has no effect on serum visfatin levels in healthy female subjects. *Cytokine* 2010;49(3):275-8.

88. Gen R, Akbay E, Muslu N, Sezer K, Cayan F. Plasma visfatin level in lean women with PCOS: relation to proinflammatory markers and insulin resistance. *Gynecol Endocrinol* 2009;25(4):241-5.

89. Hammarstedt A, Pihlajamaki J, Sopasakis VR, Gogg S, Jansson PA, Laakso M, Smith U. Visfatin is an adiopkine, but it is not regulated by thiazolidinediones. *J Clin Endocrinol Metab* 2006;91:1578-1581.

90. Yang Q, Graham TE, Mody N, et al. Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature* 2005;436:356-62.

91. Graham TE, Yang Q, Bluher M, et al. Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. *N Engl J Med* 2006;354:2552-63.

92. Cho YM, Youn B-S, Lee H, et al. Plasma retinol-binding protein-4 concentrations are elevated in human subjects with impaired glucose tolerance and type 2 diabetes. *Diabetes Care* 2006;29:2457-61.

93. Janke J, Engeli S, Boschmann M,et al. Retinol-binding protein 4 in human obesity. *Diabetes* 2006;55:2805-10.

94. Gavi S, Qurashi S, Stuart LM, Lau R, Melendez MM, Mynarcik DC, McNurlan MA, Gelato MC. Influence of age on the association of retinol-binding protein 4 with metabolic syndrome. *Obesity* 2008;16:893-895.

95. Tamori Y, Sakaue H, Kasuga M. RBP4, an unexpected adipokine. *Nat Med* 2006;12:30-31.

96. Haider DG, Schindler K, Mittermayer F, Muller M, Nowotny P, Rieger A, Luger A, Ludvik B, Wolzt M. Effect of rosiglitazine on visfatin and retinol-binding protein-4 plasma concentrations in HIV-positive patients. *Clin Pharmacol Ther* 2007;81:580-585.

97. Jia W, Wu H, Bao Y, Wang C, Lu J, Zhu J, Xiang K. Association of serum retinol-binding protein 4 and visceral adiposity in Chinese subjects with and without type 2 diabetes. *J Clin Endocrinol Metab* 2007;92:3224-3229.

98. Takebayashi K, Suetsugu M, Wakabayashi S, Aso Y, Inukai T. Retinol binding protein-4 levels and clinical features of type 2 diabetes patients. *J Clin Endocrinol Metab* 2007;92:2712-2719.

99. Yao-Borengasser A, Varma V, Bodles AM, Rasouli N, Phanavanh B, Lee MJ, Starks T, Kern LM, Spencer III HJ, Rashidi AA, McGehee Jr RE, Fried SK, Kern PA. Retinol binding protein 4 expression in humans: relationship to insulin resistance, inflammation, and response to pioglitazone. *J Clin Endocrinol Metab* 2007;92:2590-2597.

100. Cardon LR and Bell JI. Association study designs for complex diseases. *Nat Rev Genet* 2001;2:91-99.

101. Tabor et al., 2002 Tabor HK, Risch NJ, Myers RM. Candidate-gene approaches for studying complex genetic traits: practical considerations. *Nat Rev Genet* 2002;3:1-7.

102. Jiang Y, Wilk JB, Borecki I, Williamson S, DeStefano AL, Xu G, Liu J, Ellison RC, Province M, Myers RH. Common variants in the 5' region of the leptin gene are associated with body mass index in men from the National Heart, Lung, and Blood Institute Family Heart Study. *Am J Hum Genet* 2004;75:220-230.

103. Li WD, Reed DR, Lee JH, Xu W, Kilker RL, Sodam BR, Price RA. Sequence variants in the 5' flanking region of the leptin gene are associated with obesity in women. *Ann Hum Genet* 1999;63(Pt 3):227-34.

104. Chagnon YC, Wilmore JH, Borecki IB, Gagnon J, Pérusse L, Chagnon M, Collier GR, Leon AS, Skinner JS, Rao DC, Bouchard C. Associations between the leptin receptor gene and adiposity in middle-aged Caucasian males from the HERITAGE family study. *J Clin Endocrinol Metab* 2000;85(1):29-34.

105. Roth H, Korn T, Rosenkranz K, Hinney A, Ziegler A, Kunz J, Siegfried W, Mayer H, Hebebrand J, Grzeschik KH. Transmission disequilibrium and sequence variants at the leptin receptor gene in extremely obese German children and adolescents. *Hum Genet* 1998;103(5):540-6.

106. Bouatia-Naji N, Meyre D, Lobbens S, Séron K, Fumeron F, Balkau B, Heude B, Jouret B, Scherer PE, Dina C, Weill J, Froguel P. ACDC/adiponectin polymorphisms are associated with severe childhood and adult obesity. *Diabetes* 2006;55:545–550.

107. Sutton BS, Weinert S, Langefeld CD, Williams AH, Campbell JK, Saad MF, Haffner SM, Norris JM, Bowden DW. Genetic analysis of adiponectin and

obesity in Hispanic families: the IRAS Family Study. *Hum Genet* 2005;117:107–118.

108. Vimaleswaran KS, Radha V, Ramya K, Babu HN, Savitha N, Roopa V, Monalisa D, Deepa R, Ghosh S, Majumder PP, Rao MR, Mohan V. A novel association of a polymorphism in the first intron of adiponectin gene with type 2 diabetes, obesity and hypoadiponectinemia in Asian Indians. *Hum Genet* 2008;123:599–605.

109. Benzinou M, Chèvre JC, Ward KJ, Lecoeur C, Dina C, Lobbens S, Durand E, Delplanque J, Horber FF, Heude B, Balkau B, Borch-Johnsen K, Jørgensen T, Hansen T, Pedersen O, Meyre D, Froguel P. Endocannabinoid receptor 1 gene variations increase risk for obesity and modulate body mass index in European populations. *Hum Mol Genet* 2008;17:1916–1921.

110. Thomas, G. N., Tomlinson, B. & Critchley, J. A. Modulation of blood pressure and obesity with the dopamine D2 receptor gene *Taq* I polymorphism. *Hypertension* 2000;36:177–182.

111. Epstein LH, Temple JL, Neaderhiser BJ, Salis RJ, Erbe RW, Leddy JJ. Food reinforcement, the dopamine D2 receptor genotype, and energy intake in obese and nonobese humans. *Behav Neurosci* 2007;121:877–886.

112. McCarthy S, Mottagui-Tabar S, Mizuno Y, Sennblad B, Hoffstedt J, Arner P, Wahlestedt C, Andersson B. Complex *HTR2C* linkage disequilibrium and promoter associations with body mass index and serum leptin. *Hum Genet* 2005;117:545–557.

113. Pooley EC, Fairburn CG, Cooper Z, Sodhi MS, Cowen PJ, Harrison PJ. A 5-HT_{2C} receptor promoter polymorphism (HTR2C - 759C/T) is associated with obesity in women, and with resistance to weight loss in heterozygotes. *Am J Med Genet B Neuropsychiatr Genet* 2004;126B:124–127.

114. Fuemmeler BF, Agurs-Collins TD, McClernon FJ, Kollins SH, Kail ME, Bergen AW, Ashley-Koch AE. Genes implicated in serotonergic and dopaminergic functioning predict BMI categories. *Obesity* 2008;16:348–355.

115. Christensen K, Murray JC. What genome-wide association studies can do for medicine. *N Engl J Med* 2007;356:1094-1097.

116. Willer CJ, Speliotes EK, Loos RJ, Li S, Lindgren CM, Heid IM, et al. Six new loci associated with body mass index highlight a neuronal influence on body weight regulation. *Nat Genet* 2009;41(1):25-34.

117. Karns R, Zhang G, Jeran N, Havas-Augustin D, Missoni S, Niu W, Indugula SR, Sun G, Durakovic Z, Narancic NS, Rudan P, Chakraborty R, Deka R. Replication of genetic variants from genome-wide association studies with metabolic traits in an island population of the Adriatic coast of Croatia. *Eur J Hum Genet* 2010 In Press. doi 10.1038/ejhg.2010.178.

118. Quackenbush J. Computational analysis of microarray data. *Nat Rev Genet* 2001;2:418-427.

119. Soukas A, Cohen P, Socci ND, Friedman JM. Leptin-specific patterns of gene expression in white adipose tissue. *Genes Dev* 2000;14:963-980.

120. Schutz Y. Human overfeeding experiments: potentials and limitations in obesity research. *Br J Nutr* 2000;84:135-7.

121. Sims EA, Goldman RF, Gluck CM, Horton ES, Kelleher PC, Rowe DW. Experimental obesity in man. *Trans Assoc Am Physicians* 1968;81:153-70.

122. Tremblay A, Plourde G, Despres JP, Bouchard C. Impact of dietary fat content and fat oxidation on energy intake in humans. *Am J Clin Nutr* 1989;49:799-805.

123. World Health Organization. Obesity and Overweight: Fact Sheet. 2003.

124. Patterson RE, Frank LL, Kristal AR, White E. A comprehensive examination of health conditions associated with obesity in older adults. *Am J Prev Med* 2004;27(5):385-90.

125. World Health Organization Technical Report Series. Obesity: preventing and managing the global epidemic. Report of a WHO consultation. 2000;894:1-253.

126. Aronne LJ, Segal KR. Adiposity and Fat Distribution Outcome Measures: Assessment and Clinical Implications. *Obesity Research* 2002;10(1):14S-21S.

127. Smalley KJ, Knerr AN, Kendrick ZV, et al. Reassessment of body mass indices. *Am J Clin Nutr* 1990;52(3):405-8.

128. Gallagher D, Visser M, Sepulveda D, et al. How useful is body mass index for comparison of body fatness across age, sex, and ethnic groups? *Amer J Epidemiol* 1996;143(3):228-39.

129. Prentice AM, Jebb SA. Beyond body mass index. *Obes Rev* 2001;2(3):141-7.

130. Gallagher D, Heymsfield SB, Heo M. et al. Healthy percentage body fat ranges: an approach for developing guidelines based on body mass index. *Amer J Clin Nutr* 2000;72:694-701.

131. Flegal KM, Graubard BI, Williamson DF, Gail MH. Cause-specific excess deaths associated with underweight, overweight, and obesity. *JAMA* 2007;298:2028-37.

132. Yusuf S, Hawken S, Ounpuu S, et al. Obesity and the risk of myocardial infarction in 27 000 participants from 52 countries: a case-control study. *Lancet* 2005;366:1640-9.

133. Price GM, Uauy R, Breeze E, Bulpitt CJ, Fletcher AE. Weight, shape, and mortality risk in older persons: elevated waist-hip ratio, not high body mass index, is associated with a greater risk of death. *Amer J Clin Nutr* 2006;84:449-60.

134. Martin G, Loredo JC, Sun G. Lack of association of ghrelin precursor gene variants and % body fat or serum lipid profiles. *Obesity* 2008;16:908-12.

135. Randell E, Mathews M, Gadag V, *et al.* Relationship between serum magnesium values, lipids and anthropometric risk factors. *Atherosclerosis* 2008;196(1):413-9.

136. Sun G, Vasdev S, Martin G, Gadag V, Zhang HW. Altered calcium homeostasis is correlated with abnormalities of fasting serum glucose, insulin resistance and β-cell function in the Newfoundland population. *Diabetes* 2005;54:3336-9.

137. Bray GA. Contemporary diagnosis and management of obesity and the metabolic syndrome. 3rd ed. Newtown, PA: Handbooks in Health Care, 2003.

138. Norcross J, Van Loan MD. Validation of fan beam dual energy x ray absorptiometry for body composition assessment in adults aged 18-45 years. *Br J Sports Med* 2004;38:472-6.

139. Frankenfield DC, Rowe WD, Cooney RN. Limits of Body Mass Index to Detect Obesity and Predict Body Composition. *Nutrition* 2001;17:26-30.

140. Curtin F, Morabia A., Pichard C., *et al.* Body Mass Index Compared to Dual-Energy X-Ray Absorptiometry: Evidence for a Spectrum Bias. *J Clin Epidemiol* 1997;50(7):837-43.

141. Adams TD, Heath EM, LaMonte MJ, et al. The relationship between body mass index and per cent body fat in the severely obese. *Diabetes Obes Metab* 2007;9:498–505.

142. Beaufrere B, Morio B. Fat and protein redistribution with aging: metabolic considerations. *Eur J Clin Nutr* 2000;54(3):S48-S53.

143. Bray GA. Contemporary Diagnosis and Management of Obesity. Newtown, PA: Handbooks in Health Care. 1998.

144. Romero-Corral A, Somers VK, Sierra-Johnson et al. Diagnostic performance of body mass index to detect obesity in patients with coronary artery disease. *Eur Heart J* 2007;28(17):2087-93.

145. Blew RM, Sardinha LB, Milliken LA et al. Assessing the validity of body mass index standards in early postmenopausal women. *Obes Res* 2002;10(8):799-808.

146. Piers LS, Soares MJ, Frandsen SL, O'Dea K. Indirect estimates of body composition are useful for groups but unreliable in individuals. *Int J Obes Relat Metab Disord.* 2000;24(9):1145-52.

147. Roubenoff R, Kehayias JJ, Dawson-Hughes B, Heymsfield SB. Use of dual-energy x-ray absorptiometry in body composition studies: not yet a "gold standard". *Am J Clin Nutr* 1993;58:589-591.

148. Evans EM, Rowe DA, Racette SB, et al. Is the current BMI obesity classification appropriate for black and white postmenopausal women? *Int J Obes (Lond)* 2006;30:837 – 843.

149. Ode JJ, Pivarnik JM, Reeves MJ, et al. Body mass index as a predictor of percent fat in college athletes and nonathletes. *Med Sci Sports Exerc.* 2007;39(3):403-9.

150. Kim SH, Abbasi F, Reaven GM. Impact of degree of obesity on surrogate estimates of insulin resistance. *Diabetes Care* 2004;27:1998-2002.

151. Dalton M, Cameron AJ, Zimmet PZ, et al. Waist circumference, waist-hip ratio and body mass index and their correlation with cardiovascular disease risk factors in Australian adults. *J Intern Med* 2003;254:555-563.

152. Hu D, Hannah J, Gray RS, et al. Effects of obesity and body fat distribution on lipids and lipoproteins in nondiabetic merican Indians: The Strong Heart Study. *Obes Res* 2000;8:411-421.

153. Saito M, Ishimitsu T, Minami J, Ono H, Ohrui M, Matsukoka H. Relations of plasma high-sensitivty C-reactive protein to traditional cardiovascular risk factors. *Atherosclerosis* 2003;167:73-79.

154. De Lorenzo A, Martinoli R, Vaia F, Di Renzo L. Normal weight obese (NWO) women: An evaluation of a candidate new syndrome. *Nutr Metab Cardiovasc Dis* 2006;16:513-523.

155. Sims EA. Are there persons who are obese, but metabolically healthy? *Metabolism* 2001;50:1499-1504.

156. Dvorak RV, DeNino WF, Ades PA, Poehlman ET. Phenotypic characteristics associated with insulin resistance in metabolically obese but normal weight young women. *Diabetes* 1999;48:2210-2214.

157. Kennedy AP, Shea JL, Sun G. Comparison of the Classification of Obesity by BMI Versus Dual-Energy X-Ray Absorptiometry in the Newfoundland Population. *Obesity* 2009; 17(11): 2094-2099.

158. Lesser GT. Issues in body fatness. *Arch Intern Med* 2009;169(6):636.

159. Shea JL, Loredo-Osti, JC, Sun G. Association of RBP4 Gene Variants and Serum HDL Cholesterol Levels in the Newfoundland Population. *Obesity* 2010;18(7):1393-1397.

160. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985;28:412-419.

161. Grundy SM, Cleeman JI, Merz CN, et al. Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III Guidelines. *Circulation* 2004;110(2):227-239.

162. Karelis AD, Rabasa-Lhoret R. Inclusion of C-reactive protein in the identification of metabolically healthy but obese (MHO) individuals. *Diabetes Metab* 2008;34:183-184.

163. Meigs JB, Wilson PW, Fox CS, et al. Body mass index, metabolic syndrome, and risk of type 2 diabetes of cardiovascular disease. *J Clin Endocrinol Metab* 2006;91:2906-2912.

164. Roubenoff R. Sarcopenic obesity: the confluence of two epidemics. *Obes Res* 2004;12(6):887-888.

165. Bonora E, Kiechl S, Willeit J, et al. Prevalence of insulin resistance in metabolic disorders: the Bruneck Study. *Diabetes* 1998;47:1643-1649.

166. Karelis AD, Faraj M, Bastard JP, et al. The metabolically healthy but obese individual presents a favorable inflammation profile. *J Clin Endocrinol Metab* 2005;90(7):4145-4150.

167. Esteghamati A, Khalilzadeh O, Anvari M, Ahadi MS, Abbasi M, Rashidi A. Metabolic syndrome and insulin resistance significantly correlate with body mass index. *Arch Med Res* 2008;39:8

168. Shea JL, Randell E, Sun G. The prevalence of metabolically healthy obese subjects defined by BMI and dual energy x-ray absroptiometry. *Obesity* 2010; doi:10.1038/oby.2010.174.

169. Romero-Corral A, Somers VK, Sierra-Johnson J, Korenfeld Y, Boarin S, Korinek J, et al. Normal weight obesity: a risk factor for cardiometabolic dysregulation and cardiovascular mortality. *Eur Heart J* 2010;31(6):737-746.

170. Marques-Vidal P, Pecoud A, Hayoz D, Paccaud F, Mooser V, Waeber G, et al. Normal weight obesity: Relationship with lipids, glyaemic status, liver enzymes and inflammation. *Nutr Metab Cardiovasc Dis* 2009;doi:10.1016/j.numecd.2009.06.001.

171. Sun G, French CR, Martin GR, Younghusband B, Green RC, Xie Y, et al. Comparison of multifrequency bioelectrical impedance analysis with dual-energy X-ray absorptiometry for assessment of percentage body fat in a large, healthy population. *Am J Clin Nutr* 2005;81:74-78.

172. Fox CS, Masaro JM, Hoffman U, Pou KM, Maurovich-Horvat P, Liu C-Y, et al. abdominal visceral and subcutaneous adipose tissue compartments: Association with metabolic risk factors in the Framingham Heart Study. *Circulation* 2007;116(1):39-48.

173. Preis SR, Massaro JM, Robins SJ, Hoffmann U, Vasan RS, Irlbeck T, et al. Abdominal subcutaneous and visceral adipose tissue and insulin resistance in the Framingham Heart Study. *Obesity* 2010;doi:10.1038/oby.2010.59.

174. St. Onge M-P, Janssen I, Heymsfield SB. Metabolic syndrome in normal-weight Americans. *Diabetes Care* 2004;27(9):2222-2228.

175. Succurro E, Marini MA, Fronton S, Hribal ML, Andreozzi F, Lauro R, et al. Insulin secretion in metabolically obese, but normal weight, and in metabolically healthy but obese individuals. *Obesity* 2008;16:1881-1886.

176. Varma V, Yao-Borengasser A, Rasouli N et al. Human visfatin expression: relationship to insulin sensitivity, intramyocellular lipids, and inflammation. *J Clin Endocrinol Metab* 2007;92:666-672.

177. Curat CA, Wegner V, Sengenes C et al. Macrophages in human visceral adipose tissue: increased accumulation in obesity and a source of resistin and visfatin. *Diabetologia* 2006;49:744-747.

178. Berndt J, Kloting N, Kralisch S et al. Plasma visfatin concentrations and fat depot-specific mRNA expression in humans. *Diabetes* 2005;54:2911-2916.

179. Sun G, Bishop J, Khalili S, et al. Serum visfatin concentrations are positively correlated with serum triacylglycerols and down-regulated by overfeeding in healthy young men. *Am J Clin Nutr* 2007:85(2):399-404.

180. Ognjanovic S, Bao S, Yamamoto SY, Garibay-Tupas J, Samal B, Bryant-Greenwood GD. Genomic organization of the gene coding for human pre-B-cell colony enhancing factor and expression in human fetal membranes. *J Mol End*ocrinol 2001;26:107-117.

181. Adeyemo AA, Johnson T, Acheampong J, et al. A genome-wide quantitative trait linkage analysis for serum lipids in type 2 diabetes in an African population. *Atherosclerosis* 2005;181:389-397.

182. Arya R, Blangero J, Williams K, et al. Factors of insulin resistance syndrome – related phenotypes are linked to genetic locations on chromosomes 6 and 7 in non-diabetic Mexican Americans. *Diabetes* 2002;51:841-847.

183. Wu X, Cooper RS, Borecki I, et al. A combined analysis of genome-wide linkage scans for body mass index from the National Heart, Lung, and Blood Institute Family Blood Pressure Program. *Am J Hum Genet* 2002;70:1247-1256.

184. Zhang Y-Y, Gottardo L, Thompson R, et al. A visfatin promoter polymorphism is associated with low-grade inflammation and type 2 diabetes. *Obesity* 2006;14(12):2119-2126.

185. Bailey SD, Loredo-Osti JC, Lepage P, et al. Common polymorphisms in the promoter of the visfatin gene (*PBEF1*) influence plasma insulin levels in a French-Canadian population. *Diabetes* 2006;55:2896-2902.

186. Bottcher Y, Teupser D, Enigk B, et al. Genetic variation in the visfatin gene (PBEF1) and its relation to glucose metabolism and fat-depot-specific messenger ribonucleic acid exression in humans. *J Clin Endocrinol Metab* 2006;91(7):2725-2731.

187. Johansson LM, Johansson LE, Ridderstrale M. The visfatin (*PBEF1*) G-948T gene polymorphism is associated with increased high-density lipoprotein cholesterol in obese subjects. *Metabolism* 2008;57(11):1558-1562.

188. Tokunga A, Miura A, Okauchi Y, et al. The -1535 promoter variant of the visfatin gene is associated with serum triglyceride and HDL-cholesterol levels in Japanese subjects. *Endocr J* 2008;55(1):205-212.

189. Yan J-J, Tang N-P, Tang J-J, et al. Genetic variant in visfatin gene promoter is associated with decreased risk of coronary artery disease in a Chinese population. *Clin Chim Acta* 2010;411:26-30.

190. Blakemore AIF, Meyre D, Delplanque J, et al. A rare variant in the visfatin gene (NAMPT/PBEF1) is associated with protection from obesity. *Obesity* 2009;17(8):1549-1553.

191. Jian W-X, Luo T-H, Gu Y-Y, et al. The visfatin gene is associated with glucose and lipid metabolism in a Chinese population. *Diabet Med* 2006;23(9):967-973.

192. Korner A, Bottcher Y, Enigk B, Kiess W, Stumvoll M, Kovacs P. Effects of genetic variation in the visfatin gene (*PBEF1*) on obesity, glucose metabolism, and blood pressure in children. *Metabolism* 2007;56:772-777.

193. Catalan V, Gomez-Ambrosi J, Rodriguez A, et al. Association of increased visfatin/PBEF/NAMPTcirculating concentrations and gene expression levels in peripheral blood cells with lipid metabolism and fatty liver in human morbid obesity. *Nutr Metab Cardiovasc Dis* 2010 In Press. doi 10.1016/j.numecd.2009.09.008.

194. Liu SW, Qiao SB, Yuan JS, Liu DQ. Association of plasma visfatin levels with inflammation, atherosclerosis and acute coronary syndromes (ACS) in humans. *Clin Endocrinol (Oxf)* 2009;71(2):202-207.

195. Wang LS, Yan JJ, Tang NP et al. A polymorphism in the visfatin gene promoter is related to decreased plasma levels of inflammatory markers in patients with coronary artery disease. *Mol Biol Rep* 2010 In Press. doi 10.1007/s11033-010-0171-6.

196. Shea J, Randell E, Vasdev S, Wang P, Sun G. Serum retinol-binding protein 4 concentrations in response to short term overfeeding in normal weight, overweight and obese men. *Am J Clin Nutr* 2007;86(5):1310-1315.

197. Promintzer M, Krebs M, Todoric J, *et al.* Insulin resistance is unrelated to circulating retinol binding protein and protein C inhibitor. *J Clin Endocrinol Metab* 2007;92:4306-4312.

198. Meigs JB, Panhuysen CIM, Myers RH, Wilson PWF, Cupples LA. A Genome-Wide Scan for Loci Linked to Plasma Levels of Glucose and HbA_{1c} in a Community-Based Sample of Caucasian Pedigrees. *Diabetes* 2002;51:833-840.

199. Duggirala R, Blangero J, Almasy L, *et al.* Linkage of Type 2 Diabetes Mellitus and of Age at Onset to a Genetic Location on Chromosome 10q in Mexican Americans. *Am J Hum Genet* 1999;64:1127-1140.

200. Craig RL, Chu WS, Elbein SC. Retinol binding protein 4 as a candidate gene for type 2 diabetes and prediabetic intermediate traits. *Mol Genet Metab* 2007;90:338-344.

201. Kovacs P, Geyer M, Berndt J, *et al.* Effects of genetic variation in the human retinol binding protein-4 gene (*RBP4*) on insulin resistance and fat depot-specific mRNA expression. *Diabetes* 2007;56:3095-3100.

202. Munkhtulga L, Nakayama K, Utsumi N, *et al.* Identification of a regulatory SNP in the retinol binding protein 4 gene associated with type 2 diabetes in Mongolia. *Hum Genet* 2007;120:879-888.

203. Hu C, Jia R, Zhang R, *et al.* Effect of *RBP4* gene variants on circulating RBP4 concentration and type 2 diabetes in a Chinese population. *Diabet Med* 2008;25(1):11-18.

204. Liu J, Gao J, Zhang J, *et al.* Evaluation of the association between retinol binding protein 4 polymorphoisms and type 2 diabetes in Chinese by DHPLC. *Endocr* 2008;34:23-28.

205. Von Eyatten M, Lepper PM, Liu D, *et al.* Retinol-binding protein 4 is associated with components of the metabolic syndrome, but not with insulin resistance, in men with type 2 diabetes or coronary artery disease. *Diabetologia* 2007;50:1930-1937.

206. Wu Y, Li H, Loos RJF, *et al.* Genetic variation in the RBP4 gene, plasma retinol binding protein-4 levels, and hypertriglyceridemia risk in a Chinese Han Population. *J Lipid Res* 2009;50(7):1479-86

207. Van Hoek M, Dehghan A, Zillikens MC, Hofman A, Witteman JC, Sijbrands EJG. An *RBP4* promoter polymorphism increases risk of type 2 diabetes. *Diabetologia* 2008;41:1423-1428.

208. Qi Q, Yu Z, Ye X, *et al.* Elevated retinol-binding protein 4 levels are associated with metabolic syndrome in Chinese people. *J Clin Endocrinol Metab* 2007;92(12):4827-4834.

209. Ingelsson E, Sundström J, Melhus H, *et al.* Circulating retinol-binding protein 4, cardiovascular risk factors and prevalent cardiovascular disease in elderly. *Atherosclerosis* 2009;206(1):239-244.

210. Lee DC, Lee JW, Im JA. Association of serum retinol binding protein 4 and insulin resistance in apparently healthy adolescents. *Metabolism* 2007;56(3):327-331.

211. Kahn BB, Flier JS. Obesity and insulin resistance. *J Clin Invest* 2000;106:473-481.

212. Mlinar, B, Marc J, Janez A, Pfeifer M. Molecular mechanisms of insulin resistance and associated diseases. *Clinica Chimica Acta* 2007;375:20-35.

213. Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab* 2004;89:2548-2556.

214. Ruan H, Lodish HF. Insulin resistance in adipose tissue: direct and indirect effects of tumor necrosis factor-alpha. *Cytokine Growth Factor Rev* 2003;14:447-455.

215. Stefan N, Hennige AM, Staiger H, et al. High circulating retinol-binding protein 4 is associated with elevated liver fat, but not with total-, subcutaneous-, visceral-, or intramyocellular fat in humans. *Diabetes Care* 2007;30(5):1173-1178.

216. Gavi S, Stuart LM, Kelly P, et al. Retinol-binding protein 4 is associated with insulin resistance and body fat distribution in non-obese subjects without type 2 diabetes. *J Clin Endocrinol Metab* 2007;92(5):1886-1890.

217. Mauriege P, Despres JP, Marcotte M, et al. Adipose tissue lipolysis after long-term overfeeding in identical twins. Int J Obes Relat Metab Disord 1992;16:219-25.

218. Imbeault P, Doucet E, Mauriege P. Difference in leptin response to a high fat meal between lean and obese men. *Clinical Science* 2001;101:359-365.

219. Weyer C, Vozarova B, Ravussin E, Tataranni PA. Changes in energy metabolism in response to 48 h of overfeeding and fasting in Caucasians and Pima Indians. *Int J Obes Relat Metab Disord* 2001;25:593-600.

220. Kolaczynski JW, Ohannesian JP, Considine RV, Marco CC, Caro JF. Response of leptin to short-term and prolonged overfeeding in humans. *J Clin Endocrinol Metab* 1996;81:4162-4165.

221. Blaner WS, Goodman DS. Purification and properties of plasma retinol-binding protein. *Methods Enzymol* 1990;189:193-206.

222. Tsutsumi C, Okuno M, Tannous L, et al. Retinoids and retinoid-binding protein expression in rat adipocytes. *J Biol Chem* 1992;267:1805-1810.

223. Zovich DC, Orologa A, Okuno M, et al. Differentiation-dependent expression of retinoid-binding proteins in BFC-1β adipocytes. *J Biol Chem* 1992;267:13884-13889.

224. Christiansen E, Garby L, Sorensen TIA. Quantitative analysis of the energy requirements for development of obesity. *J Theor Biol* 2005;234:99-106.

225. Panagiotakos DB, Pitsavos C, Yannakoulia M, Chrysohoou C, Stefanadis C. The implication of obesity and central fat on markers of chronic inflammation: The ATTICA study. *Atheroscelrosis* 2005;183:308-315.

226. Robertson MD, Henderson RA, Vist GE, Rumsey RDE. Plasma ghrelin response following a period of acute overfeeding in normal weight men. *Int J Obes Relat Metab Disord* 2004;28:727-733.

227. Silha JV, Krsek M, Skrha JV, Sucharda P, Nyomba BLG, Murphy LJ. Plasma resistin, adiponectin and leptin levels in lean and obese subjects: correlations with insulin resistance. *Eur J Endocrinol* 2003;149:331-335.

228. Tataranni PA, Ravussin E. Effect of fat intake on energy balance. *Ann N Y Acad Sci* 1997;819:37-43.

229. Farooqi IS, O'Rahilly S. New advances in the genetics of early onset obesity. *Int J Obes* 2005;29(10);1149-1152.

230. Schousboe K, Willemsen G, Kyvik KO, et al. Sex differences in heritability of BMI: a comparative study of results from twin studies in eight countries. *Twin Res* 2003;6(5):409-421.

231. Halaas JL, Gajiwala KS, Maffei M, et al. Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* 1995;269(5223):543-546.

232. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 1993;259(5091):87-91.

233. Gomez-Ambrosi J, Catalan V, Diez-Caballero A, et al. Gene expression profile of omental adipose tissue in human obesity. *FASEB J* 2004;18(1):215-217.

234. Taleb S, Van Haaften R, Henegar C, et al. Microarray profiling of human white adipose tissue after exogenous leptin injection. *Eur J Clin Invest* 2006;36:153-163.

235. Lee YH, Nair S, Rousseau E, et al. Microarray profiling of isolated abdominal subcutaneous adipocytes from obese vs non-obese Pima Indians: increased expression of inflammation-related genes. *Diabetologia* 2005;48:1776-1783.

236. Duff E, Li CL, Hartzell DL, Choi YH, Della-Fera MA, Baile CA. Ciliary neurotrophic factor injected icv induces adipose tissue apoptosis in rats. *Apoptosis* 2005;9:629-634.

237. Reusch JE, Klemm DJ. Inhibition of cAMP-response element-binding protein activity decreases protein kinase B/Akt expression in 3T3-L1 adipocytes and induces apoptosis. *J Biol Chem*. 2002;277(2):1426-1432.

238. Urs S, Smith C, Campbell B, et al. Gene expression profiling in human preadipocytes and adipocytes by microarray analysis. *J Nutr* 2004;134(4):762-770.

239. Henegar C, Tordjman J, Achard V, et al. Adipose tissue transcriptomic signature highlights the pathological relevance of extracellular matrix in human obesity. *Genome Biol* 2008;9(1):R14.

240. Taleb S, Lacasa L, Bastard J-P, et al. Cathepsin S, a novel marker of adiposity: relevance to atherogenesis. *FASEB J* 2005;19:1540-1542.

241. Bluher M, Williams CJ, Kloting N, et al. Gene expression of adiponectin receptors in human visceral and subcutaneous adipose tissue is related to insulin resistance and metabolic parameters and is altered in response to physical training. *Diabetes Care* 2007; 30(12):3110-3115.

242. Louis E, Raue U, Yang Y, Jemiolo B, Trappe S. Time courses of proteolytic, cytokine, and myostatin gene expression after acute exercise in human skeletal muscle. *J Appl Physiol* 2007;103(5):1744-1751.

243. Lakka HM, Tremblay A, Despres JP, Bouchard C. Effects of long-term negative energy balance with exercise on plasma lipid and lipoprotein levels in identical twins. *Atherosclerosis* 2004;172(1):127-133.

244. Vitkova M, Klimcakova E, Kovacikova M, et al. Plasma levels and adipose tissue messenger rinbonucleic acid expression of retinol-binding protein 4 are reduced during calorie restriction in obese subjects but are not related to diet-induced changes in insulin-sensitivity. *J Clin Endocrinol Metab* 2007;92(6):2330-2335.

245. Das SK, Gilhooly CH, Golden JK, et al. Long-term effects of 2 energy-restricted diets differing in glycemic load on dietary adherence, body composition, and metabolism in CALERIE: a 1-y randomized controlled trial. *Am J Clin Nutr* 2007;85(4):1023-1030.

246. Minehira K, Vega N, Vidal H, Acheson K, Tappy L. Effect of carbohydrate overfeeding on whole body macronutrient metabolism and expression of lipogenic enzymes in adipose tissue of lean and overweight humans. *Int J Obes* 2004;28:1291-1298.

247. Schneider J, Buness A, Huber W, et al. Systematic analysis of T7 RNA polymerase based in vitro linear RNA amplification for use in microarray experiments. *BMC Genomics* 2004;5:29.

248. Stoltzman CA, Peterson CW, Breen KT, Muoio DM, Billin AN, Ayer DE. Glucose sensing by MondoA:Mlx complexes: A role for hexokinases and direct regulation of thioredoxin-interacting protein expression PNAS 2008;105(19):6912-7.

249. Aujla SJ, Chan YR, Zheng M, et al. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia *Nat Med* 2008;14(3):275-281.

250. Seelan RS, Khalyfa A, Lakshmanan J, Casanova MF, Parthasarathy RN. Deciphering the lithium transcriptome: Microarray profiling of lithium-modulated gene expression in human neuronal cells. *Neuroscience* 2008;151(4):1184-1197.

251. Benjamini Y and Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)* 1995;57(1):289-300.

252. Reiner A, Yekutieli D, Benjamini Y. Identifying differentially expressed genes using false discovery rate controlling procedures. *Bioinformatics* 2003;4:R7.

253. Lopez IP, Marti A, Milagro FI, et al. DNA microarray analysis of genes differentially expressed in diet-induced (cafeteria) obese rats. *Obes Res* 2003;11(2):188-194.

254. Scaglia H, Igal RA. Stearoyl-CoA desaturase is involved in the control of proliferation, anchorage-independent growth, and survival in human transformed cells. *J Biol Chem* 2005;280(27):25339-25349.

255. Sass-Kuhn SP, Moqbel R, MackayJA, Cromwell O, Kay AB. Human granulocyte/pollen-inding protein. Recognition and identification as transferrin. *J Clin Invest* 1984;73(1):202-210.

256. Kim JH, Kim Y, Lee SD. Selective activation of phospholipase D2 by unsaturated fatty acid. *FEBS Lett* 1999;454:42-46.

257. Roche E, Buteau J, Aniento I, Reig JA, Soria B, Prentki M. Palmitate and oleate induce the immediate-early response genes c-fos and nur-77 in the pancreatic beta-cel line INS-1. *Diabetes* 1999;48:2007-2014.

258. Listenberger LL, Han X, Lewis SE, et al. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proc Natl Acad Sci* 2003;100:3077-3082.

259. Dobrzyn A and Ntambi JT. Stearoyl-CoA desaturase as a new drug target for obesity treatment. *Obes Rev* 2005;6:169-174.

260. Ausk KJ, Ioannou GN. Is obesity associated with anemia of chronic disease? A population-based study. *Obesity* 2008;16:2356-2361.

261. Paris A, Strukelj B, Pungercar J, Renko M, Dolenc I, Turk V. Molecular cloning and sequence analysis of human preprocathepsin C. *FEBS Lett* 1995;369(2-3):326-330.

262. Sun XJ, Wang LM, Zhang Y, et al. Role of IRS-2 in insulin and cytokine signaling. *Nature* 1995; 377(6545):173-177.

263. Lautier C, El Mkadem SA, Renard E, et al. Complex haplotypes of IRS2 gene are associated with severe obesity and reveal heterogeneity in the effect of Gly1057Asp mutation. Hum Genet 2003;113(1):34-43.

264. Mammarella S, Romano F, Di Valerio A, et al. Interaction between the G1057D variant of IRS-2 and overweight in the pathogenesis of type 2 diabetes. *Hum Mol Genet* 2000;9(17):2517-2521.

265. Rowles J, Scherer SW, Xi T, et al. Cloning and characterization of PDK4 on 7q21.3 encoding a fourth pyruvate dehydrogenase kinase isoenzyme in human. *J Biol Chem* 1996;271(37):22376-22382.

266. Jeoung NH, Wu P, Joshi MA, et al. Role of pyruvate dehydrogenase kinase isoenzyme 4 (PDHK4) in glucose homeostasis during times of starvation. *Biochem J* 2006;397(3):417-425.

267. Da Silva LA, De Marcucci OL, Carmona A. Adaptive changes in total pyruvate dehydrogenase activity in lipogenic tissues of rats fed high-sucrose or high-fat diets. *Comp Biochem Physiol Comp Physiol* 1992;103(2):407-411.

268. Meugnier E, Bossu C, Oliel M, Jeanne S, Michaut A, Sothier M, Brozek J, Rome S, Laville M, Vidal H. Changes in gene expression in skeletal muscle in response to fat overfeeding in lean men. *Obesity* 2007;15(11):2583-2594.