THE PPAR PATHWAY TO OBESITY AND TYPE-2 DIABETES: A MULTI-LOCUS APPROACH TO UNDERSTANDING COMPLEX DISEASE

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Many common diseases such as obesity and type-2 diabetes have a significant genetic component that contributes to susceptibility. Peroxisome proliferator activated receptors (PPARs) are nuclear receptors that heterodimerize with the retinoid X receptors (RXRs) to influence the expression of many genes involved in adipocyte differentiation and lipid metabolism such as the fatty acid binding proteins (FABPs) and the uncoupling proteins (UCPs). Genetic variation in any of these gene families could potentially alter metabolic traits related to obesity and type-2 diabetes. The goal of this project is to identify genetic variation in the PPARs and RXRs and then to determine if this variation is associated to quantitative traits related to obesity and type-2 diabetes using a multi-locus analysis approach. In this study, three sets of regression models were constructed: the first containing polymorphisms in just the PPARs or RXRs; the second with variants from all four gene families; and the third using polymorphisms from the gene isoforms showing the highest level of expression in each of three tissues. Some of the models were only able to account for small portions of the particular trait variation; however, many of the models accounted for a large amount of variation in the trait, up to 23.4% in the Hispanic female model for fasting free fatty acids. Multi-locus genotypes, as opposed to single locus effects, were found to be the best predictors of variation in almost all of the final models. These analyses confirmed the importance of

gene-gene interactions on traits related to obesity and type-2 diabetes such as fasting free fatty acids and cholesterol; therefore, multiple polymorphisms should be considered together to fully understand their influence on a quantitative trait.

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I. Specific Aims

Peroxisome proliferator activated receptors (PPARs) are nuclear receptors that are activated by fatty acids and their derivatives. They act as transcription factors to influence the expression of many genes involved in fatty acid metabolism and adipocyte differentiation. PPARs must form heteroduplexes with the retinoid X receptors (RXRs) before binding to the PPAR response elements (PPREs) in the 5' promoter sequence of the genes that they regulate. Sequence variations in either the PPAR or RXR genes could alter the transcriptional activity, heteroduplex formation, ligand binding, DNA binding or overall stability of the molecules. Genetic variation could also alter regulation of the genes the PPAR:RXR heterodimer controls such as the fatty acid binding proteins (FABPs) or the uncoupling proteins (UCPs). Any of these changes could alter the balance between energy intake and energy expenditure contributing to obesity and/ or type-2 diabetes. Therefore, it is of interest to analyze genetic variation in these genes to determine if they are important factors contributing to obesity or diabetes in the general population. Also, since the RXRs, FABPs, and UCPs are all involved in the PPAR regulatory pathway, there is a potential for interactions between genetic variations in any of these genes that could further contribute to the obese or diabetic phenotype. The specific aims for this project are:

1. To identify common single nucleotide polymorphisms in the promoter, coding sequence or flanking sequences of the genes PPARα, PPARβ, PPARγ, RXRα, and RXRβ.

2. To genotype a sample of 764 individuals participating in the San Luis Valley Diabetes Study for the variations identified in the genes of interest.

3. To test for associations between single locus genotypes and quantitative traits related to obesity and type-2 diabetes and to test for gene-gene interactions by building regression models including genotypic data from polymorphisms in the PPARs, RXRs, FABPs, and UCPs.

II. Background and Significance

A. Genetics of Complex Disease

Many common diseases including obesity, diabetes, and coronary artery disease have a significant genetic component that contributes to susceptibility. It is vital that we understand the biological basis of susceptibility for these common disorders in order to develop rational strategies for treatment and prevention. By identifying genetic factors that influence the risk for common disorders, we can gain a clearer understanding of their etiology and develop more effective interventions. However, common disorders present a new challenge to geneticists. These disorders have both genetic and environmental factors contributing to disease risk and severity. In most cases, the genetic component is thought to be polygenic with many genes having a small effect on risk. In addition, disease susceptibility is genetically heterogeneous both within and between ethnic groups. Gene-gene and gene-environment interactions are also likely to play an important role in the development of these disorders.

Traditional approaches to finding susceptibility loci for complex diseases have not been as successful as when they are applied to monogenic disorders. In a review of 101 linkage studies looking at complex disorders, more than 66% failed to find significant linkage based on the criteria proposed by Lander and Kruglyak (Lander, 1995; Altmuller, 2001). Even when linkage was detected, the significant results within a given disease were often inconsistent (Altmuller, 2001). New techniques need to be developed that are better able to model a polygenic trait. Several groups have found

multiple polymorphisms within a region to be associated with disorders like type-2 diabetes and Crohn's disease, highlighting the need to look at various sites in combination (Gura, 2001). Researchers are in the process of developing new strategies to do just that. The combinatorial partitioning method (CPM) compares all possible genotypic groupings (or partitions) of multiple loci to variation in a quantitative trait in order to determine the most predictive partitions (Nelson, 2001). Unfortunately, questions remain as to the biological significance of these partitions and, as this is a new technology, its availability to the scientific community is limited. Another method based on the CPM, multifactor-dimensionality reduction, is applicable to case-control studies and is computationally more simple but suffers from the same limitations as the CPM (Ritchie, 2001). In addition, scientists have recently begun a new project to construct a haplotype map of the human genome in order to reduce the number of polymorphisms that need to be typed while still retaining most of the genetic information (Helmuth, 2001). In the future, these techniques will likely help us to elucidate the genetics of complex disease, but for now variations on more traditional methods should be explored.

1. Obesity

Obesity is a very common and important health problem in the world, especially in the US. Over one third of Americans are considered overweight or obese by current clinical guidelines (body weight ≥ 120% of ideal body weight) (Solomon, 1997). When height is taken into consideration, approximately 20% of US men and 25% of US

women are considered obese, with a body-mass index (BMI, calculated as kg/m^2) greater than 30 (Kopelman, 2000). Obesity has a severe impact on public health because of its association with other diseases such as hypertension, cardiovascular disease, cancer, and type-2 diabetes (Friedman, 2000). The risk for developing most of these conditions increases not only with greater amounts of excess adipose tissue but also with a central deposition of the fat, a region linked to increased lipolysis and fatty acid production (Pi-Sunyer, 1993;Jensen, 1997).

Obesity occurs when energy intake exceeds energy expenditure and the surplus energy is stored as fat in adipose tissue. In addition to environmental influences such as dietary content and physical activity, there are many physiological pathways that regulate energy balance. Insulin and leptin, two adiposity hormones, circulate at levels corresponding to an individual's body fat and stimulate the central nervous system (CNS) to reduce energy intake thereby helping to regulate weight gain when adipose stores are at sufficient levels (Schwartz, 2000). However, many obese individuals are both insulin and leptin resistant and require higher levels of the hormones, and therefore adipose tissue, to produce a response by the CNS (Friedman, 2000). Increased adipose tissue lipolysis and subsequent elevation of free fatty acids is also associated with insulin resistance (Kopelman, 2000). In addition, energy expenditure in the form of heat is under close physiological control and disruptions in these thermogenic pathways are thought to contribute to the development of obesity (Lowell, 2000).

Mutations in genes such as leptin, the leptin receptor and the melanocortin-4 receptor can lead to monogenic forms of obesity, but these simple genetic forms of obesity are very rare in the general population (Barsh, 2000). Many potential susceptibility loci for obesity have been identified through various forms of linkage studies. Human chromosome regions such as 2p (pro-opiomelanocortin), 7q15 (neuropeptide Y), 7q31 (leptin), 10p, and 20q13 have been linked to obesity traits (Bray, 1999; Hager, 1998; Lee, 1999). When potential human homologs for mouse qualitative traits are included in the list of potential obesity susceptibility loci, every human chromosome except the Y chromosome shows at least one region of interest (Barsh, 2000). Another approach to finding genes contributing to obesity is to identify polymorphisms in candidate genes known to regulate energy balance then look for associations with obese phenotypes. The candidate gene approach has identified a long list of possible obesity related genes, but the results of these candidate gene association studies have been inconsistent among various populations and in some cases the functional significance of the identified polymorphism is questionable (Barsh, 2000).

2. Type-2 Diabetes

Type-2 diabetes mellitus or non-insulin dependant diabetes mellitus occurs when glucose homeostasis is altered due to impaired insulin action on peripheral tissues and/ or abnormal insulin secretion (Kahn, 1998). Type-2 diabetes is the major cause of blindness, renal failure and lower limb amputations in adults and is showing an

increasing prevalence in most populations (O'Rahilly, 1997). Obesity, family history, diet and physical inactivity are all major risk factors for developing type-2 diabetes Metabolic changes such as insulin resistance, dyslipidaemia and high blood pressure are other risk factors that often appear before the clinical disease is evident (Groop, 1997). Elevated levels of free fatty acids are also a strong predictor of diabetes and correlate with hepatic glucose output, a major cause of diabetic hyperglycemia (Bergman, 2000; Saltiel, 1996). In addition, high levels of circulating free fatty acids have been shown to cause pancreatic β cell apoptosis in obese rats linking increased adipocyte lipolysis to diabetic pancreatic dysfunction (Shimabukuro, 1998).

Several human monogenic forms of diabetes have been identified including: maturity-onset diabetes of the young (MODY) which can be caused by mutations in the glucokinase gene; extreme insulin resistance caused by a defective insulin receptor gene; and the diabetes-deafness and optic atrophy syndrome which is due to defects in mitochondrial genes (Kahn, 1996). However, studies of animal models have demonstrated the polygenic nature of this disease. Mice with a phenotype similar to human type-2 diabetes generally have mutations in two or more genes (Hussain, 1997). In addition, studies in the Pima Indian population of Arizona have shown the importance of gene-environment interactions in the development of type-2 diabetes in humans which further complicates the search for genetic causes of this disease (Pratley, 1998).

Thiazolidinediones (TZDs) are a class of insulin sensitizing compounds that include the drugs ciglitazone, troglitazone, and pioglitazone (Saltiel, 1996). TZDs are

capable of improving insulin action in a variety of insulin resistant animal models, independent of the cause. In humans, TZDs result in a 20% to 40% reduction in insulin resistance although their effect on glucose disposal seems variable (Olefsky, 2000). TZDs also improve many of the abnormalities associated with the insulin resistance syndrome by lowering triglyceride levels, increasing HDL levels, and lowering blood pressure (Olefsky, 2000). It has been found that TZDs act as agonists for the peroxisome proliferator activated receptor gamma, a member of the nuclear receptor superfamily (Spiegelman, 1998).

B. Nuclear Receptors

The nuclear receptor superfamily is a group of proteins that act as transcription factors to alter gene expression when bound to specific ligands. They are involved in a wide variety of functions such as lipid metabolism, embryonic development, and virtually all endocrine pathways. The physical structure of most nuclear receptors is very similar and include four major regions: an NH_2-terminal region with ligand-independent activation properties, a core with two zinc finger motifs for specific DNA binding, a hinge region for protein flexibility, and a COOH-terminal region with several binding domains for ligands, dimers and activators (Chawla, 2001). The superfamily can be broken down into two groups: type I are the steroid receptors such as the estrogen receptor and androgen receptor while type II contains the orphan receptors (liver X receptors (LXRs), peroxisome proliferator activated receptors (PPARs), etc.) as well as the thyroid hormone receptors (TRs), Vitamin D receptor (VDR), retinoic acid receptors (RARs),

and the retinoid X receptors (RXRs) (Kastner, 1995). Type I receptors form homodimers and bind to palindromic repeats only in the presence of ligand while type II receptors can form either homodimers or, more often, heterodimers and bind to direct repeats with or without ligand (McKenna, 1999). Many of the type II nuclear receptors form heterodimers with the RXRs in order to bind with their appropriate response elements and the PPARs, in particular, are only functional as heterodimers (Mangelsdorf, 1995).

1. Retinoid X Receptors

There are three retinoid X receptor genes in humans, RXRα, β, and γ, found on chromosomes 9, 6 and 1 respectively (Li, 2000; Numasawa, 1999; Rebhan, 1997). In adults, RXRβ is expressed ubiquitously, with RXRα found mainly in the liver, kidney and skin and RXRγ in muscle and heart (Rowe, 1997). The RXRs can form homodimers as well as heterodimers with other nuclear receptors like PPARs, RARs, LXRs, VDR and TRs. Interestingly, the crystal structure of the RXRα:PPARγ heterodimer suggests that the heterodimeric state is more permissive than the homodimer (Gampe, 2000).

The only known natural ligand for the RXRs is 9-cis retinoic acid, a metabolite of vitamin A, although the presence of this ligand is not required for most RXR heterodimers to be active (Mangelsdorf, 1995). Several other substances, such as phytanic acid, methoprene acid, are also capable of activating the RXRs and a series of synthetic drugs called rexinoids have been developed to influence lipid homeostasis

through the RXRs (Chawla, 2001). In apolipoprotein E knockout mice, activation of RXRs by rexinoids caused a dramatic reduction in atherosclerotic lesion size at least partly through the PPAR and LXR signaling pathways (Claudel, 2001).

The RXRβ gene contains 10 exons and spans 6.2kb at 6p21.3 near HLA class II. Numasawa et al screened the coding sequence of the RXRβ gene to look for variation associated with ectopic bone formation and discovered three polymorphisms in or near the gene (Numasawa, 1999). The first site, a T to A substitution, is in the 3' untranslated region of exon 10. The other variants are in the intergeneic region between RXRβ and collagen 112A at +140bp (A to T) and +561bp (C insertion/deletion) after the 3' end of exon 10. The allele frequencies for these three polymorphisms in Japanese controls are given in Table 1.

Table 1: Allele Frequencies for RXRβ Polymorphisms

Polymorphism	Allele Frequency	# of Individuals
RXRb10 (T/A)	q=0.07 (A)	158
RXRb+140 (A/T)	q=0.23 (T)	158
RXRb+561 (C/CC)	q=0.17 (CC)	131

2. PPAR:RXR Heterodimer

The PPAR:RXR heterodimer exists in both an active and inactive state. When inactive, it is bound to corepressors such as the nuclear receptor corepressor (N-CoR) or the silencing mediator for retinoid and thyroid hormone receptor (SMRT) (DiRenzo, 1997). In the presence of ligand for either PPAR or RXR, the corepressors dissociate so that the ligand can bind and activate the complex. Activity of the complex is thought to increase in the presence of both a PPAR and a RXR ligand although both are not necessary. PPAR ligands include fatty acids and their derivatives as well as several synthetic compounds while the only known natural ligand for RXR is 9-cis retinoic acid (Desvergne, 1999). PPARs can also show enhanced activity in the presence of coactivators that phosphorylate a specific site on the protein. At least two of the three isotypes (PPARα and PPARγ) show increased activity when phosphorylated in response to insulin (Juge-Aubry, 1999; Shalev, 1996). When the PPAR:RXR complex is activated, it binds to a PPAR response element (PPRE) in the 5'region of target genes to induce transcription. PPREs consist of two direct repeats of AGGTCA separated by one base, called a DR-1 element, although the 5' flanking region is also important for proper binding since the PPAR:RXR complex is polar and binds in a specific direction (Desvergne, 1998).

3. Peroxisome Proliferator Activated Receptors

Peroxisome proliferator activated receptors regulate a number of cellular functions from fatty acid oxidation to adipocyte differentiation. They were first identified as proteins that induce peroxisome proliferation in rodent liver cells in response to exogenous chemicals, many of which are hepatocarcinogens. There are three PPAR isotypes; α, β (or δ) and γ, which have somewhat different expression patterns and functions. All three types have similar gene structures consisting of six coding exons and two 5' untranslated exons. The PPARγ gene has a third 5' exon that is specific for the γ2 isoform and encodes for an additional 28 amino acids (Beamer, 1997; Fajas, 1997). In addition to directly regulating gene expression by complexing with the RXRs, PPARs can also act as negative regulators of gene transcription by interacting with other transcription factors, like NF-κB, to form inactive complexes thereby inhibiting the normal function of the other protein (Gervois, 2000).

a) PPARα

PPARα is found primarily in the liver and regulates genes involved with fatty acid utilization. The gene for PPARα is located on chromosome 22 and has one main isoform (Sher, 1993). A splice variant lacking exon 6 is found in the liver, but its function is unknown (Palmer, 1998). Other than the liver, PPARα is also expressed at significant levels in skeletal muscle, kidney, heart and small intestine (Auboeuf, 1997; Mukherjee, 1997). Elevated glucocorticoids cause an increase in PPARα expression levels (Auboeuf, 1997; Mukherjee, 1997) and physical activity also seems to generate

higher levels of the protein (Horowitz, 2000). Insulin causes activation of the protein through phosphorylation, but down regulates PPARα expression (Desvergne, 1999; Shalev, 1996). Ethanol, however, seems to reduce activity and could relate PPARα to the development of fat accumulation in alcoholic liver diease (Torra, 2001). Activation of PPARα by fibrates, a common hypolipidemic drug, causes a decrease in circulating lipid levels in humans (Gervois, 2000). On the other hand, PPARα null mice show hyperlipidemia and obesity associated with ageing in a sexually dimorphic pattern with females showing higher levels for the traits (Costet, 1998). Under fasting conditions, PPARα null mice show hypoglycemia, hypothermia, increased plasma free fatty acids levels and an accumulation of lipids in the liver due to their inability to degrade fatty acids (Pineda Torra, 2001).

PPARα regulates the expression of genes involved in the peroxisomal and mitochondrial β-oxidation pathways such as acyl-CoA oxidase, enoyl-CoA hydratase/dehydrogenase multifunctional enzyme, keto-acyl-CoA thiolase, malic enzyme, medium chain acyl-CoA dehydrogenase, and mitochondrial hydroxy methylglutaryl-CoA synthase (Schoonjans, 1996a). PPARα also regulates fatty acid transporter protein (FATP), fatty acid translocase (FAT), fatty acid binding proteins (FABPs) and uncoupling proteins 2 and 3 (UCP2 and UCP3) (Bernlohr, 1997: Brun, 1999; Desvergne, 1999; Motojima, 1998). By altering transcription of these genes, activated PPARα leads to increased breakdown of triglycerides and fatty acids, increased cellular fatty acid uptake, and reduced triglyceride and fatty acid synthesis (Schoonjans, 1996b).

In a study of 165 diabetic individuals (Mexican American and Caucasian), Au, *et. al.* screened the PPARα gene and found several single nucleotide polymorphisms (Au, 1998). Many of the SNPs were at very low frequencies and/ or conservative or intronic; however, two missense mutations were identified. The first, is a C to G substitution in codon 162 of exon 5 which changes a leucine to a valine at a frequency of q=0.054 for the G allele. The other variant is a substitution of a T (valine) for a C (alanine) in codon 223 of exon 6 with the rare allele at a frequency of q=0.015 (Au, 1998). The valine allele of the L162V polymorphism has been associated with increased levels of apolipoprotein B, total cholesterol, HDL cholesterol and apolipoprotein A1 in type-2 diabetics and increased Apo B and LDL cholesterol in non-diabetic individuals (Vohl, 2000; Flavell, 2000). This allele has also been reported to show ligand-concentration dependant variation in activation. The response of V162 in the absence of ligand and at low ligand levels was much lower than the L162 form, but at high ligand levels, the V162 variant showed greater transactivation activity than L162 (Sapone, 2000; Flavell, 2000).

b) PPARβ

Less is known about PPARβ, also referred to as PPAR δ, NUC1 and FAAR. The expression of PPARβ is widespread and it is found at moderate levels in most tissues, with high levels of the protein found in the placenta and large intestine (Auboeuf, 1997; Mukherjee, 1997). The gene for PPARβ is located on chromosome 6 and encodes one known isoform. PPARβ is thought to help regulate many of the genes that the other two PPARs influence including the FABPs, FAT and acyl-CoA synthase in the intestines

(Bernlohr, 1997; Desvergne, 1999). The PPARβ-null mouse shows the importance of this protein in normal development. Mice lacking the PPARβ protein are growth retarded, have low myelination in their central nervous system and show reduced adipocyte mass (Chawla, 2001). This loss of adipocye mass is likely due to a direct effect of PPARβ on adipogenesis. Overexpression of PPARβ in preadipose cells causes an increase in the adipogenic response to fatty acids while a dominant negative mutant form of the receptor shows an extremely muted response to fatty acids (Bastie, 2000; Jehl-Pietri, 2000). PPARβ has also been found to be a direct regulator of PPARγ gene expression (Hansen, 2001; Bastie, 1999). Therefore, PPARβ activation by nutritional fatty acids appears to induce PPARγ expression that then stimulates adipogenesis upon activation.

c) PPARγ

PPARγ is an important regulator of adipocyte differentiation and function. The gene for PPARγ is located on chromosome 3 and codes for three distinct isoforms (Green, 1995). PPARγ1 and PPARγ3 are regulated by separate promoters, but differ only in their transcription initiation start site so the mature proteins are identical. Recently, a fourth isoform, PPARγ4, was identified with a separate promoter just 5' of exon 1 which also results in a mature protein identical to the γ1 and γ3 isoforms (Sundvold, 2001). These isoforms are located in several tissues including adipose tissue, liver, muscle and heart. The PPARγ2 isoform is regulated by its own promoter

and has a unique first exon. It is only found at low levels in adipocytes and possibly skeletal muscle cells. In adipocytes, PPARγ is activated by fatty acids and stimulates genes involved in fatty acid release, transport, and synthesis such as lipoprotein lipase (LPL), FAT, FATP, the FABPs, acyl-CoA synthase, malic enzyme and phosphoenolpyruvate carboxykinase (PEPCK) (Motojima, 1998; Schoonjans, 1996a). It also reduces the expression of leptin, a potent satiety factor (Schoonjans, 1997). Expression of the insulin sensitive glucose transporter, GLUT4, and the UCPs are also influenced by PPARγ (Kelly, 1998; Wu, 1998).

Expression of PPARγ is induced by both insulin and corticosteroids (Vidal-Puig, 1997), but is down-regulated by TNF-α, IFNγ and other inflammatory cytokines which causes de-differentiation of adipocytes (Xing, 1997; Tanaka, 1999; Hogan, 2001). In mice, a high fat diet will also induce PPARγ expression (Vidal-Puig, 1996). Increased levels of PPARγ have also been detected in the skeletal muscle of obese subjects both with and without NIDDM in relation to BMI and fasting insulin (Kruszynska, 1998). On the other hand, heterozygous PPARγ-deficient mice show adipocyte hypertrophy and normal insulin sensitivity even when fed a high fat diet (Kubota, 1999). PPARγ null mice are non-viable due to placental and cardiac defects (Barak, 1999). Even though PPARγ is of obvious importance in lipid and glucose homeostasis, it has also been implicated in many other cellular functions including carcinogenesis, inflammation, and atherosclerosis due to its interactions with many of the compounds listed above (Auwerx, 1999).

Several genetic variations have been described in the human PPARγ gene. The P115Q conversion, first identified in 4 morbidly obese subjects, causes defective phosphorylation of the protein resulting in accelerated adipocyte differentiation (Ristow, 1998). Two other mutations (P467L and V290M) were found in 3 individuals (in 2 families) with severe insulin resistance, dyslipidaemia and hypertension. Both of these mutations have been shown to cause impaired ligand-dependant transactivation and inhibit wild-type PPARγ in a dominant negative manner (Barroso, 1999). Two other more common polymorphisms, a silent C to T substitution in exon 6 and a C (proline) to G (alanine) substitution at codon 12, have also been reported in PPARγ (Vigouroux, 1998; Yen, 1997). The frequencies of these polymorphisms in several populations are shown in Table 2 below. There is evidence that suggests that the mutant protein produced by the alanine allele at codon 12 has reduced transcriptional and adipogenic, activity *in vitro*, which could lead to lower adipose tissue mass (Masugi, 2000). In addition, the alanine allele was found to be associated with lower lipolysis and greater insulin sensitivity in a group of lean non-diabetic subjects (Stumvoll, 2001).

Table 2: Previously reported frequencies for the PPARγ polymorphisms

Polymorphism	Frequency of Less Common Allele	Population
hPPARg2 (Pro12Ala)	0.12	26 Diabetic Caucasian Americans (Yen, 1997)
	0.10	36 Mexican Americans (Yen, 1997)
	0.03	53 African Americans (Yen, 1997)
	0.01	50 Chinese (Yen, 1997)
	0.13	107 Finns (Valve, 1999)
	0.03	215 Japanese Men (Mori, 1998)
	0.18	312 Italians (Mancini, 1999)
	0.15	973 Elderly Finns (Deeb, 1998)
	0.11	686 Caucasian Americans (Beamer, 1998)
	0.11	839 French (Meiraeghe, 2000)
PPARg6	0.17	26 Diabetic Caucasian Americans (Yen, 1997)
	0.21	107 Finns (Valve, 1999)
	0.14	820 French (Meiraeghe, 1998)

d) Genetic Association Studies of P12A

Many studies have examined the association between the P12A polymorphism and various measures of obesity or diabetes, but the results have been variable. Four groups have shown an association between the Ala allele and increased BMI in Caucasian or Mexican American samples (Beamer, 1998; Cole, 2000, Meiraeghe, 2000; Valve, 1999) while another study reports that the Ala allele is associated with lower BMI in a large sample of Finnish individuals (Deeb, 1998) and five other studies found no association with BMI (Douglas, 2001; Mancini 1999; Meirhaeghe, 1998; Mori,

1998; Mori, 2001a). Ek, *et al* found that the Ala allele was related to higher BMI in a sample of obese Danish men, but lower BMI in the non-obese controls (Ek, 1999). Some of these apparent inconsistencies can be explained by variations in study design, study population, or methods of analysis and many of these studies lacked a sufficient number of Ala homozygotes to accurately compare them to the other genotypes. The study samples varied in age, gender, ethnicity, and phenotypic definitions that may also account for some of the discrepancies. In addition, as the Ek study shows, other variables seem to be influencing the phenotypic expression of this polymorphism that simple analyses do not take into account. By incorporating dietary measures into their model, Luan, *et al* were able to detect an interaction between the ratio of polyunsaturated fat to saturated fat and the Pro12 Ala polymorphism in relation to BMI and fasting insulin (Luan, 2001). These results may also explain some of the inconsistencies of previous studies and support the use of more sophisticated analysis techniques that can incorporate environmental variables.

Unlike the reports for BMI, a more consistent finding from many groups has been that the Ala allele is associated with a decreased risk of type-2 diabetes. Most notably, in a large study of Scandinavian individuals, the P12A variation was the only polymorphism out of 16 common SNPs that were previously reported to influence diabetes risk to show a reproducible association with decreased diabetes risk. Because the detrimental allele is the more common proline ($p \sim 0.85$) the modest genotype relative risk of 1.25 for this sample is equivalent to a population attributable risk of 25% (Altshuler, 2000). A large case-control study of Japanese type-2 diabetics versus non-

diabetics also supports the conclusion that the A12 allele is protective against diabetes (Hara, 2000). The protective effect of the alanine allele correlates with the in vitro findings of lower transactivating activity and increased insulin sensitivity. However, even this association is not without dispute. In a study of the Canadian Oji-Cree, the alanine allele of the P12A polymorphism was found to be associated with increased risk for type-2 diabetes in women, but not in men (Hegele, 2000). Thus, it is likely that the Pro12Ala variation either has different consequences in distinct ethnic groups or else is in varying linkage disequilibrium with another polymorphism that is also influencing measures of obesity or diabetes.

C. Fatty Acid Binding Proteins

The cytoplasmic fatty acid binding proteins (FABPs) are among the most abundant proteins in cells that metabolize lipids. They bind to long chain fatty acids with high affinity that can vary with chain length and degree of saturation (Bernlohr, 1997). The FABPs are involved in fatty acid cellular uptake and intracellular transport and may protect the cell from potentially detrimental effects of fatty acids by sequestering them (Glatz, 1995). In addition, liver FABP (FABP1) appears to be involved in the signaling pathway for PPARα in the liver by delivering a fatty acid to the PPAR protein through a direct interaction (Wolfrum, 2001). Other FABPs have also been found to localize to the nucleus in a ligand dependant manner suggesting that they may also act in a similar manner to FABP1 and could be major players in PPAR signaling and regulation of fatty acid levels (Glatz, 2001).

There are at least eight distinct types of FABPs including FABP1 (liver), FABP2 (intestine), FABP3 (heart and skeletal muscle), FABP4 (adipose), FABP5 (epidermal), FABP7 (brain), myelin FABP and ileal FABP (Glatz, 1995). Most of these proteins are only expressed in a single tissue as noted above; however, FABP1 is expressed in several tissues other than liver and FABP3 is expressed in many tissues in addition to heart and muscle (Bernlohr, 1997). The gene structure of four exons separated by three introns is conserved among the all of the FABPs. The 5' promoter region for at least four of the FABPs (1, 2, 3, and 4) contain PPAR response elements suggesting direct regulation of these genes by the PPARs (Bernlohr, 1997).

Several FABP knockout mice have been created to better understand the function of these proteins. The FABP2 null mouse develops hyperinsulinemia independent of weight gain. The male mice also show increased levels of serum triacylglycerols and gain more weight than normal mice suggesting that FABP2 may be influenced by sex hormones (Vassileva, 2000). Similarly, a polymorphism has been identified in the human FABP2 gene at codon 54 that substitutes a threonine for an alanine and has been associated with insulin resistance and dislipidemias (Glatz, 2001). The FABP3 deficient mice show decreased fatty acid uptake into heart and skeletal muscle that is compensated for by increased glucose uptake and oxidation (Binas, 1999). Mice that lack FABP4 have no obvious phenotype, but when fed a high fat diet, they had lower insulin levels than control mice on the same diet (Shaughnessy, 2000).

D. Uncoupling Proteins

The uncoupling proteins (UCPs) were first discovered in mitochondria from brown adipose tissue (BAT). There, they divert energy from ATP synthesis by diffusing the proton gradient across the mitochondrial inner membrane to produce heat (Gura, 1998). Through this pathway, BAT is able to help regulate thermogenesis and fat storage by burning calories instead of producing ATP. In addition to UCP1, the BAT isoform, two other major UCP isoforms have been identified, UCP2 and UCP3. UCP2 and 3 have reduced thermogenic capabilities in BAT and are thought to have additional or separate functions such as regulation of fatty acid oxidation or reactive oxygen species production (Boss, 2000). All three UCPs are regulated by the PPARs and therefore, fatty acids, although the response varies depending on the PPAR and UCP isotypes. Two other isoforms have recently been identified in the brain, UCP4 and UCP5 or brain mitochondrial carrier protein-1, but little is known about their function or regulation other than that they are capable of uncoupling activity (Adams, 2000).

1. UCP1

The gene for UCP1 is located on chromosome 4 and contains 6 exons (Dalgaard, 2001). As previously noted, UCP1 is exclusively expressed in brown adipose tissue which is well developed in human infants and adult rodents although only trace amounts of BAT are detectable in adult humans (Pecqueur, 2001). Mice deficient for UCP1 are cold intolerant, but not obese although they show increased expression of

UCP2 which could be compensating for the lack of UCP1 (Boss, 2000). Fasting causes a decrease in UCP1 mRNA expression in rats (Sivitz, 1998).

Because UCP1 is only expressed in BAT, it is difficult to determine the importance of UCP1 in human disorders such as obesity and type-2 diabetes since affected individuals should not have much, if any, BAT during the development of the disease. Two polymorphisms in linkage disequilibrium, an A to C transition in exon 1 and a Met229Leu substitution in exon 5, were both associated with susceptibility to type-2 diabetes (Mori, 2001b). Another variant was identified in exon 2 of the UCP1 gene that changes an alanine (G) to a threonine (A) at codon 64 (Urhammer, 1997a). Also, an A to G substitution has been identified in the 5' region of the UCP1 gene that has been associated with reduced basal metabolic rate and ability to lose weight in obese subjects when present with the Trp64Arg polymorphism in the β3-adrenergic receptor (Valve, 1998; Fogelholm, 1998). These studies suggest that UCP1 may be important in adult onset disorders and should not be discounted simply because of its limited expression.

2. UCP2

The UCP2 and UCP3 genes are both located on chromosome 11 separated by approximately 7kb and contain 8 and 7 exons, respectively (Dalgaard, 2001). UCP2 is expressed ubiquitously but the highest levels are found in white adipose tissue (Dalgaard, 2001). Non-specific activation of PPARs in a myocyte cell line was found to

slightly increase UCP2 expression while specific activation of PPARγ caused reduced levels of UCP2 mRNA (Cabrero, 2000). As further evidence of the complexity of the PPARs regulation of UCP2, increased free fatty acid levels have been found to correspond to higher UCP2 mRNA levels in rat muscles (Samec, 1999). Obese individuals have been found to show increased levels of UCP2 protein in skeletal muscle that coincided with a reduced ability to use lipids (Simoneau, 1998). UCP2 null mice have no apparent physical phenotype and respond normally to cold exposure or a high fat diet, possibly due to compensation by UCP1 in white adipose tissue. They are, however, resistant to infection by *Toxoplasma gondii*, a normally lethal pathogen, and show an 80% increase in macrophage reactive oxygen species compared to wild-type suggesting increased immunity in the absence of UCP2 (Arsenijevic, 2000).

Several variations in the UCP2 gene have been identified. Urhammer, et al, identified a substitution of a valine for an alanine at codon 55 in exon 4 (Urhammer, 1997b). Also, a 45bp insertion/ deletion in the 3' UTR of exon 8 has been associated with increased BMI in one study, but not in another (Evans, 2000; Dalgaard, 1999). Recently, Esterbauer *et al* identified 5 polymorphisms in the 5' promoter region of the UCP2 gene. They found that for one of these, a G to A substitution at –866bp, the A allele was associated with increased expression in vitro and reduced risk of obesity in middle-age subjects (Esterbauer, 2001).

3. UCP3

UCP3 is expressed at the highest levels in skeletal muscle and BAT, but is also found in the heart (Samec, 1998). Increases in free fatty acid levels induce UCP3 mRNA levels in rat muscle, probably through activation of the PPARs (Samec, 1999). Carbacyclin, a non-selective PPAR ligand was found to increase UCP3 mRNA levels in a myocyte cell line while specific activators of either PPARα or PPARγ had no effect implicating PPARβ as the major regulator in this cell type (Nagase, 1999). In another study, PPARγ specific activators were found to reduce UCP3 mRNA levels in a different myocyte cell line while PPARα specific and non-specific activators had no effect (Cabrero, 2000). Taken together, these results suggest that either the myocyte cell lines have different responses to PPAR stimulation or, more likely, that there is a complex interaction between the PPAR isotypes which have complementary effects on UCP3 expression. This is a plausible explanation knowing that PPARβ can alter PPARγ expression as well as UCP3 expression and variation in ligand affinity between the isotypes would complicate comparison of studies using different PPAR activators. In contrast to the findings in skeletal muscle, PPARα appears to be a potent stimulator of UCP3 in rodent cardiac muscle (Young, 2001). Also, PPARα and PPARγ show a synergistic induction of UCP3 expression in rodent BAT (Teruel, 2000).

Mice with a deficiency in UCP3 are not obese on either a normal or high fat diets, although they do have reduced proton leak across the mitochodrial membrane. UCP3 knock-out mice also show increased levels of reactive oxygen species in skeletal muscle, similar to UCP2 null mice (Dalgaard, 2001). On the other hand, UCP3

overexpressing mice are hyperphagic yet leaner than wild-type control animals. While this overexpression is specific to the skeletal muscle, the phenotype is seen outside of this tissue in the form of reduced adipose tissue mass and lower insulin, glucose and cholesterol levels (Clapham, 2000).

Many polymorphisms have been described in UCP3. Argyropoulos *et. al.* identified a V102I polymorphism in exon 3, a nonsense mutation in exon 4 (R143X), and a splice variant in exon 6 in a group of obese and/ or diabetic African Americans (Argyropoulos, 1998). The R143X mutation and a missense mutation (R70W) were both found to have severe to total loss of uncoupling activity while the V102I and splice variant had normal activity (Brown, 1999). Two additional polymorphisms were identified, a T to C substitution in codon 99 of exon 3 and a C to T substitution in codon 210 of exon 5, that both conserve a tyrosine so are likely non-functional (Otabe, 1999). Also, a C to T substitution at –55bp in the 5' region of the UCP3 gene has been associated with fasting glucose levels in males and percent body fat in females (Yanagisawa, 2001).

E. The San Luis Valley Diabetes Study

The San Luis Valley Diabetes Study (SLVDS) is a geographically based case-control study designed to identify risk factors for type-2 diabetes, heart disease and obesity in a population of Hispanic and non-Hispanic whites living in Southern Colorado. The study is based in two rural counties, Alamosa and Conejos, in the San Luis Valley

of Colorado. These counties are 43.6% Hispanic and have low immigration rates with most families having lived there for several generations. Diabetics were identified through medical records and advertisment. Then controls were selected from general household interviews to reflect the distribution of the diabetic cohort. The SLVDS control cohort is comprised of 1280 individuals (1107 normal glucose tolerance and 173 impaired glucose tolerance) on whom metabolic, anthropometric and interview data was collected over the course of three possible visits. Informed consent was obtained from all subjects and the University of Colorado Health Sciences Center institutional review board approved all protocols. Participants in the control cohort were 20-74 years old, living in the San Louis Valley and had no reported history of diabetes at baseline. Phase I and II clinic visits (baseline) were conducted between 1984 and 1988, Phase III between 1988 and 1992, and Phase IV from 1997 to 1998. The age adjusted prevalence of type-2 diabetes (%) calculated from the Phase I data is shown in Table 3 (Hamman, 1989).

Table 3: Age adjusted prevalence of type-2 diabetes (%) in the SLVDS

	Male	**Female**
Hispanic	9.9%	10.7%
Non-Hispanic	4.5%	3.5%

F. Summary

This review shows the obvious importance of the PPAR transcriptional pathway in regulating adiposity and glucose homeostasis. The PPARs control genes involved in lipid and glucose homeostasis and are important regulators of adipogenesis. Since the RXRs are the obligate binding partners for the PPARs and the FABPs are couriers for the PPAR ligands, they also become key components in these pathways. In addition, the UCPs, which are regulated by the PPARs, are directly involved in energy regulation. Figure 1 depicts the relationship between these four genes families. The purpose of this project is to examine the effects of polymorphisms in these genes, as single locus effects and two locus genotypic interactions, on measures of obesity and type-2 diabetes in a large sample of Hispanic and non-Hispanic white individuals from the San Luis Valley Diabetes Study.

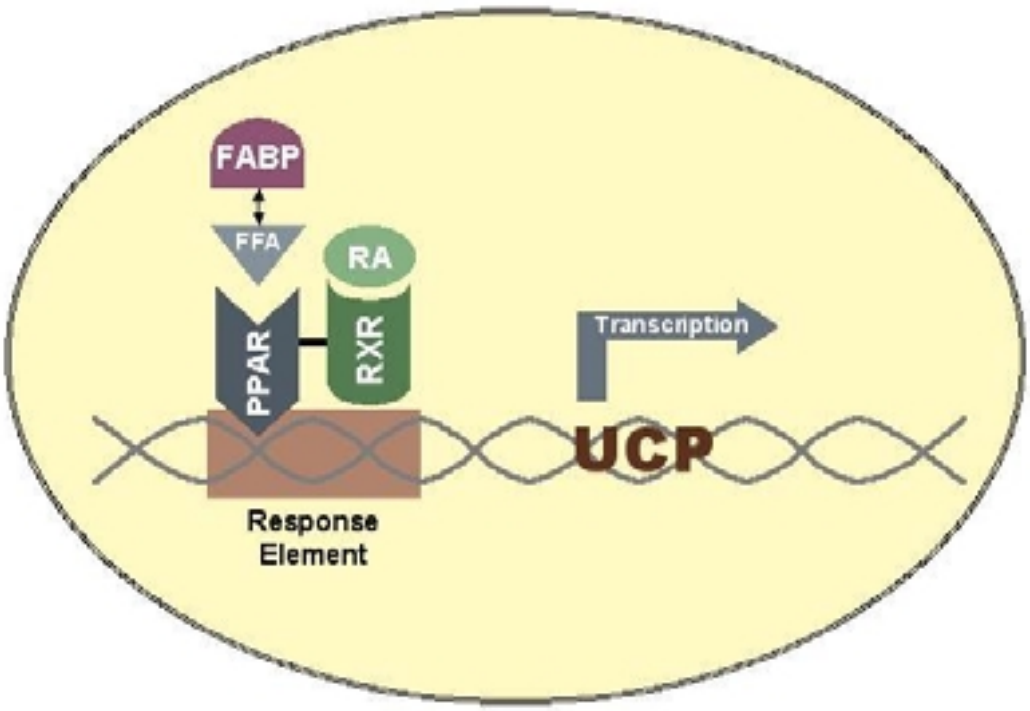


Figure 1: Diagram of the PPAR Transcriptional Pathway

III. Methods and Materials

A. General Protocols

1. PCR

PCR reactions were optimized for each specific primer set, but a general protocol was used in all cases. All PCR reactions consisted of 0.5μM of each primer, 1x PCR buffer (Gibco BRL), 1.25mM dNTPs (Gibco BRL), 5% DMSO (if necessary), 0.625U of Taq DNA polymerase (Gibco BRL), and ~50 to 100ng of genomic DNA. The $MgCl_2$ concentration was specific for each primer set. The reactions were amplified using either a Hybaid Touchdown Thermal Cycler or an MJ PTC-100 Programable Thermal Controller at the following conditions: initial denaturation at 95^{o}C for 3 min.; 35 cycles of 95 ^{o}C for 30 sec., the annealing temperature (specific for each primer set) for 30 sec., and extension at 72 ^{o}C for 30 sec.; followed by a 5 min. extension at 72 ^{o}C. Amplification of PCR products was confirmed by electrophoresis on a 1% agarose gel. Optimization of particular primer sets was achieved by testing each primer set at three different salt concentrations (1.0M, 1.5M and 2.5M $MgCl_2$) and at various annealing temperatures estimated from the predicted melting temperatures of the primers.

2. Direct Sequencing

Sequencing reactions consisted of 4µl of ABI Prism dRhodamine Dye Terminator Ready Reaction Mix, 4µl of 0.8mM primer and 4µl of PCR product. The reactions were run for 25 cycles on either a PE GeneAmp 9600 thermal cycler or an MJ PTC-100 Programable Thermal Controller under the following conditions: 96^{o}C for 10 seconds, 50^{o}C for 5 seconds, and 60^{o}C for 4 minutes. The DNA was then precipitated with 30µl of a solution of 70% ethanol and 0.5mM $MgCl_2$ for at least 2 hours at 4^{0}C before being spun down at 2000 rpm for 30 minutes in an IEC Centra-7 centrifuge. The ethanol was then discarded and 3.5µl of formamide dye (see Appendix A) was added to each tube. The products were then separated on an ABI Prism 377 DNA Sequencer and the output was analyzed using Sequencher v3.1.1 by Genecodes.

3. Single Stranded Conformational Polymorphism Analysis

Single stranded conformational polymorphism analysis (SSCP) was used as an alternate way to detect sequence variation in the genes of interest to ensure that all variants were found (see Appendix A). SSCP analysis followed the protocol of Razzaghi and Kamboh (2001). The PCR product (7.5µl) was combined with 3.75µl of denaturing solution and incubated at 42^{o}C for 10 minutes. The reactions were immediately transferred to ice where 3.75µl of formamide dye was added to each reaction. Each sample was then loaded into a well of a 20% 15-Well TBE Pre-Cast polyacrylamide gel (Novex) and run at 150V at 4^{o}C on a Mighty Small II Vertical

Electrophoresis Unit (Hoefer) until both dye fronts were run off. The gels were then stained with SYBR Green II RNA Gel Stain (Molecular Probes Inc.) for 20 to 30 minutes in the dark before being photographed.

4. Oligonucleotide Ligation Assay

Polymorphisms that did not alter a restriction enzyme site were genotyped using the oligonucleotide ligation assay (OLA) (see Appendix B) (Nickerson, 1990). First, the PCR product was diluted 1:1 with 0.1% triton in deionized water. A ligation reaction mixture was then made for each allele consisting of 2mM NAD, 2x ligase buffer, 0.058% triton water, 25mM KCl, 0.1 μM of the oligonucleotide unique to the allele, 0.1 μM of the common oligonucleotide and 0.1unit of ampligase. The unique oligonucleotides were labeled on the 5' end with biotin while the common oligonucleotide was labeled 3' with digoxygenin. Two reactions were created for each PCR product, one for each allele, by mixing 10μl of one ligation reaction mixture with 10μl of the diluted PCR product and repeating this in the adjacent well with the other ligation reaction mix. Ligation reactions were then carried out for ten cycles at 93^{o}C for 30 seconds and 58^{o}C for 2 minutes on a Hybaid Touchdown Thermal Cycler. In a reaction where the DNA matches the unique oligonucleotide, the unique and common oilgos are ligated together to form a longer oligonucleotide labeled with biotin (5') and digoxigenin (3'). If the DNA allele does not match the unique oligonucleotide, the probes will not be ligated and will remain separated from each other. Next, 10μl of stop solution was added and the entire reaction was transferred to 96-well flat bottom plates that were coated with strepavidin

and blocked with BSA. The strepavidin captures the biotinylated oligonucleotides and binds them to the plate while the unligated common oligos remain free in solution. After incubating for ~30 minutes at room temperature, the plates were washed two times with 0.1M NaOH wash and twice with 0.1M Tris wash. Then, 40μl of anti-digoxigen antibody solution was added to each well and allowed to sit for 25 to 30 minutes before being washed again six times with 0.1M Tris wash. An ELISA substrate was then added (25μl) and after 15 minutes an equal amount of amplifier was also added. A solution of sulfuric acid at a concentration 0.3M was used to stop the ELISA color change reaction when the plates were readable. Wells containing ligated oligonucleotides (unique + common) show a positive reaction (pink color) and are considered to contain DNA with the allele specified in the ligation reaction mixture. On the other hand, the digoxigenin labeled common probe is washed away when it is not ligated to the unique oligo so these wells show a negative reaction (clear color). A negative reaction is read as a lack of the allele specified in the ligation reaction.

B. Screening and Identification of Variation

1. PPARβ

No variation had been previously reported in the PPARβ gene so it was screened using single-stranded conformational polymorphism analysis (SSCP) and direct sequencing. Primers were designed in the introns of the gene to cover the translated regions of the sequence and in the promoter region to cover approximately 1kb of sequence immediately 5' of exon 1A (see Table 4). Sequence and genomic structure were derived from the human clone 109F14 on chromosome 6p21.2-21.3 (Genbank accession #AL022721, http://www.ncbi.nlm.nih.gov). Several exons were amplified in multiple overlapping pieces in order to keep the PCR products within a size range for efficient SSCP. Standard PCR reaction conditions were used (see Table 4). Screening was preformed on 11 control samples in order to detect common variations.

Table 4: Primers and PCR conditions used for amplification of PPARβ

Fragment Name	Gene Region	Forward and Reverse Primer	Anneal -ing Temp.	MgCl Conc.	DMSO
PPARB p1	5’	F-cgcaggctccgcagaattct R-ctccaggaggcgtggtgattg	60°C	1.0M	
PPARB p2	5’	F-cgagacgtcaccgcgctg R-gagcgccaggtccagtagaaca	64°C	2.5M	
PPARB p3	5’	F-gatgcggacgtagcagctaag R-gacctagcactgggttaagagtc	58°C	1.5M	
PPARB p4	5’	F-ggtacgtgacttgcagtgacaag R-gcgtattgcataagcacatgg	58°C	1.5M	
PPARB 2	Exon 2	F–tcagaggaacaactggcata R–ctctccaccacatttctaccaa	52 °C	2.5M	
PPARB 3-1	Exon 3	F–ctgtggctgctccatggct R–tcctcttccttgtcactgcctc	56°C	2.5M	Yes
PPARB 3-2	Exon 3	F–actgcactgggctgtgcct R–ccgaggggagatcagccatg	58 °C	1.0M	Yes
PPARB 4	Exon 4	F–gaccacagcctcagtggccagc R–ggcctggcagcatgtggagc	64 °C	2.5M	
PPARB 5	Exon 5	F–ctgtgtggagccagcagccc R–ctccctcctcctggtggcctt	66 °C	2.5M	
PPARB 6-1	Exon 6	F–ggtcggccacctgtgggttgt R–cctgcctgggctccttgctg	53 °C	2.5M	Yes
PPARB 6-2	Exon 6	F–ccaggatgctgccaggccaa R–tgaaggccttctccaagcacatctacaat	60 °C	2.5M	Yes
PPARB 7-1a	Exon 7	F–ctcaatgatatcactgaagggtttgcg R–gcgtgcacgaggccatcttc	60 °C	1.0M	Yes
PPARB 7-1b	Exon 7	F–gactgggtgtgccaggccag R–ctaagtttgaatttgctgtcaacg	57 °C	1.5M	Yes
PPARB 7-2a	Exon 7	F–ccgtccttgttcacgatagaggc R–gctgccagtgcaccacagtg	58 °C	1.5M	Yes
PPARB 7-2b	Exon 7	F–ggtagaagacgtgcacgctgatct R–ggtctcccgaggcctgatctc	58 °C	1.5M	Yes
PPARB 8-1	Exon 8	F–cattggagtctgcagggaggc R–acctgcatgccaaccaccct	63 °C	1.5M	Yes
PPARB 8-2	Exon 8	F–gcaggtcagccatcttctgca R–ggagctccactgcctttctgag	58 °C	1.5M	Yes

Primer sequences are listed 5’ to 3’ for both forward (F) and reverse (R) primers.

2. RXRα

No variation had been previously reported in RXRα. It was screened for common variations using SSCP and direct sequencing. PCR products were designed to cover the coding sequence of the gene in 200 to 250bp amplimers based on sequence obtained from Genbank (accession #AQ917567, AQ917569, AQ917571, AQ917573, AQ917575, AQ917577, AQ917579, AQ917581, AQ917566, AQ917568, AQ917570, AQ917572, AQ917574, AQ917576, AQ917578, AQ917580 and NM_002957). Twelve to sixteen normal control samples were screened by both detection methods for exons 2 through 10. See Table 5 for the PCR conditions and primer sequences.

Table 5: Primers and PCR conditions used for amplification of RXRA

Fragment Name	Gene Region	Forward and Reverse Primer	Annealing Temp.	MgCl Conc.
RXRA 2a	Exon 2	F-ctgcactgaccactctcctgc R-gagctgatgaccgagaaaggc	58°C	1.0M
RXRA 2b	Exon 2	F-cagctgcattctcccatcag R-ctgcagttgaagccacagcc	60°C	1.0M
RXRA 3	Exon 3	F-ggacatagggacaaacctggtg R-cctgcaatggcctgacaac	54°C	1.5M
RXRA 4	Exon 4	F-tcaagcggacggtgcgca R-ccatctcgggtgtccacgca	58°C	1.0M
RXRA 5	Exon 5	F-gctgagcgtggggctcacct R-gaggcatgtgaggctgcccac	64°C	1.5M
RXRA 6	Exon 6	F-ggactgaatgtcctgctcttct R-tggtacgtgtcccatctgc	54°C	1.5M
RXRA 7	Exon 7	F-gcctggagacagctgagtgactg R-ggcggaggtgaccgaagc	54°C	1.5M
RXRA 8	Exon 8	F-tggtgagggctgcgacctaac R-acggggccagaagcctcaa	54°C	1.5M
RXRA 9	Exon 9	F-ccagctgagggttctgacc R-gagacaagagcctgggtctg	58°C	1.0M

Primer sequences are listed 5’ to 3’ for both forward (F) and reverse (R) primers.

3. Promoter Regions

The 5' regions of the PPARα and PPARγ genes were also screened for variation. Approximately 1kb of sequence immediately 5' of exon 1 for PPARα and exon B (γ2 specific) for PPARγ was screened using SSCP and direct sequencing. Four overlapping primer sets were designed for each of the regions based on the reported sequence (Genbank accenssion #Z94161 and AB00520). At the time of the screening, the PPARγ1 promoter had not been characterized and only 125bp of sequence was available for primer design so this region was not investigated in this project. Twelve control individuals were screened to identify common variation. PCR reaction conditions are reported in Table 6.

Table 6: Primers and PCR conditions used for amplification of the PPAR promoter regions

Fragment Name	Gene Region	Forward and Reverse Primer	Annealing Temp.	MgCl Conc.
PPARA p1	α promoter	F-agggccctgagcttcagcct R-agcctccttgggataagggtgc	64°C	1.5M
PPARA p2	α promoter	F-caaggaggctgggagaggagg R-accccaaccgggcacaact	60°C	1.5M
PPARA p3	α promoter	F-ggcagggccgaccctcctgac R-gaccccggacaggctgcgct	64°C	1.5M
PPARA p4	α promoter	F-acccggcccagcgcagcct R-gcggtcgccgactcagaaggtgct	64°C	1.0M
PPARG2 p1	γ2 promoter	F-gtcttgactcatgggtgtattc R-agtgtatcagtgaaggaatcgc	60°C	1.5M
PPARG2 p2	γ2 promoter	F-cagctggctcctaataggaca R-ctatctagcaaaagatcaatccgt	60°C	2.5M
PPARG2 p3	γ2 promoter	F-tacagttcacgcccctcac R-ggagagatgggaataaacacag	60°C	2.5M
PPARG2 p4	γ2 promoter	F-agattcaaccaggaatagacacc R-cggtgacccacatgttcag	60°C	2.5M

Primer sequences are listed 5' to 3' for both forward (F) and reverse (R) primers.

C. Genotyping

1. Study Population

The San Luis Valley Diabetes Study (SLVDS) was designed to explore the risk factors for type-2 diabetes in Hispanic and Non-Hispanic White individuals living in rural southern Colorado. Metabolic, anthropometric and interview data was collected over the course of three possible visits. The Phase I and Phase II clinic visits are considered the baseline visits and were conducted between 1984 and 1988. The Phase III and Phase IV follow up clinic visits were conducted from 1988 to 1992 and 1997 to 1998, respectively (see Figure 2). The SLVDS control cohort consisted of 1270 subjects who had no previous history of diabetes and were not diabetic based on a 75g oral glucose tolerance test at the time of the baseline clinic exam (WHO, 1985). Individuals were only included in the genotyping if they participated in the Phase Four visit and had DNA available. Development of type-2 diabetes was possible during the 9 to 14 years between the baseline visit, when the controls were classified as non-diabetic, and the Phase IV visit. Individuals who were diagnosed with type-2 diabetes during the Phase IV visit were not excluded from this study. Of the 764 individuals who were included in the genotyping, 321 subjects were Hispanic (H) and 443 were Non-Hispanic whites (NH) based on self-reported ethnicity.

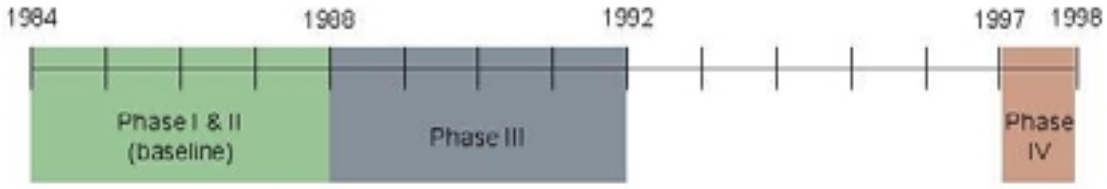


Figure 2: Timeline of the SLVDS clinic visits

2. PPARα

Several polymorphisms have been identified in PPARα by Au, et. al. (1998). One of these sites is a C (leucine) to G (valine) substitution found at nucleotide 700 of the mRNA that corresponds to codon 162 in exon 5 of the PPARα gene (Genbank accession #L02932). Another site, found in exon 6 at nucleotide 896 of the mRNA, is a T to C substitution that changes a valine to an alanine at codon 223 (Genbank accession #L02932). These particular sites were chosen for further analysis in this study because they showed the highest frequencies of the exonic polymorphisms that were reported. Neither of these polymorphisms altered a restriction enzyme site so they were genotyped using OLA. PCR conditions and primer sequences can be found in Table 7. These polymorphisms will be referred to as PPARa5 (exon 5 variant) and PPARa6 (exon 6 variant) in this report.

Table 7: Primers and PCR conditions for OLA genotyping for PPARα variants

		PPARa5	PPARa6
PCR Conditions	Forward PCR Primer	ttctttcggcgaacgattcg	ctgaaagcagaaattcttacctgt
	Reverse PCR Primer	cgttgtgtgacatcccgacag	ttgttactggcctttcctgag
	Annealing Temp.	56°C	56°C
	MgCl Conc.	2.5M	2.5M
	DMSO	Yes	Yes
OLA Primers	Common Detection Primer	tttctgtcgggatgtcacac-**D**	catcctctcaggaaaggcca-**D**
	Allele Specific Primer #1	**B**-ttgtcgatttcacaagtgcC	**B**-aacaaggtcaaagcccgggT
	Allele Specific Primer #2	**B**-ttgtcgatttcacaagtgcG	**B**-aacaaggtcaaagcccgggC

Primer sequences are listed 5' to 3' for both forward (F) and reverse (R) primers.
Abbreviations: **B** – biotin; **D** – digoxigenin

3. PPARβ

A silent C to T substitution located at nucleotide 72054 (Genbank accession # AL022721) was detected in exon 6 of the PPARβ gene. This polymorphism, called PPARb6, was typed using an engineered *Nde*I restriction enzyme site (see Table 8). The digestion reaction consisted of 2U of *Nde*I, 1x NEB buffer 4 and 10μL of PCR product. The reaction was left at 37°C overnight and then run out on a 2% agarose gel to separate the fragments. A fragment of 236bp corresponded to the C allele and two fragments of 213bp and 23bp corresponded to the T allele.

Table 8: Primers and PCR conditions for RFLP genotyping

Variant	Forward and Reverse Primers	Anneal-ing Temp.	MgCl Conc.
PPARb6	F-ctcccggagtcaggcaggcg R-ctggtggcagggctgac	58°C	1.5M
PPARg2	F-gccaattcaagcccagtc R-gatatgtttgcagacagtgtatcagtgaaggaatcgctttccg	50°C	1.5M
PPARg6	F-gatgagttgcttggtagagctg R-cgggtgaagactcatgtctgt	58°C	1.5M
RXRb10 & RXRb+140	F-tgagaagtccagggcagaac R-ctgcagaaggaggtgaagt	60°C	1.5M

Primer sequences are listed 5' to 3' for both forward (F) and reverse (R) primers.

4. PPARγ

Two polymorphisms have been previously reported in the PPARγ gene. The first site is in the γ2 specific exon (exon B), at nucleotide 892 (Genbank accession #AB005520). The more common C allele corresponds to a proline at amino acid position 12 while the G allele encodes for an alanine. This polymorphism, henceforth referred to as PPARg2, was detected through an engineered *Bst*UI restriction enzyme digestion (2.5U of *Bst*UI, 1xNEB buffer 2, and 10μL of PCR product) that was incubated at 60°C overnight. The digested DNA fragments were resolved on a 2% agarose gel and visualized under ultra violet light in the presence of ethidium bromide (see Table 8). The resulting fragments were either 270bp (C Allele) or 227bp and 43bp (G Allele) when compared to a DNA size standard.

The other polymorphism in PPARγ is located in exon 6 at nucleotide 268 (Genbank accession #AB005526). This C to T substitution, referred to as PPARg6 in this report, leads to the introduction of a *Nla*III restriction site that was used to detect the polymorphism (see Table 8). The digestion reaction contained 2U of *Nla*III, 1xNEB buffer 4, 1μg BSA, and 10μL of PCR product that was then kept at 37°C overnight. The resulting fragments were then viewed on a 2% agarose gel. The C allele resulted in a fragment of 313bp and the T allele gave fragments of 270bp and 43bp.

5. RXRβ

Two polymorphisms have been reported in the RXRβ gene, one in the 3' untranslated region of exon 10 and one 140bp after the 3' end of the gene. These sites will be referred to as RXRb10 and RXRb+140 in this report. The two sites are close enough together to be amplified in one product of 442bp (see Table 8). The first site, a T to A substitution at nucleotide 7266 introduces a unique *Dde*I restriction site (Genbank accession #AF120161). Fifteen μL of RXRβ PCR product was digested with 1U of *Dde*I and 1x Promega buffer D. This results in fragments of size 440bp for the T allele and 319bp and 121bp for the A allele. The A to T substitution at nucleotide 7412 introduces a second *Mbo*I site into the PCR fragment that can be used for detection (Genbank accession #AF120161). This digestion reaction contained 1U of *Mbo*I, 1x React 2 buffer and 15μL of the RXRβ PCR product. Fragments of size 398bp and 42bp correspond to the A allele and of size 225bp, 173bp, and 42bp for the T allele.

D. Data Analysis

1. Genotype Coding

Several numerical variables were used to represent the genotype data (see Table 9). First, a three category variable was made for each polymorphism using 1 for the common homozygote, 2 for the heterozygote and 3 for the less common homozygote. Because all of the polymorphisms have low frequencies, variables were created for each site with 2 categories where the heterozygous individuals were combined with those homozygous for the less common allele. These assume a dominant effect of the common allele and were labeled as PPARa5d, PPARa6d, PPARb6d, etc. Also, for some of the regression analyses, PPARb6 and RXRb+140 were common enough to look at all three genotypes so they were coded into 2 separate dummy variables. The first of these had the common homozygote equal to 1 and all other genotypes equal to 0 and were labeled as PPARb6v1 or RXRb140v1. The second variable, PPARb6v2 or RXRb140v2, had the heterozygotes equal to 1 and all other genotypes as 0.

Table 9: Genotype Variable Coding for Regression Analysis

Genotype	Three Category Variable	Dominant Variable	First Dummy Variable	Second Dummy Variable
Common Homozygote	1	1	1	0
Heterzygote	2	0	0	1
Less Common Homozygote	3	0	0	0

2. Variables Used in Analysis

Three variables were initially chosen as outcomes for this study: body mass index (BMI), fat mass (FM) as measured by DEXA, and fasting free fatty acid levels (FFA). BMI, calculated as weight in kilograms divided by the square of height in meters (kg/m^2), is a standard measure of general obesity used in many studies and is included in this analysis to allow comparison with the findings of other studies. Fat mass is a more direct measure of adiposity and as the PPARs, PPARγ in particular, are known to influence adipogenesis and fat storage, it is of obvious interest in this study. In the SLVDS, fat mass has been measured by dual energy x-ray absorptiometry (DEXA) using a LUNAR DPX-L scanner, a method that measures body fat within soft tissues independent of age, body water or the chemical content of the tissue (Pierson, 1991). The PPAR:RXR heterodimers are also major regulators of fatty acid metabolism so

genetic variation in these genes could alter blood levels of free fatty acids. Fasting free fatty acid levels (μmol/L) were measured from blood samples.

Other variables were included in the analysis as possible predictors, including percent body fat, lean body mass, HOMA IR, total cholesterol, triglycerides, systolic blood pressure, diastolic blood pressure, smoking, physical activity, total caloric intake, total fat intake, ethnicity, age, and sex. Percent body fat was calculated as the percent fat mass (measured by DEXA) of the whole body mass. Lean body mass represents the amount of non-fat mass in an individual's body and is calculated as the difference between the total body mass and the fat mass in grams measured by DEXA. HOMA IR is a measure of insulin resistance based on the fasting insulin and glucose levels [HOMA IR= (fasting insulin * fasting glucose)/ 22.5]. It is highly correlated with insulin and glucose levels and was used instead of the fasting, 1 hour and 2 hour insulin and glucose levels (Matthews, 1985). Total cholesterol (mg/dl) and triglycerides (mg/dl) were measured from blood samples. The two separate readings were averaged for the systolic and diastolic blood pressure measurements (mM). Smoking status was classified in three categories: 1 for non-smokers, 2 for current smokers, and 3 for ex-smokers. When smoking was used as an adjustment in the regression models, it was recoded into two categories (1=non-smoker, 2=current smokers and ex-smokers) and was referred to as "ever smoke". METS was used for the physical activity data and is equal to the total physical activity over one week measured in kCal/kg/hr. Dietary measures were based on a recall interview of food intake for the previous 24 hours. Ethnicity was based on self-declared status as either a Hispanic or non-Hispanic white.

Skin reflectance measured by a portable spectrophotometer was used as a surrogate for ethnicity during certain analyses (Gardner, 1984). Age at the time of the Phase IV clinic visit was used.

3. Descriptive Statistics

Allele frequencies were estimated for all typed polymorphisms and then tested for fit to the expectations of Hardy-Weinberg equilibrium by the X^2 test using Microsoft Excel. The EH program was used to determine estimated haplotype frequencies with association (Ott, 2001). D' was then calculated between each pair of loci as a measure of linkage disequilibrium (Hartl, 2000; Devlin, 1995).

All other statistical analyses were done using the SPSS statistical analysis software package, version 10.0 for Macintosh. All descriptive analyses were done on two sets of the data: the entire data set and the data set split by ethnicity. This results in three groups of individuals: un-split, Hispanics, and non-Hispanics.

Descriptive statistics were calculated for all of the variables. Frequencies were determined for each of the categorical variables (genotypes, sex, ethnicity, and smoking) that were then graphed as bar charts. The X^2 test was used to determine if there were any ethnic differences in the frequencies of the categorical variables. Several statistical measures were calculated for the continuous variables including mean, standard deviation, standard error of the mean, skewness, kurtosis, and range.

Histograms of the distributions for each of the continuous variables were also graphed and a t-test was used to determine if there were any differences between the ethnic groups. Outcome variables with a skewness or kurtosis value greater than one were transformed with the natural log and the descriptive statistics were re-calculated.

Outliers were determined based on the descriptive statistics and histograms. Seventeen extreme outliers who were likely the result of incorrect measurements or data entry errors were excluded from all further analyses. These individuals were ID# 252, 749, 3077, 3169, 3194, 3137, 3195, 3243, 3348, 3467, 3592, 3645, 3757, 3759, 3776, 3878, and 3953. Exclusion of these individuals did not substantially change the descriptive statistics for any of the variables.

4. Univariate Analysis of Predictor Variables

All of the predictor variables were tested for associations with the outcome variables (BMI, FM, FFA). One-way analysis of variance (ANOVA) was used to test for associations between the outcome variables and the categorical variables. Linear regression was used to test the relationship between the continuous variables and the outcome variables.

In order to determine colinearity between the predictor variables, all of the predictor variables were tested against each other. A X^2 test was used to look for relationships between pairs of categorical variables. Associations between categorical

variables and continuous variables were determined with one-way ANOVA. Bivariate correlations were calculated for all combinations of continuous variables.

5. New Outcome Variables

The results of the univariate analyses suggested that other variables might be of more interest than the outcome variables originally selected. The ANOVA tests did not show any significant relationships between the genotype variables and two of the outcomes, BMI and fat mass, for any of the groups. Fasting free fatty acids did show significant associations with some of the polymorphisms so it was retained as an outcome variable. As there were other continuous variables, notably fasting glucose and cholesterol, found in the predictor correlation analyses that also showed association with some of the variants, these variables were used as outcomes in the regression analyses. Both of these variables are plausible outcomes based on the known biology of the PPARs and RXRs as these genes are involved in regulation of both cholesterol and glucose metabolism. Fasting glucose was not normally distributed so it was transformed using the natural log.

6. Univariate Analysis with New Outcome Variables

Because sex was also an important predictor for the outcome variables, the next set of analyses was done split by sex and ethnicity as well as un-split and split by only ethnicity. This resulted in seven groups of individuals: un-split SLVDS (U), Hispanics

(H), non-Hispanics (NH), Hispanic males (H M), Hispanic females (H F), non-Hispanic males (NH M), and non-Hispanic females (NH F).

One-way ANOVA was used to determine which genotypes were associated with the outcomes in each of the seven groups. Then, all pair-wise combinations of the polymorphisms were tested by two-way ANOVA to determine any interesting interaction effects. The results of these analyses were then used to build regression models for each of the outcomes in each of the groups as described below.

7. Regression Modeling

Models were constructed for each outcome in each group of individuals by following a series of steps (see Figure 3). Step 1 was to pick the polymorphisms to include in each of the preliminary models. Loci were included in the regression model if they were significantly associated with the outcome ($p<0.05$) by one-way ANOVA or if they were part of an interaction term with a p-value of less than 0.20 from a two-way ANOVA test. All possible pair-wise combinations of the single loci picked for each model were also included in the preliminary model as interactions. The interaction terms were calculated by multiplying the two single locus genotype variables together. This resulted in interaction terms that contrasted the double common homozygotes with all other genotype combinations.

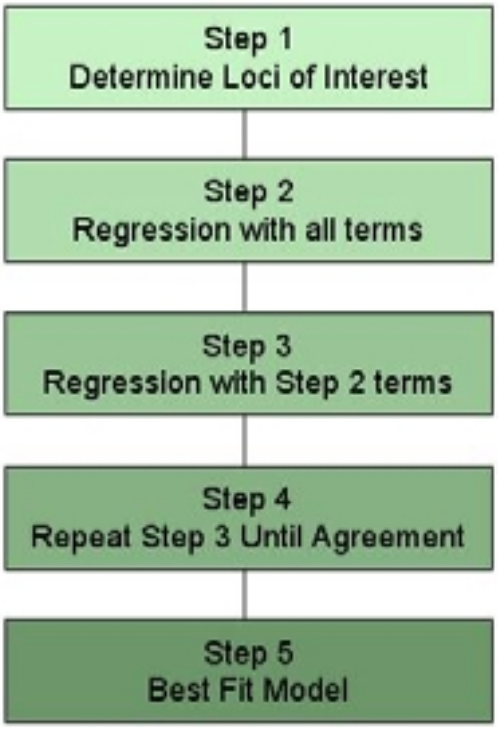


Figure 3: Diagram of Regression Modeling Process

The next step (Step 2) was to use linear regression to test the preliminary models. All models were tested using both a stepwise and backward approach to modeling. The stepwise approach entered significant terms one-by-one and re-tested each term after the addition of a new term. The backward approach entered all of the terms into the model then removed the non-significant terms one-by-one. Entry and removal criteria were set at a p-value of 0.05 and 0.051, respectively. Cases were excluded listwise from the analysis. Residual statistics and descriptive statistics were calculated for each model. In step 3, the regression models were re-tested but only

included the terms that remained in either the stepwise or backward model from step 2. If the models did not agree after this step, they were re-tested with the remaining terms from the models in step 3. This was continued until the stepwise and backward models retained the same terms (Step 4). At times, the backward models retained terms that the stepwise did not and no agreement could be reach using the above criteria. In this case, the backward model was used as the final model because it allows for multiple terms to be retained that are dependant on each other.

Finally, step 5 was to construct a final model for each of the groups and outcomes. Terms were entered into the final model if they appeared in the final backward and stepwise model as well as any corresponding dummy variable. Each of the terms in the final model was graphed to visualize the effect on the outcome variable. It should be noted that no correction was made for multiple testing in this analysis as it is exploratory. Results from this study were based on consistencies within the analysis as a whole and should be replicated in other studies for confirmation.

8. Combined Analysis with UCPs and FABPs

Models were constructed that combined the previously developed models for the PPARs and RXRs with models including polymorphisms in the UCP and FABP genes. Coleen Damcott went through a similar process as described above to construct models for fasting free fatty acids and cholesterol in the SLVDS sample split by sex and ethnicity (Damcott, unpublished data, 2001; see Appendix F). The eleven sites used for

this analysis were UCP1-2, UCP2-4, UCP2-8, UCP3-5, UCP3p-55, FABP2pID, FABP2p-834, FABP3pID, FABP3p-313, FABP4-2b, and FABP4p-376.

The combined models were constructed for fasting free fatty acids and cholesterol in the four ethnic/sex groups since there were corresponding models from the PPAR/RXR and UCP/FABP analyses. Sites were included for this analysis if they met any of the following criteria: 1) Present in the final model from the previous analysis for that outcome, 2) Oneway ANOVA with a p-value <0.05, or 3) Interaction term from previous paired ANOVA with a p-value of <0.15. The sites which met one of these criteria were then tested against all of the sites in the other group by 2-way ANOVA (PPARs and RXRs vs. UCPs and FABPs). A list of possible terms for the regression analysis was constructed that included the sites that met the inclusion criteria for the 2-way ANOVAs and the sites from the 2-way ANOVA that had an interaction term with a p-value <0.15. Sites were excluded from this list based on their p-values and the number of times they appeared, until a reasonable number of terms (<40) were left to test in the regression model. More weight was given to sites and interactions with a p-value <0.05. The list of terms included in the regression analysis always included main effect terms and interactions from the previous models and all possible interactions between the two sets of polymorphisms (PPARs and RXRs vs. UCPs and FABPs). The same process for determining the final model in the previous analysis was used to make final models for each of the ethnic/ sex groups for both outcome variables. Final models included both sets of any dummy variables. The terms from the final models were graphed to visualize the effect.

9. Tissue Specific Covariate Models

Tissue specific regression models were also constructed for several adjusted outcome variables. Models were constructed for the muscle, adipose tissue and intestines using polymorphisms from the genes that have the highest expression in that particular tissue. Muscle models included terms from PPARα, UCP3, and FABP3. Adipose models included PPARγ, UCP 1, UCP2, and FABP4 sites. Intestinal models tested sites from PPARβ, UCP2, and FABP2. The outcome variables tested in this analysis were fat mass and lean mass. These variables were adjusted for skin reflectance, smoking (ever smoke), age and physical activity by saving the unstandardized residuals from a regression model of the outcome versus those covariates. The covariates were entered into the adjustment model regardless of their effect to ensure that as much environmental variation as possible was removed. Skin reflectance was used as a measure of ethnicity in order to account for the admixture between Europeans and Native Americans that is present in the Hispanic population. The other covariates were included in this analysis due to their potential effect on the outcomes and their lack of an obvious genetic component. The mean value for each unadjusted outcome was then added to the unstandardized residuals to compute the adjusted values. From this point on, all analyses were carried out on the SLVDS data set, split by sex.

Regression models were then constructed for each outcome in each tissue with the tissue specific sites noted above as well as all possible interactions. The models were tested in both a stepwise and backward manner until they matched at which point,

they were considered final models. In some cases, the backward models kept sets of terms that were dependant on each other and that the stepwise models did not consider. When this occurred, the backward model was considered the final model. After the models were constructed for each tissue, overall models were constructed based on the final models for each tissue. All of the terms that appeared in each final tissue specific model plus all single locus terms that appeared in interactions were tested for the overall models. Models were constructed for each of the outcome variables in both sexes. All terms from the models were then graphed to visualize the effects.

E. Other Analysis

1. PPARb6

During the analysis to construct multi-locus models, PPARb6 was noted to have a particularly strong effect by itself on fasting glucose, fasting insulin and HOMA IR as tested by one-way analysis of variance. To further explore this effect, regression models were constructed for ln(fasting glucose), ln(fasting insulin) and ln(HOMA IR) containing PPARb6, sex and skin reflectance as a measure of ethnicity. Stepwise and backward approaches were used to test the regression models until they resulted in the same model that was then considered the final model.

2. Analysis of PPARγ Polymorphisms

The two polymorphisms in PPARγ, PPARg2 and PPARg6, have been associated with various measures of obesity and type-2 diabetes in other studies that have assumed the effect is attributable to the PPARg2 amino acid substitution. A separate set of analysis was done to determine the relative contribution of each of these polymorphisms to several obesity and diabetes related traits. The outcome variables (fasting glucose, fasting insulin, HOMA IR, fasting free fatty acids, fat mass, and percent fat) were adjusted for skin reflectance using linear regression then the mean of the unadjusted outcome was added to the unstandardized residuals to get the adjusted values for each outcome. One-way analysis of variance was then used to test the effect of each site on the adjusted outcomes for males and females. Next, regression models were constructed for the traits showing association with at least one of the polymorphisms to determine the effect of each variant alone and in combination. The outcomes used in this analysis were fasting glucose, fasting insulin and HOMA IR which were adjusted for skin reflectance, fat mass, smoking (ever smoke) and age. Separate models were tested for PPARg2 alone, PPARg6 alone and both sites plus the interaction.

IV. Results

A. Screening and Identification of Variation

1. PPARβ

The PPARβ gene was screened for variation using single-stranded conformational polymorphism analysis (SSCP) and direct sequencing. Exons 2, 3, 4, 5, 6, 7 and 8 were screened in eleven samples and one polymorphism was identified in exon 6 (see Figure 4 and Figure 5). This silent C to T substitution, PPARb6, is located at nucleotide 72054 (Genbank accession # AL022721) and was genotyped using an engineered *Nde*I restriction enzyme site. Of the eight screening samples with reliable genotypes there were four individuals with the TT genotype, three heterozygous individuals and one CC individual giving an estimated allele frequency of 0.31 for the less common C allele.

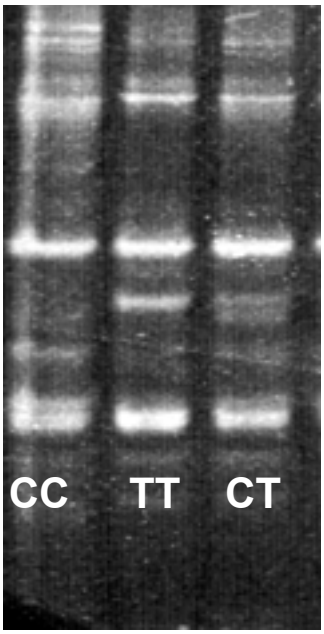


Figure 4: SSCP Gel for PPARb6 Variant

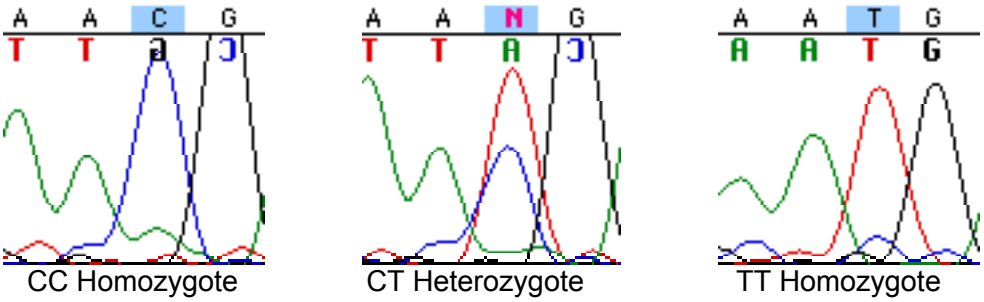


Figure 5: Sequence Chromatograms of the PPARb6 Variant

2. RXRα and promoter regions

The gene for RXRα was screened for common variations using SSCP and direct sequencing. Exons 2 through 9 were successfully screened in 12 to 16 samples and no polymorphisms were identified. Approximately 1kb of the region 5' of the first exon was screened for PPARα, PPARβ and PPARγ (γ2 promoter). No polymorphisms were identified in any of these promoter regions in 16 individuals screened.

B. Genotyping

Seven polymorphisms were identified either from previous reports or from the screening described above, and these were genotyped in 764 individual from the SLVDS population (see Figure 6 and Figure 7). There were two sites in PPARα (PPARa5 and PPARa6) one site in PPARβ (PPARb6) two sites in PPARγ (PPARg2 and PPARg6) and two sites in RXRβ (RXRb10 and RXRb+140). All seven of the typed sites conformed well to Hardy-Weinberg expectations in both the Hispanic (H) and Non-Hispanic (NH) subsets of the SLVDS population (see Table 10 and Table 11). The allele frequencies for the PPARa5 T (Val) allele, typed by OLA, were 0.08 in NH and 0.11 in H. The PPARa6 G (Ala) allele, also typed by OLA, was very rare in the NH group at a frequency of 0.009 and was slightly more common in the H group (q=0.04) although no GG homozygotes were identified in either ethnic group. PPARb6 was typed using an engineered NdeI enzyme site and the resulting allele frequency for the T allele was 0.127 for NH and 0.137 for H. The two sites in PPARγ were each typed by

restriction enzyme digestion and showed similar frequencies in the Hispanics where q=0.12 for both sites, but were somewhat different in the Non-Hispanics with the rare allele at a frequency of 0.12 for PPARg2 and 0.15 for PPARg6. The variants in RXRβ, typed by restriction enzyme digestion, showed the highest frequencies of the typed polymorphisms with the A allele of RXRb10 at q=0.13 in NH and q=0.20 in H while the T allele of RXRb+140 showed a frequency of 0.27 in NH and 0.30 in H. Linkage disequilibrium (D') was then calculated between each pair of polymorphisms (see Table 12).

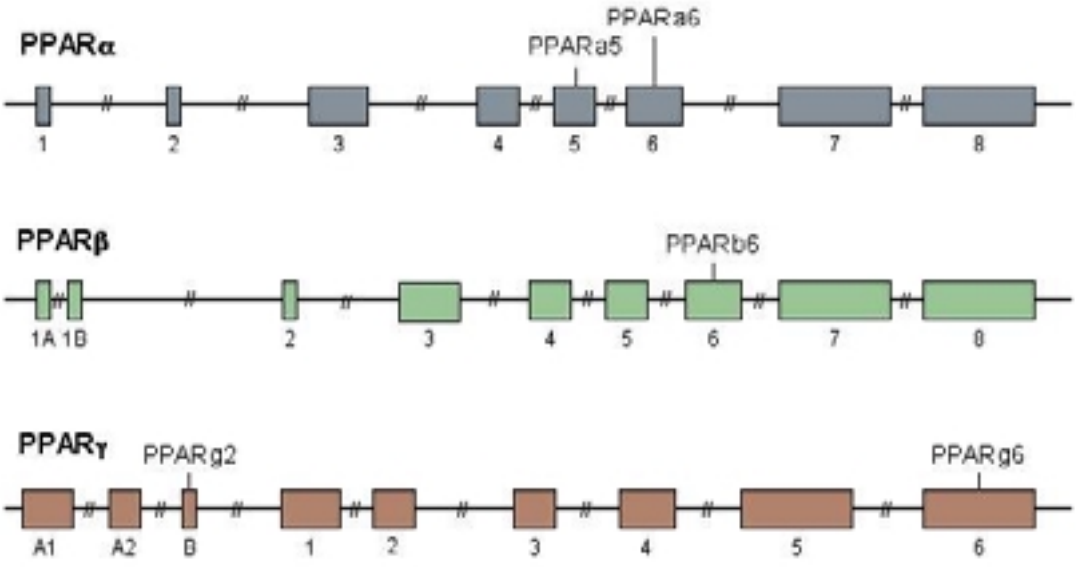


Figure 6: Locations of polymorphisms in the PPARs

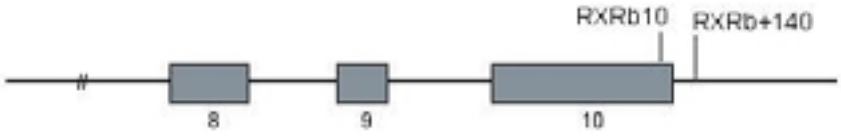


Figure 7: Locations of Polymorphisms in RXRβ

Table 10: Allele frequencies for PPAR and RXR polymorphisms in the SLVDS population

Polymorphic Site	Allele	Overall Frequency	Frequency		Method of Detection
			Hispanics	Non-Hispanics	
PPARa5	C (Leu)	0.905	0.886	0.918	Oligonucleotide Ligation Assay
	G (Val)	0.095	0.114	0.082	
PPARa6	T (Val)	0.980	0.965	0.991	Oligonucleotide Ligation Assay
	C (Ala)	0.020	0.035	0.009	
PPARb6	C	0.869	0.863	0.873	*NdeI* Digestion
	T	0.131	0.137	0.127	
PPARg2	C (Pro)	0.883	0.884	0.882	*BstUI* Digestion
	G (Ala)	0.117	0.116	0.118	
PPARg6	C	0.861	0.882	0.848	*NlaIII* Digestion
	T	0.139	0.118	0.152	
RXRb10	T	0.842	0.800	0.871	*DdeI* Digestion
	A	0.158	0.200	0.129	
RXRb+140	A	0.721	0.704	0.732	*MboI* Digestion
	T	0.279	0.296	0.268	

Table 11: Genotype Frequencies for PPAR and RXR polymorphisms in the SLVDS population

Polymorphic Site	Hispanics				Non-Hispanics			
	11	12	22	HWE*	11	12	22	HWE*
PPARa5 (C/G)	0.84	0.15	0.01	1.00	0.78	0.22	0.003	0.22
PPARa6 (T/C)	0.98	0.02	0	0.98	0.93	0.07	0	0.81
PPARb6 (C/T)	0.77	0.20	0.03	0.12	0.76	0.21	0.03	0.09
PPARg2 (C/G)	0.78	0.21	0.01	1.00	0.78	0.20	0.01	1.00
PPARg6 (C/T)	0.72	0.25	0.03	0.88	0.77	0.22	0.01	0.86
RXRb10 (T/A)	0.76	0.23	0.01	0.89	0.63	0.33	0.04	0.90
RXRb+140 (A/T)	0.55	0.37	0.08	0.63	0.52	0.36	0.12	0.17

*HWE: p-value from χ^2 test of Hardy Weinberg Equilibrium

Table 12: D' measurements of linkage disequilibrium between typed polymorphisms

	PPARa5	PPARa6	PPARb6	PPARg2	PPARg6	RXRb10	RXRb+140
PPARa5	-	0.9633	0.0504	0.0608	0.2063	0.0441	0.1024
PPARa6	0.9633	-	0.1498	0.1357	0.1049	0.0064	0.2023
PPARb6	0.0504	0.1498	-	0.1084	0.2096	0.1076	0.0282
PPARg2	0.0608	0.1357	0.1084	-	0.7670	0.1188	0.0066
PPARg6	0.2063	0.1049	0.2096	0.7670	-	0.1156	0.0261
RXRb10	0.0441	0.0064	0.1076	0.1188	0.1156	-	0.9995
RXRb+140	0.1024	0.2023	0.0282	0.0066	0.0261	0.9995	-

C. Analysis of Multi-Locus Models

1. Descriptive Statistics

Descriptive statistics were calculated for all variables used in the analysis (see Appendix C). The frequencies of the categorical variables were determined and no significant differences between Hispanics and Non-Hispanics were found for sex or smoking status using a χ^2 test (see Table 13). The study population consisted of 42% Hispanic and 58% Non-Hispanic individuals with 47% males and 53% females in the entire sample. Only 15%of the subjects reported that they were current smokers while 36% were classified as ex-smokers and 49% as non-smokers.

Table 13: Frequencies of categorical variables in SLVDS population

	Catagory	Unsplit	Non-Hispanics	Hispanics	p-value (NH vs H)
Smoking Status	Non-smoker	49.3%	50.9%	47.0%	0.271
	Current smoker	14.7%	13.0%	17.1%	
	Ex-smoker	36.0%	36.1%	35.9%	
Sex	Male	47.2%	48.5%	45.4%	0.398
	Female	52.8%	51.5%	54.6%	
Ethnicity	Non-Hispanic	58.0%			
	Hispanic	42.0%			

Extreme outliers were identified through examination of the distributions for the continuous variables. A total of 17 samples were classified as extreme outliers and were removed from all subsequent analyses. In many of the cases, these extreme values were likely the result of incorrect measurements or data entry errors as they were biologically implausible. After removal of the outliers, mean, median, maximum, minimum, range, standard error of the mean, standard deviation, skewness and kurtosis were calculated for all of the continuous variables in the entire SLVDS sample as well as in each ethnic group. The average age of the study group was 63 years and was between 34 and 88 years of age. Fasting glucose, fasting insulin, and HOMA IR had highly skewed distributions (see Table 14). When these variables were used as the outcome of a linear regression analysis, they were transformed using the natural log to reduce their skewness.

Table 14: Descriptive statistics for continuous variables in the SLVDS population after removal of outliers

Variables	Mean (SD)	Skewness	Kurtosis	N
BMI (kg/m^2)	27.29 (4.86)	0.755	1.402	746
Fat Mass (kg)	26.21 (9.29)	0.457	0.447	697
% Body Fat (%)	35.45 (9.63)	-0.110	-0.545	697
Lean Mass (kg)	44.67 (10.83)	0.428	-0.863	697
Fasting FFA (μmol/L)	598 (238)	0.660	0.342	745
Cholesterol (mg/dl)	201 (36)	0.366	0.699	745
Triglycerides (mg/dl)	178 (92)	1.797	4.475	745
Fasting Glucose (mg/dl)	100 (22)	3.663	18.980	745
ln(Fasting Glucose)	4.59 (0.17)	2.091	7.614	745
Fasting Insulin (mg/dl)	11.08 (6.58)	2.021	5.321	745
ln(Fasting Insulin)	2.27 (0.50)	0.494	0.012	745
HOMA IR	17.37 (13.02)	2.445	7.558	745
ln(HOMA IR)	2.66 (0.60)	0.515	0.159	745
Systolic BP (mm)	127 (18)	1.009	1.766	745
Diastolic BP (mm)	74 (9)	0.322	0.770	745
Caloric Intake (cal)	1931 (846)	0.979	1.568	713
Fat Intake (g)	76 (42)	1.278	2.683	712
Physical Activity (kCal/kg/hr)	282.6 (61.9)	1.937	3.940	725
Age (years)	63 (12)	-0.176	-0.756	746
Skin Reflectance (%)	32.64 (3.83)	-0.402	0.155	744

Analysis of variance was used to test for ethnic differences in the continuous variables after removal of the outliers (see Table 15). Hispanic individuals showed significantly higher values for fasting free fatty acids (H=640, NH=568; $p<0.001$), triglycerides (H=190, NH=169; $p=0.002$), fasting glucose (H=104, NH=98; $p<0.001$), fasting insulin (H=12.3, NH=10.2; $p<0.001$) and HOMA IR (H=19.9, NH=15.5; $p<0.001$). In contrast, Non-Hispanic individuals had higher caloric intake (NH=2016, H=1810;

p=0.001) and fat intake (NH=79, H=71; p=0.015). Skin reflectance was higher in the Non-Hispanic subjects as expected (NH=34.3, H=30.4; p<0.001), as was age (NH=64, H=62; p=0.047).

Table 15: Comparison of mean values (SD) for continuous variables in the SLVDS population split by ethnicity after removal of outliers

Variables	Non-Hispanics		Hispanics		p-value
	Mean (SD)	N	Mean (SD)	N	
BMI (kg/m^2)	27.12 (4.76)	433	27.53 (5.00)	313	0.257
Fat Mass (kg)	26.56 (9.16)	407	25.71 (9.46)	290	0.238
% Body Fat (%)	34.92 (9.40)	407	36.20 (9.92)	290	0.084
Lean Mass (kg)	46.48 (11.36)	407	42.12 (9.52)	290	<0.001
Fasting FFA (μmol/L)	568 (223)	432	640 (252)	313	<0.001
Cholesterol (mg/dl)	201±37	432	200 (35)	313	0.868
Triglycerides (mg/dl)	169 (87)	432	190 (97)	313	0.002
ln(Triglycerides)	5.02 (0.45)	432	5.14 (0.46)	313	0.002
Fasting Glucose (mg/dl)	98 (19)	432	104 (26)	313	<0.001
ln(Fasting Glucose)	4.57 (0.16)	432	4.62 (0.20)	313	<0.001
Fasting Insulin (mg/dl)	10.21 (5.91)	432	12.27 (7.25)	313	<0.001
ln(Fasting Insulin)	2.20 (0.48)	432	2.37 (0.51)	313	<0.001
HOMA IR	15.54 (11.30)	432	19.90 (14.73)	313	<0.001
ln(HOMA IR)	2.57 (0.57)	432	2.78 (0.62)	313	<0.001
Systolic BP (mm)	127 (17)	433	126 (19)	312	0.979
Diastolic BP (mm)	74 (9)	433	74 (9)	312	0.831
Caloric Intake (cal)	2016 (826)	421	1810 (862)	292	0.001
Fat Intake (g)	79 (41)	421	71 (43)	291	0.015
Physical Activity (kCal/kg/hr)	283.7 (61.2)	425	281.1 (63.0)	300	0.517
Age (years)	64 (11)	433	62 (12)	313	0.047
Skin Reflectance (%)	34.25 (2.98)	431	30.42 (3.76)	313	<0.001

2. Univariate Analysis of Outcome and Predictor Variables

Univariate analyses were carried out to compare all outcome variables with the predictor variables. Analysis of variance was used to test for associations between the outcomes and the categorical variables (including genotype) while linear regression was used for the continuous variables. The genotypic means for each of the outcome variables are given in Tables 16 through 22. There were no significant associations between any of the polymorphisms and BMI or fat mass although FFA and PPARb6 were associated in the entire sample as well as in the Hispanic sub-group (see Table 18). The CC genotype of PPARb6 was 107μmol/L higher than the TT genotype in the unsplit sample (CC=608, CT=555, TT=497; p=0.011). Even more strikingly, there was a difference of 185μmol/L between the two homozygotes in the Hispanics (CC=660, CT=558, TT=471; p=0.003). This effect appeared to be additive in both cases with the T allele lowering FFA by ~56μmol/L and ~92μmol/L in the unsplit sample and Hispanic sub-sample, respectively. The Non-Hispanic individuals showed a similar decrease in FFA although the difference between the homozygous groups was only 54μmol/L and was not significant (CC=569, CT=551, TT=515; p=0.624).

Table 16: Means for PPARa5 Genotypes vs. Selected Variables in the SLVDS

PPARa5	Unsplit SLVDS			Hispanics			Non-Hispanics		
	11	12	22	11	12	22	11	12	22
BMI (kg/m^2)	27.4	26.8	27.3	27.7	27.0	21.7	27.2	26.6	29.1
FM (kg)	26.2	26.0	28.2	25.8	25.7	21.0	26.6	26.4	30.6
FFA (μmol/L)	595	607	780	640	638	732	565	574	797
FG (mg/dl)	100	101	94	104	103	83	98	99	97
Chol (mg/dl)	201	198	203	199	205	152	**203**	**191**	**221**
N	607	134	4	243	69	1	364	65	3

* Values in **bold** show significantly different means between the two genotype groups (11 vs. 12+22; $p<0.05$).
* Abbreviations: BMI=body mass index; FM=fat mass; FFA=fasting free fatty acids; FG=fasting glucose; Chol=total cholesterol

Table 17: Means for PPARa6 Genotypes vs. Selected Variables in the SLVDS

PPARa6	Unsplit SLVDS		Hispanics		Non-Hispanics	
	11	12	11	12	11	12
BMI (kg/m^2)	27.2	28.3	27.5	28.4	27.1	28.1
FM (kg)	26.2	28.3	25.6	28.0	26.5	28.8
FFA (μmol/L)	597	566	641	593	567	500
FG (mg/dl)	100	107	104	103	**97**	**119**
Chol (mg/dl)	201	192	**201**	**185**	201	211
N	700	28	283	20	417	8

* Values in **bold** show significantly different means between the two genotype groups (11 vs. 12; $p<0.05$).
* Abbreviations: BMI=body mass index; FM=fat mass; FFA=fasting free fatty acids; FG=fasting glucose; Chol=total cholesterol

Table 18: Means for PPARb6 Genotypes vs. Selected Variables in the SLVDS

PPARb6	Unsplit SLVDS			Hispanics			Non-Hispanics		
	11	12	22	11	12	22	11	12	22
BMI (kg/m^2)	27.4	27.3	25.6	27.7	27.8	24.5	27.3	27.0	26.4
FM (kg)	26.4	26.6	23.1	25.9	26.2	22.2	26.7	26.9	23.7
FFA (μmol/L)	**608**	**555**	**497**	**660**	**558**	**471**	569	551	515
FG (mg/dl)	**102**	**97**	**91**	105	100	92	<u>99</u>	<u>95</u>	<u>90</u>
Chol (mg/dl)	201	197	205	202	195	186	201	198	218
N	525	139	19	222	60	8	303	79	11

* Values in **bold** show significantly different means between the three genotype groups ($p<0.05$) while values that are <u>underlined</u> show significantly different means only when two genotype groups are tested (11 vs. 12+22; $p<0.05$)
* Abbreviations: BMI=body mass index; FM=fat mass; FFA=fasting free fatty acids; FG=fasting glucose; Chol=total cholesterol

Table 19: Means for PPARg2 Genotypes vs. Selected Variables in the SLVDS

PPARg2	Unsplit SLVDS			Hispanics			Non-Hispanics		
	11	12	22	11	12	22	11	12	22
BMI (kg/m^2)	27.4	27.3	27.1	27.4	28.0	27.4	27.3	28.0	27.4
FM (kg)	26.1	26.7	27.8	27.3	26.8	26.9	26.8	26.2	30.0
FFA (μmol/L)	591	608	534	625	674	683	566	563	415
FG (mg/dl)	101	101	105	104	103	127	98	99	88
Chol (mg/dl)	201	198	216	201	196	199	201	199	229
N	540	141	9	225	57	4	315	84	5

* Abbreviations: BMI=body mass index; FM=fat mass; FFA=fasting free fatty acids; FG=fasting glucose; Chol=total cholesterol

Table 20: Means for PPARg6 Genotypes vs. Selected Variables in the SLVDS

PPARg6	Unsplit SLVDS			Hispanics			Non-Hispanics		
	11	12	22	11	12	22	11	12	22
BMI (kg/m^2)	27.5	27.0	26.6	27.5	27.7	27.9	27.4	26.7	26.1
FM (kg)	26.4	26.0	25.6	25.6	26.6	27.0	27.1	25.6	25.1
FFA (μmol/L)	593	594	580	620	689	670	573	540	552
FG (mg/dl)	101	100	94	104	103	96	98	97	94
Chol (mg/dl)	201	201	201	201	196	188	201	204	205
N	511	159	13	215	58	3	296	101	10

* Abbreviations: BMI=body mass index; FM=fat mass; FFA=fasting free fatty acids; FG=fasting glucose; Chol=total cholesterol

Table 21: Means for RXRb10 Genotypes vs. Selected Variables in the SLVDS

RXRb10	Unsplit SLVDS			Hispanics			Non-Hispanics		
	11	12	22	11	12	22	11	12	22
BMI (kg/m^2)	27.3	27.5	29.0	27.7	27.5	28.0	27.1	27.4	30.7
FM (kg)	26.4	26.1	24.8	25.7	25.7	24.8	26.8	26.5	24.8
FFA (μmol/L)	588	616	646	637	647	710	561	584	530
FG (mg/dl)	101	100	100	105	103	98	99	97	104
Chol (mg/dl)	200	202	203	200	198	195	200	205	219
N	427	163	14	154	82	9	273	81	5

* Abbreviations: BMI=body mass index; FM=fat mass; FFA=fasting free fatty acids; FG=fasting glucose; Chol=total cholesterol

Table 22: Means for RXRb+140 Genotypes vs. Selected Variables in the SLVDS

RXRb+140	Unsplit SLVDS			Hispanics			Non-Hispanics		
	11	12	22	11	12	22	11	12	22
BMI (kg/m^2)	27.3	27.2	27.7	27.6	27.2	28.5	27.1	27.3	25.8
FM (kg)	25.9	26.8	24.9	25.7	25.7	23.8	26.0	27.5	25.8
FFA (μmol/L)	588	605	582	622	667	609	563	567	559
FG (mg/dl)	100	102	99	102	108	98	98	99	100
Chol (mg/dl)	198	203	203	**193**	**208**	**202**	201	200	203
N	333	218	55	138	84	25	195	134	30

* Values in **bold** show significantly different means between the three genotype groups ($p<0.05$).
* Abbreviations: BMI=body mass index; FM=fat mass; FFA=fasting free fatty acids; FG=fasting glucose; Chol=total cholesterol

Correlations among predictor variables were also tested to determine co-linearity (see Appendix D). Bivariate correlations were calculated for each pair of continuous variables and the X^2 test was used for each pair of categorical variables including the genotype data. In the case of the genotype data, the X^2 test approximates a test for linkage disequilibrium (Schneider, 1995). One-way ANOVA was used to test for associations between categorical and continuous variables. Sex was found to be an important predictor for all of the outcome variables except BMI (see Table 23).

Table 23: Mean values (SD) for outcome variables in males and females

Outcomes	Males		Females		p-value
	Mean (SD)	N	Mean (SD)	N	
BMI (kg/m^2)	27.3 (4.1)	352	27.3 (5.4)	394	0.893
FM (kg)	23.2 (7.6)	330	28.9 (9.8)	367	<0.001
FFA (μmol/L)	523 (211)	351	665 (241)	394	<0.001
FG (mg/dL)	104 (24)	351	97 (19)	394	<0.001
Chol (mg/dL)	195 (36)	351	206 (36)	394	<0.001

* Abbreviations: BMI=body mass index; FM=fat mass; FFA=fasting free fatty acids; FG=fasting glucose; Chol=total cholesterol

Analysis of variance was used to look for correlations between the genotype variables and the continuous predictor variables. This resulted in several significant results. First, in the Non-Hispanic group, when the PPARa5 GG individuals were combined with the heterozygous subjects they showed significantly lower cholesterol levels compared to the CC homozygotes (CC=203, CT+TT=192; p=0.036) (see Table 16). The Non-Hispanics also showed a significant increase of 22mg/dL for fasting glucose levels when they were heterozygous for PPARa6 (TT=97, TC=119; p<0.001) (Table 17). Neither of these effects was seen in the Hispanics. On the other hand, heterozygosity for PPARa6 was significantly associated with lower cholesterol levels in Hispanics (TT=201, TC=185; 0.045), but not in the Non-Hispanic individuals (see Table 17). In addition to FFA, PPARb6 was also associated with fasting glucose levels. This effect was significant in the unsplit sample where the CC homozygotes showed an increase of 11mg/dL over the TT homozygotes (CC=102, CT=97, TT=91; p=0.013). In

the ethnic sub-groups, the trend remained although it was not significant (NH: CC=99, CT=95, TT=90; p=0.075; H: CC=105, CT=100, TT=92; p=0.138) (see Table 18). Presence of the T allele for the RXRb+140 polymorphism was associated with significantly higher cholesterol levels in the Hispanics (AA=193, AT=208, TT=202; p=0.007), but no effect was seen in the Non-Hispanics (see Table 22). The PPARg2, PPARg6 and RXRb10d polymorphisms were not associated with any of the continuous predictor variables (see Table 19, Table 20, and Table 21). Due to the fact that two of the original outcomes (BMI and FM) did not appear to be related to any of these polymorphisms in our data set, they were excluded from subsequent analyses while fasting glucose and cholesterol were added as new outcome variables.

3. Univariate Analysis for New Outcome Variables

In addition to the analysis of variance results shown above, each of the new outcome variables was tested for associations with the genotypic data in the four ethnic/ gender sub-groups: NH males, NH females, H males and H females. The PPARb6 variant was again related to FFA in Hispanics, but only in the females (CC=728, CT+TT=584; p=0.003). Both groups of females also showed a significant association between PPARg6 and FFA, although in different directions, with the Non-Hispanic females showing a decrease of 81μmol/L in the presence of the T allele (NH: CC=651, CT+TT=580; p=0.049) while the T allele increased FFA in Hispanic females by 114μmol/L (H: CC=675, CT+TT=789; p=0.021). Fasting glucose levels were associated with PPARa6 (TT=100, TC=128; p<0.001) and PPARb6d (CC=103, CT+TT=96;

p=0.045) in the Non-Hispanic males and RXRb+140 (AA=104, AT=118, TT=103; p=0.045) in the Hispanic male group. The Hispanic males also showed an association between RXRb+140 and cholesterol levels with presence of the T allele increasing cholesterol levels by ~20mg/dL (AA=186, AT=204, TT=206; p=0.013).

Associations between all pair-wise combinations of the polymorphisms and each of the outcome variables were tested by two-way ANOVA and several significant interactions were found ($p<0.05$). In Non-Hispanics, two interactions (PPARa5d*PPARb6d and RXRb10d*RXRb+140d) were associated with FFA. The PPARa5d*PPARb6d interaction was also significantly associated with FFA in the Non-Hispanic Males. Fasting glucose was associated with the greatest number of interactions with two terms in the unsplit group (PPARa6*PPARg2d and PPARa6*PPARg6d), three terms in the Non-Hispanics (PPARa6*PPARg2d, PPARa6*PPARg6d and PPARa6*RXRb+140), two terms in the Hispanic group (PPARa5d*PPARg2d and RXRb10d*RXRb+140d), one term for Non-Hispanic females (PPARa5d*RXRb+140d) and two terms in the Hispanic males (PPARa5d*PPARg2d and PPARg2d*PPARg6d). Cholesterol also showed significant associations with the PPARa5d*PPARb6d interaction (in Hispanics and Hispanic Females) and PPARa5d*RXRb10d (in Non-Hispanic Females). These terms, other interactions with a p-value less than 0.20 and the significant terms from the single locus analysis were considered potentially interesting for the regression analysis and were used to build the regression models that were initially tested (see Table 24, Table 25, and Table 26).

Table 24: List of interesting terms used to build regression models for fasting free fatty acids.

FFA	Unsplit			
	PPARb6 PPARa5d*PPARb6d PPARb6*RXRb+140d PPARg6d*RXRb+140d RXRb10d*RXRb+140d			
Non-Hispanics		Hispanics		
PPARa5d*PPARb6d PPARa5d*PPARg6d PPARa5d*RXRb+140d PPARa6*PPARg2d PPARb6d*RXRb10d **RXRb10d*RXRb+140**		**PPARb6d** PPARa6*PPARg6d		
Non-Hispanic Males	Non-Hispanic Females	Hispanic Males	Hispanic Females	
PPARa5d*PPARb6d PPARg2d*PPARg6d RXRb10d*RXRb+140d	**PPARg6d** PPARa5d*PPARb6d PPARa5d*RXRb+140d RXRb10d*RXRb+140d	No terms	**PPARb6d** **PPARg6d** PPARa5d*RXRb+140d	

*Terms in **bold** are significantly associated with the outcome variable ($p<0.05$)
*Interesting terms were defined as having either a one-way ANOVA result with $p<0.05$ or a two-way ANOVA interaction with $p<0.20$.

Table 25: List of interesting terms used to build regression models for fasting glucose

FG	Unsplit
	PPARb6 PPARa5d*PPARa6 PPARa5d*PPARg2d **PPARa6*PPARg2d** **PPARa6*PPARg6d** PPARa6*RXRb+140 PPARg2d*PPARg6d RXRb10d*RXRb+140

Non-Hispanics		Hispanics	
PPARa6 **PPARb6d** PPARa6*PPARb6d **PPARa6*PPARg2d** **PPARa6*PPARg6d** **PPARa6*RXRb+140**		**PPARa5d*PPARg2d** PPARg2d*PPARg6d **RXRb10d*RXRb+140d**	
Non-Hispanic Males	Non-Hispanic Females	Hispanic Males	Hispanic Females
PPARa6 **PPARb6d**	PPARa5d*PPARg2d **PPARa5d*RXRb+140d** PPARb6d*PPARg6d	**RXRb+140** **PPARa5d*PPARg2d** PPARb6d*RXRb10d **PPARg2d*PPARg6d**	PPARg2d*PPARb6d

*Terms in **bold** are significantly associated with the outcome variable ($p<0.05$)
*Interesting terms were defined as having either a one-way ANOVA result with $p<0.05$ or a two-way ANOVA interaction with $p<0.20$.

Table 26: List of interesting terms used to build regression models for total cholesterol

Chol	Unsplit
	PPARa6*PPARb6d PPARg2d*PPARg6d

Non-Hispanics	Hispanics
PPARa5d PPARa5d*RXRb10d PPARa6*PPARg6d PPARg6d*RXRb10d	**PPARa6** **RXRb+140** **PPARa5d*PPARb6d** PPARa5d*RXRb+140d PPARg2d*PPARg6d

Non-Hispanic Males	Non-Hispanic Females	Hispanic Males	Hispanic Females
PPARb6d*RXRb10d PPARg6d*RXRb10d RXRb10d*RXRb+140d	**PPARa5d*RXRb10d** RXRb10d*RXRb+140d	**RXRb+140** PPARa5d*PPARb6d PPARa5d*RXRb+140d	**PPARa6** **PPARa5d*PPARb6d** PPARb6d*PPARg2d PPARb6d*PPARg6d PPARg2d*PPARg6d PPARg6d*RXRb10d

*Terms in **bold** are significantly associated with the outcome variable ($p<0.05$)
*Interesting terms were defined as having either a one-way ANOVA result with $p<0.05$ or a two-way ANOVA interaction with $p<0.20$.

4. Regression Modeling

Regression models were built to test the polymorphisms that were found to be potentially interesting in the analysis described above. Final models were constructed for each outcome in all seven of the groups (see Table 27, Table 28, Table 29 and Appendix E). Most of the models were reduced to one or two significant terms, either single locus effects or interactions, which explained between 1% and 4% of the variation in the unadjusted outcome variables. For example, the final model for fasting glucose in the Non-Hispanic females showed that the PPARb6d*PPARg6d interaction was the only significant predictor (p=0.008) and explained 3.5% of the trait variation (see Table 30). Therefore, the double common homozygotes (CC/CC) are expected to have a mean FG of 96.32mg/dL compared to 90.92mg/dL for all other genotypes combinations (see Figure 8).

Table 27: Regression Model Summaries for Fasting Free Fatty Acids (μmol/L) vs. PPARs and RXRs

Group	Terms	r^2	p-value
Unsplit	PPARb6	0.013	0.011
Non-Hispanics	RXRb+140 RXRb10*RXRb+140	0.016	0.061
Hispanics	PPARb6 PPARa6*PPARg6	0.052	0.001
Non-Hispanic Males*	PPARa5*PPARb6	0.018	0.066
Non-Hispanic Females	No Model	N/A	N/A
Hispanic Males	No Model	N/A	N/A
Hispanic Females	PPARb6 PPARa5*RXRb+140	0.110	P=0.001

Table 28: Regression Model Summaries for Fasting Glucose (mg/dL) vs. PPARs and RXRs

Group	Terms	r^2	p-value
Unsplit	PPARa6 PPARb6 PPARg6 PPARa6*PPARg6	0.026	0.002
Non-Hispanics	PPARa6 PPARa6*PPARb6	0.030	0.003
Hispanics	RXRb10*RXRb+140	0.017	0.024
Non-Hispanic Males	PPARb6	0.027	0.024
Non-Hispanic Females	PPARb6*PPARg6	0.035	0.008
Hispanic Males	PPARg6 PPARa5*hPPARg2 PPARa5*PPARg6 PPARg2*PPARg6 PPARg2*RXRb+140 PPARg6*RXRb+140	0.170	0.002
Hispanic Females	No Model	N/A	N/A

Table 29: Regression Model Summaries for Cholesterol (mg/dL) vs. PPARs and RXRs

Group	Terms	r^2	p-value
Unsplit	No Model	N/A	N/A
Non-Hispanics	PPARa5d*PPARa6	0.012	0.022
Hispanics	PPARa6*RXRb+140	0.040	0.002
Non-Hispanic Males	No Model	N/A	N/A
Non-Hispanic Females	RXRb10*RXRb+140	0.026	0.034
Hispanic Males	RXRb+140	0.071	0.003
Hispanic Females	PPARg2 PPARa6*RXRb10 PPARg2*PPARg6 PPARg6*RXRb10	0.108	0.009

Table 30: Regression Model for ln(Fasting Glucose) in Non-Hispanic Females

Term	β	p-value
Constant	4.510	0.000
PPARb6d*PPARg6d	0.0577	0.008

r^2=0.035
p-value=0.008

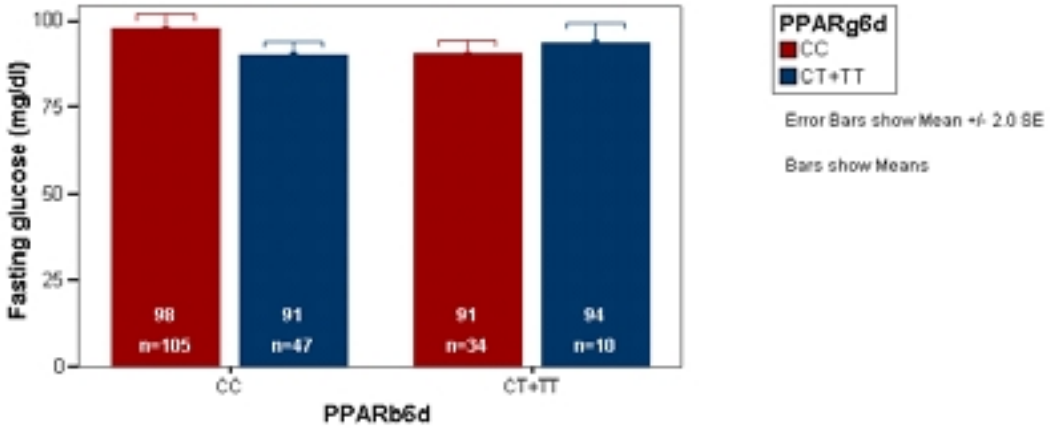


Figure 8: PPARb6d*PPARg6d vs. Fasting Glucose in Non-Hispanic Females

There were, however, several models that accounted for an even higher percentage of variation in the outcomes. The highest r^2 value was achieved in the fasting glucose model for Hispanic males that explained 17% of the variation and included six predictors (see Table 31). The significant terms consisted of a single locus effect by PPARg6d and various interactions between PPARa5d, PPARg2d, PPARg6d and RXRb+140d. The final model for cholesterol in Hispanic males included only RXRb+140d as a significant predictor, yet accounts for over 7% of the trait variation (r^2=0.071, p=0.003). This model predicts that presence of the T allele increases total cholesterol levels by 18.7mg/dL over the AA individuals (see Table 32 and Figure 9). Another final model that was constructed for Hispanic females was able to explain 11% of the variation in fasting free fatty acids with PPARb6d andPPARa5d*RXRb+140d as predictors (p=0.001) (see Table 33). In this model, subjects homozygous for the PPARb6 C allele show an increase of 156μmol/L for FFA versus individuals who have at least one of the rare alleles at each of the three sites (-T/-G/-T for b6/a5/b140) while those who are homozygous for the common allele of both PPARa5d and RXRb+140d have FFA levels 109μmol/L below the rare allele carriers. Individuals homozygous for the common alleles at each of the three sites show FFA levels slightly above the rare allele carriers (+47μmol/L) (see Figure 10).

Table 31: Regression Model for ln(Fasting Glucose) in Hispanic Males

Term	β	p-value
Constant	4.630	0.000
PPARg6d	0.677	<0.001
PPARa5d*PPARg2d	0.670	<0.001
PPARa5d*PPARg6d	-0.646	<0.001
PPARg2d*PPARg6d	-0.646	<0.001
PPARg2d*RXRb+140d	-0.754	<0.001
PPARg6d*RXRb+140d	-0.711	0.001

r^2=0.170
p-value=0.002

Table 32: Regression Model for Cholesterol in Hispanic Males

Term	β	p-value
Constant	204.596	0.000
RXRb+140d	-18.659	0.003

r^2=0.071
p-value=0.003

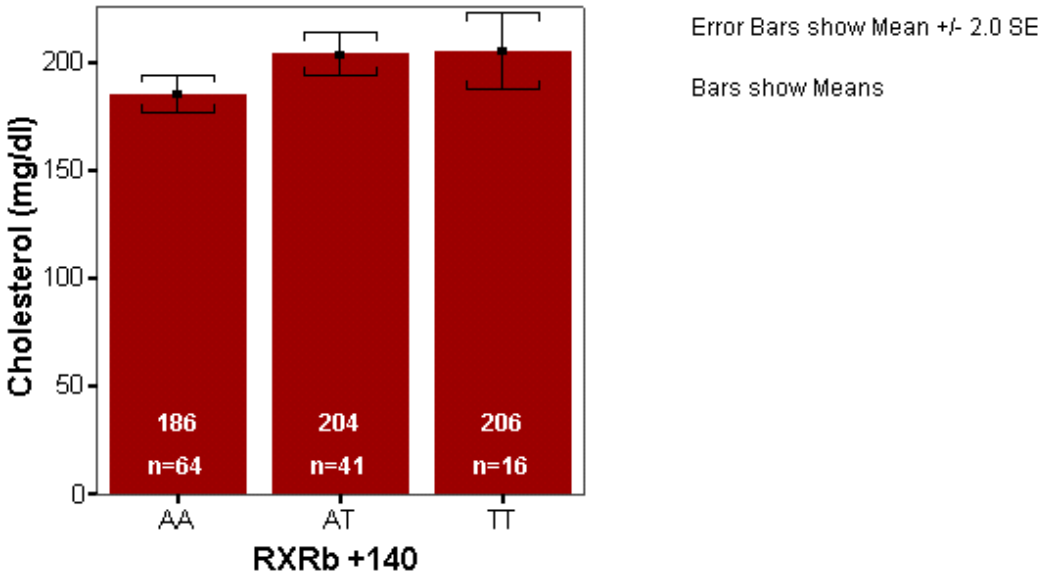


Figure 9: RXRb+140 vs. Cholesterol in Hispanic Males

Table 33: Regression Model for Fasting Free Fatty Acids in Hispanic Females

Term	β	p-value
Constant	632.443	0.000
PPARb6d	156.324	0.005
PPARa5d*RXRb+140d	-109.391	0.014

r^2=0.110
p-value=0.001

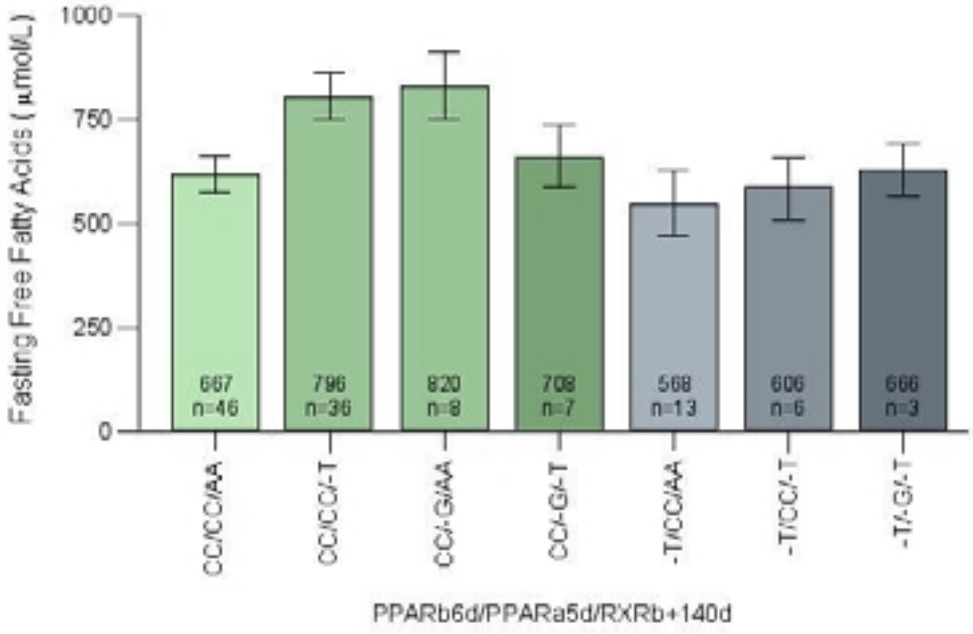


Figure 10: Multi-Locus Genotype Means for PPARb6d/PPARa5d/RXRb+140d vs Fasting Free Fatty Acids in Hispanic Females

5. Combined Analysis with UCPs and FABPs

Because of the known relationships between the PPARs, RXRs, UCPs, and FABPs, regression models were constructed using polymorphisms from all of these gene families. Regression models, similar to those described above, were constructed based on genotypic data for twenty polymorphisms in the UCP and FABP genes, provided by Coleen Damcott (Damcott, 2002). The allele frequencies of these sites are reported in Table 34. Several of these polymorphisms are in high linkage disequilibrium; therefore, many of the sites were not used in the analysis. Five

variations in the FABP2 promoter in complete linkage disequilibrium were represented in the analysis as FABP2pID, with the I allele referring to the more common G/G/D/I/I haplotype. The other sites used to represent another related variation were UCP1-2 for UCP1-5, UCP3p-55 for UCP3-3a, FABP2p-834 for FABP2-2, and FABP4p-376 for FABP4-2a. In addition to these five polymorphisms and the seven previously discussed PPAR or RXR variants, six other sites (UCP2-4, UCP2-8, UCP3-5, FABP4-2b, FABP3pID and FABP3p-313) were considered when building the combined regression models (see Figure 11 and Figure 12). Six of the UCP or FABP sites (UCP2-4, UCP2-8, UCP3-5, FABP2pID, FABP2p-834, and FABP3pID) were found to be at a high enough frequency for all three genotypes to be considered separately in the regression models so two dummy variables were created according to the criteria previously described (see Section III.D.1).

Table 34: Allele frequencies for Polymorphisms in the UCPs and FABPS

Polymorphic Site	Allele	Overall Frequency	Frequency		Method of Detection
			Hispanics	Non-Hispanics	
UCP1 Exon 2	G (Ala)	0.900	0.883	0.911	*HhaI* Digestion
	A (Thr)	0.100	0.117	0.089	
UCP1 Exon 5	A (Met)	0.899	0.888	0.911	*HindIII* Digest
	T (Leu)	0.101	0.112	0.089	
UCP2 Exon 4	C (Ala)	0.547	0.537	0.553	*EaeI* Digestion
	T (Val)	0.453	0.463	0.447	
UCP2 Exon 8	Del	0.665	0.679	0.656	2% Agarose Gel (45bp InsDel)
	Ins	0.335	0.321	0.345	
UCP3 Exon 3a	T	0.747	0.768	0.731	*DrdI* Digestion
	C	0.235	0.232	0.269	
UCP3 Exon 3b	G (Val)	0.995	0.992	0.998	*AspI* Digestion
	A (Ile)	0.005	0.008	0.002	
UCP3 Exon 5	T	0.545	0.557	0.536	*RsaI* Digestion
	C	0.455	0.443	0.464	
FABP2 Exon 2	G (Ala)	0.715	0.690	0.733	*HhaI* Digestion
	A (Thr)	0.285	0.310	0.267	
FABP4 Intron 2a	T	0.857	0.909	0.819	*AflIII* Digestion
	G	0.143	0.091	0.181	
FABP4 Intron 2b	Ins	0.879	0.837	0.908	*PvuII* Digestion
	Del	0.121	0.163	0.092	
UCP3 Promoter (-55bp)	C	0.782	0.805	0.765	*AvaI* Digestion
	T	0.218	0.195	0.235	
FABP2 Promoter (-834bp)	C	0.709	0.684	0.727	*BanII* Digestion
	T	0.291	0.316	0.273	
FABP2 Promoter (-778bp)	G	0.592	0.607	0.581	3% Agarose Gel: 7bp overall size difference in Ins/Del polymorphisms. Other genotypes inferred based on linkage disequilibrium.
	T	0.408	0.393	0.419	
FABP2 Promoter (-260bp)	G	0.592	0.607	0.581	
	A	0.408	0.393	0.419	
FABP2 Promoter (-169bp)	Del	0.592	0.607	0.581	
	Ins	0.408	0.393	0.419	
FABP2 Promoter (-166bp)	Ins	0.592	0.607	0.581	
	Del	0.408	0.393	0.419	
FABP2 Promoter (-136bp)	Ins	0.592	0.607	0.581	
	Del	0.408	0.393	0.419	
FABP3 Promoter (-493bp)	Del	0.586	0.565	0.600	Direct Sequencing
	Ins	0.414	0.435	0.400	
FABP3 Promoter (-313bp)	C	0.771	0.742	0.790	Direct Sequencing
	T	0.229	0.258	0.210	
FABP4 Promoter (-376bp)	A	0.856	0.894	0.829	Fluorescence Polarization
	C	0.144	0.106	0.171	

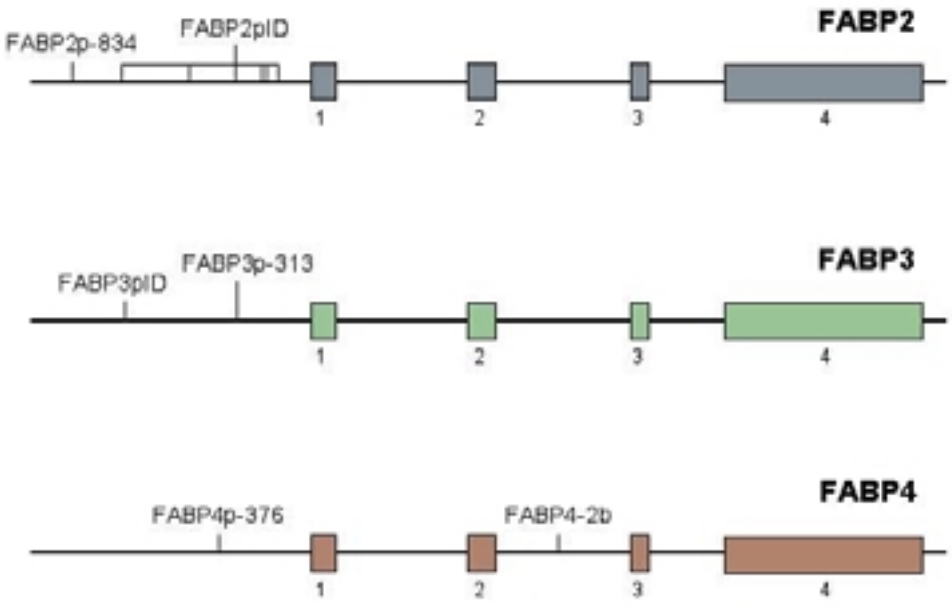


Figure 11: Locations of Polymorphisms in the FABPs

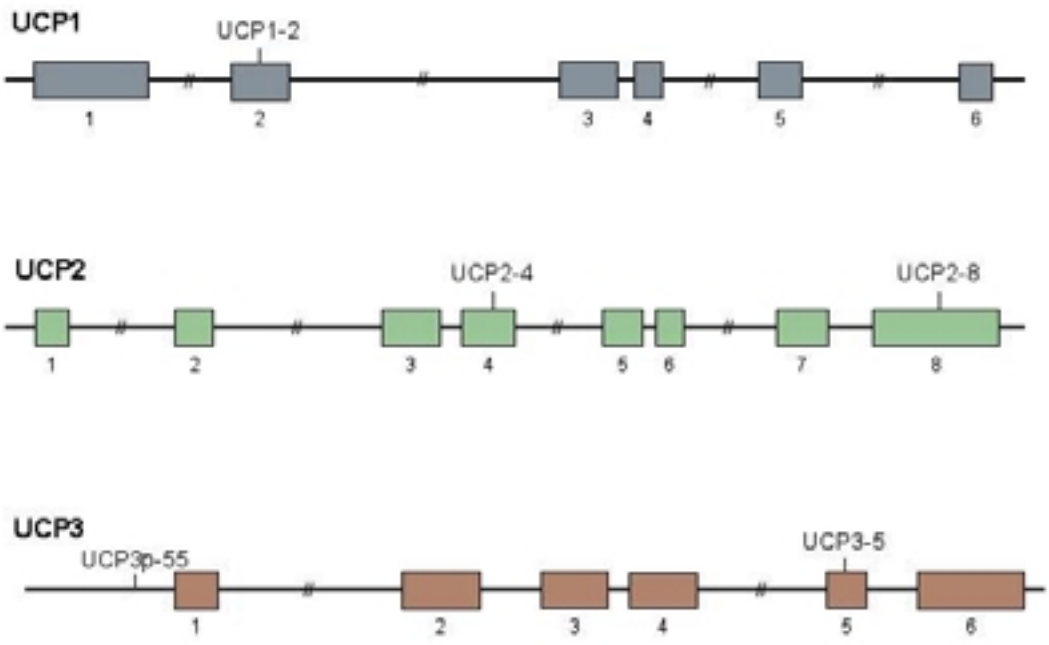


Figure 12: Locations of Polymorphisms in the UCPs

Regression models were constructed to predict fasting free fatty acids and cholesterol in each of the four ethnic/sex groups. The r^2 values for the eight models ranged from 0.054 up to 0.234 and most only contained two or three terms (see Table 35, Table 36 and Appendix G). All of these final models, except the Non-Hispanic female model for cholesterol, were able to explain a greater amount of the trait variation than the sum of the two corresponding models from the PPAR/RXR and UCP/FABP analyses. For example, the fasting free fatty acids model in Non-Hispanic females from the UCP/FABP analysis showed that the UCP2-4*UCP3-5 interaction was the only significant predictor of FFA levels with an r^2 value of 0.058 (see Appendix F, Table 68). The PPAR/RXR analysis did not predict any of the polymorphisms to be associated with fasting free fatty acids levels in Non-Hispanic females (see Table 27). However, when all eighteen of the sites are considered together, interactions between four of these sites (PPARa5d, PPARg6d, UCP3-5 and FABP3pID) are able to account for almost 14% of the variation in FFA levels (see Table 35).

Table 35: Combined Regression Model Summaries for Fasting Free Fatty Acids (μmol/L)

Group	Terms	r^2	p-value
Non-Hispanic Males*	PPARa5d*PPARb6d PPARb6d*FABP3pID	**0.054**	0.018
Non-Hispanic Females	PPARg6d PPARa5d*FABP3pID PPARg6d*UCP3-5 PPARg6d*FABP3pID	**0.139**	<0.001
Hispanic Males	UCP2-4 UCP2-8	**0.101**	0.008
Hispanic Females	UCP1-2 FABP3pID UCP1-2*RXRb+140d UCP1-2*UCP3p-55 UCP1-2*FABP3pID UCP3p-55*PPARb6	**0.234**	<0.001

*r^2 is in bold if it is more than the sum of the corresponding models from the PPAR/RXR and UCP/FABP analyses

Table 36: Combined Regression Model Summaries for Cholesterol (mg/dL)

Group	Terms	r^2	p-value
Non-Hispanic Males	PPARb6d PPARg6d PPARg6d*RXRb10d PPARb6d*UCP1-2 PPARg6d*FABP2p-834 RXRb10d*UCP1-2	**0.094**	0.025
Non-Hispanic Females	FABP2pID PPARa5d*UCP3-5	0.064	0.009
Hispanic Males	RXRb+140 UCP1-2 UCP1-2*UCP2-8	**0.197**	>0.001
Hispanic Females	UCP2-8 UCP3-5*FABP3pID PPARg6*UCP2-8	**0.157**	0.002

*r^2 is in bold if it is more than the sum of the corresponding models from the PPAR/RXR and UCP/FABP analyses

The final model for cholesterol in Hispanic males accounted for almost 20% of the variation in unadjusted total cholesterol levels (r^2=0.197) and included RXRb+140d, UCP1-2d and UCP1-2d*UCP2-8 as significant predictors (p<0.001) (see Table 37). The T allele of RXRb+140d was again shown to increase cholesterol levels by ~22mg/dL compared to AA homozygotes and presence of the A allele for UCP1-2 decreased cholesterol levels by 47mg/dL versus the GG individuals. However, the UCP1-2 GG individuals showed a modified effect when combined with the UCP2-8 Del allele which lowered cholesterol levels by ~55mg/dL (see Figure 13). Therefore, the subjects with a multi-locus genotype of GG/II/-T for UCP1-2/UCP2-8/RXRb+140 were predicted to have the highest mean cholesterol levels of 257mg/dL by this model.

Table 37: Combined Regression Model for Cholesterol in Hispanic Males

Term	β	p-value
Constant	204.596	0.000
RXRb+140d	-22.228	<0.001
UCP1-2d	47.337	0.002
UCP1-2d*UCP2-8v1	-52.358	<0.001
UCP1-2d*UCP2-8v2	-57.761	<0.001

r^2=0.197
p-value<0.001

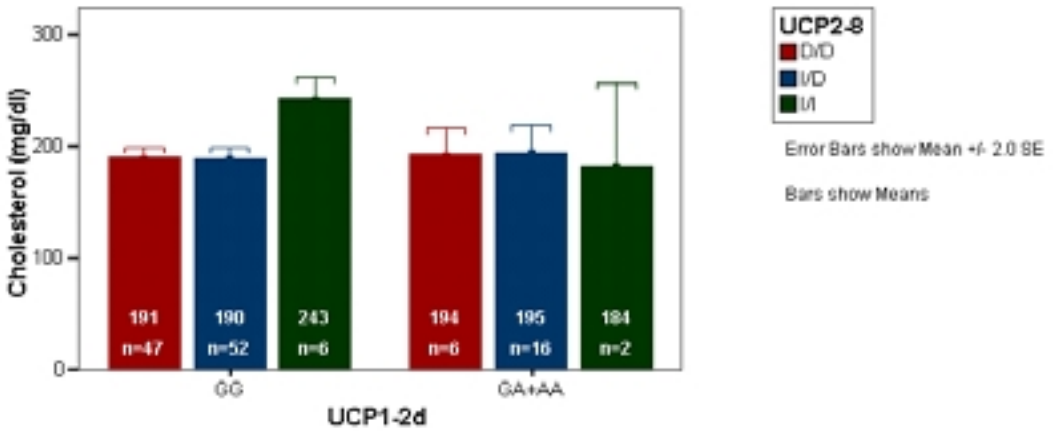


Figure 13: UCP1-2 genotype modification by UCP2-8 for cholesterol in Hispanic Males

The final model constructed for fasting free fatty acids in Hispanic females was able to account for 23.4% of the trait variation (see Table 38). This model included six terms with UCP1-2d showing the greatest effect on FFA levels where the GG homozygotes were predicted to have cholesterol levels elevated by 315μmol/L. This effect, however, was highly modified by three other polymorphisms so that in individuals homozygous for the common alleles at RXRb+140, UCP3p-55 and FABP3pID, the UCP1-2 GG genotype was predicted to have an FFA level of 554μmol/L while presence of the UCP1-2 A allele would increase this to 913μmol/L. In addition, individuals who were homozygous for the common alleles of UCP3p-55 and PPARb6 also were predicted to have 132μmol/L higher free fatty acid levels. Therefore, GG/DD/AA/CC/-T

(UCP1-2/FABP3pID/RXRb+140/UCP3p-55/PPARb6) was the multi-locus genotype with the lowest predicted mean FFA levels of 422μmol/L while –A/ DD/AA/CC/CC was the genotype with the highest predicted value of 913μmol/L (see Figure 14 and Figure 15).

Table 38: Combined Regression Model for Fasting Free Fatty Acids (μmol/L) in Hispanic Females

Term	β	p-value
Constant	557.985	0.000
UCP1-2d	315.908	<0.001
FABP3pIDv2	223.693	0.003
UCP1-2d*RXRb+140d	-168.160	0.001
UCP1-2d*UCP3p-55d	-182.675	0.006
UCP1-2d*FABP3pIDv2	-324.455	<0.001
UCP3p-55d*PPARb6d	131.697	0.018

r^2=0.234
p-value<0.001

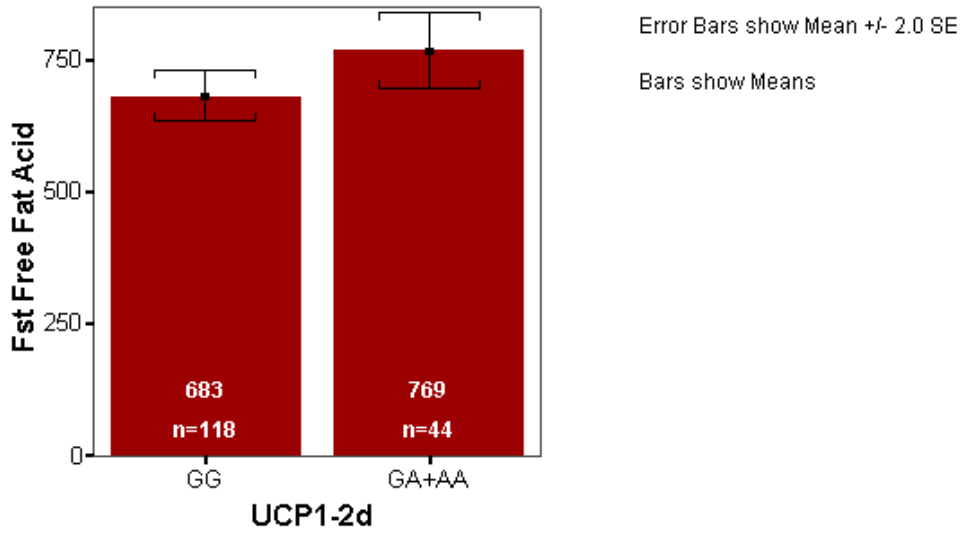


Figure 14: UCP1-2d vs fasting free fatty acid levels in Hispanic Females

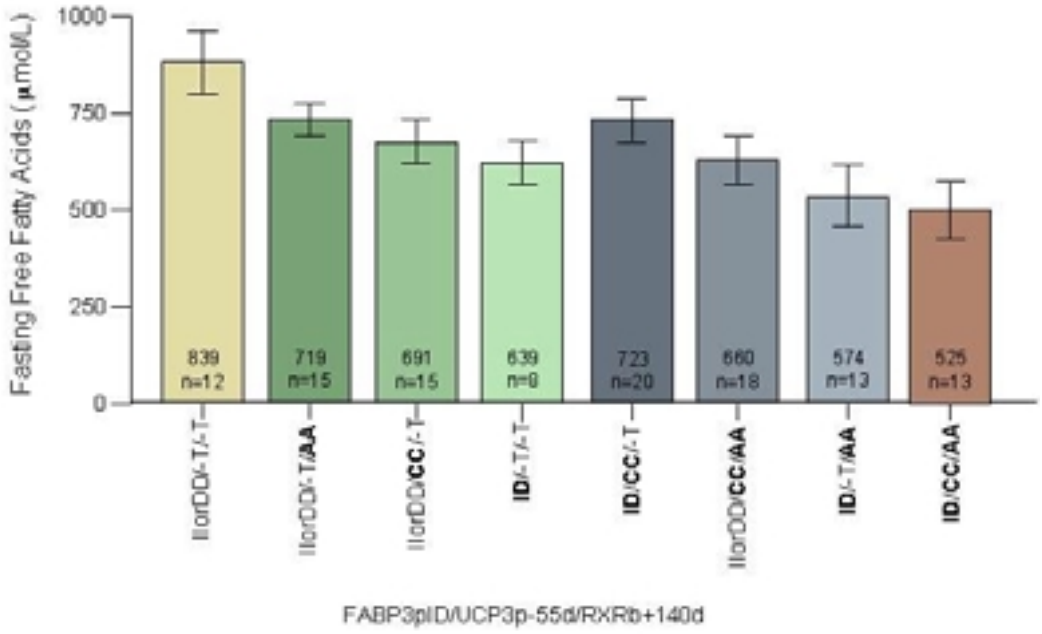


Figure 15: FABP3pID, UCP3p-55d and RXRb+140d modification of UCP1-2 GG Hispanic Females for fasting free fatty acid levels

6. Tissue Specific Analysis

Regression models were next tested that contained polymorphisms in the gene isoforms with the highest level of expression in several different tissues. Muscle models included sites in PPARα, FABP3 and UCP3. Adipose models looked at PPARγ, FABP4, UCP1 and UCP2 variations. Intestinal models tested PPARβ, FABP2 and UCP2 polymorphisms. After adjustment for several covariates, final models for fat mass and lean mass were found in both males and females (see Table 39, Table40, Table41 and Table 42). Similar models were also constructed for several lipid measures, but are not presented here in detail (Damcott, 2002) (see Appendix H). Two of the muscle models (FM in females and LM in males) showed only one significant interaction term while no terms were kept for the other two models (FM in males and LM in females). The adipose models were somewhat more complex yet still did not explain much of the trait variation; the exception was the female model for lean mass that had an r^2 of 0.056. On the other hand, the intestinal models accounted for the highest portion of the variation in lean mass for both sexes and in fat mass for males.

Table 39: Fat Mass Tissue Specific Regression Model Summary for Males

Tissue	Genotype Terms	p-value	r^2
Muscle	No Model	N/A	N/A
Adipose	PPARg2*FABP4-2b UCP1-2*FABP4-2b	0.017	0.027
Intestine	UCP2-8*FABP2p-ID UCP2-8*FABP2p-834 FABP2p-ID*FABP2p-834	0.003	0.057
Overall	UCP2-8 PPARg2*FABP4-2b UCP1-2*FABP4-2b UCP2-8*FABP2p-ID UCP2-8*FABP2p-834	<0.001	0.095

Table 40: Fat Mass Tissue Specific Regression Model Summary for Females

Tissue	Genotype Terms	p-value	r^2
Muscle	UCP3-5*FABP3p-313	0.001	0.033
Adipose	No Model	N/A	N/A
Intestine	No Model	N/A	N/A
Overall	UCP3-5*FABP3p-313	0.001	0.033

Table 41: Lean Mass Tissue Specific Regression Model Summary for Males

Tissue	Genotype Terms	p-value	r^2
Muscle	PPARa5*UCP3-5	0.025	0.016
Adipose	UCP2-4*FABP4-2b	0.033	0.015
Intestine	PPARb6*UCP2-4 UCP2-4*FABP2p-834 UCP2-8*FABP2p-834	0.002	0.058
Overall	PPARb6*UCP2-4 UCP2-4*FABP4-2b UCP2-4*FABP2p-834 UCP2-8*FABP2p-834	0.001	0.074

Table 42: Lean Mass Tissue Specific Regression Model Summary for Females

Tissue	Genotype Terms	p-value	r^2
Muscle	No Model	N/A	N/A
Adipose	FABP4p-376 UCP1-2*UCP2-8 UCP1-2*FABP4p-376 UCP2-4*UCP2-8	0.003	0.056
Intestine	UCP2-4 UCP2-8 PPARb6*UCP2-8 UCP2-4*FABP2p-834	<0.001	0.085
Overall	UCP2-4 FABP2p-834 FABP4p-376 PPARb6*UCP2-8 UCP1-2*UCP2-8 UCP1-2*FABP4p-376 UCP2-4*UCP2-8 UCP2-4*FABP2p-834	<0.001	0.155

Overall tissue models were then tested to determine which tissue was the most important contributor to fat mass or lean mass variation. The muscle model in females was the only tissue with a final model so it was considered the overall final model as well. This model showed that individuals homozygous for the common alleles of UCP3-5 and FABP3p-313 had a 4kg increase in fat mass over the other genotype groups (r^2=0.033, p=0.001) (see Table 43). The male overall model for fat mass, on the hand, was a combination of the adipose and intestinal models yet was able to account for more of the variation in fat mass than the other two models separately (see Table 44). The adipose contribution consisted of interactions between three polymorphisms, FABP4-2bd, PPARg2d and UCP1-2d. In the individuals homozygous for the FABP4-2b insertion allele, homozygosity for the common allele of PPARg2d and UCP1-2d seem to compensate for one another. However, in the FABP4-2b deletion carriers, presence of the less common alleles for either PPARg2d or UCP1-2d increases fat mass in an additive manner (see Figure 16). Homozygosity for the D allele of UCP2-8 was found to decrease fat mass by ~4kg although this effect was highly modified by the intestinal FABP2 polymorphisms. FABP2pID and FABP2p-834 were predicted to have opposite modifications of the UCP2-8 effect; an effect that is increasingly disparate with addition of FABP2pID D alleles (see Figure 17 and Figure 18).

Table 43: Overall Tissue Specific Regression Model for Fat Mass in Females

Term	β	p-value
Constant	27590	0.000
UCP3-5*FABP3p-313	4092	0.001

r^2=0.032
p-value=0.001

Table 44: Overall Tissue Specific Regression Model for Fat Mass (g) in Males

Term	β	p-value
Constant	25940	0.000
UCP2-8v1	-3955	0.007
PPARg2d*FABP4-2bd	-2877	0.007
UCP1-2d*FABP4-2bd	2405	0.027
UCP2-8v2*FABP2pIDv1	-6604	0.002
UCP2-8v2*FABP2pIDv2	-4587	0.002
UCP2-8v1*FABP2p-834v1	3747	0.005
UCP2-8v2*FABP2p-834v1	4240	0.013

r^2=0.095
p-value<0.001

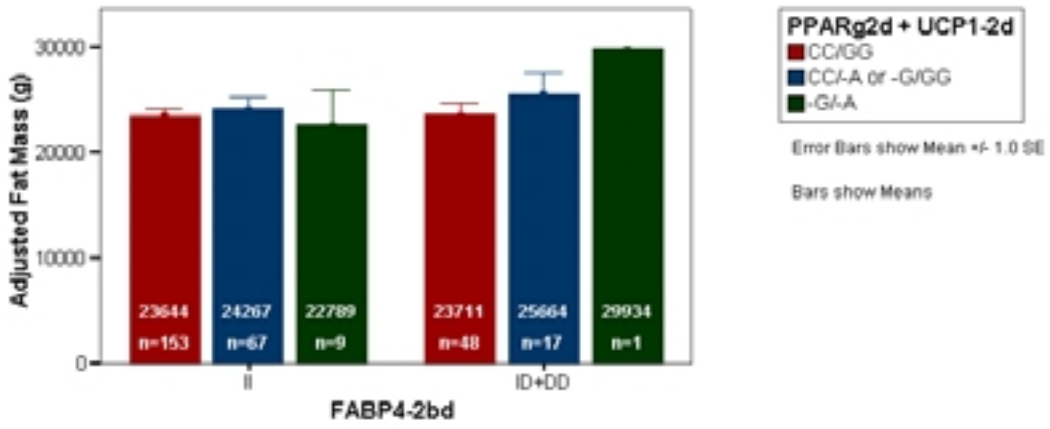


Figure 16: FABP4-2bd modification by PPARg2d and UCP1-2d for adjusted fat mass in males

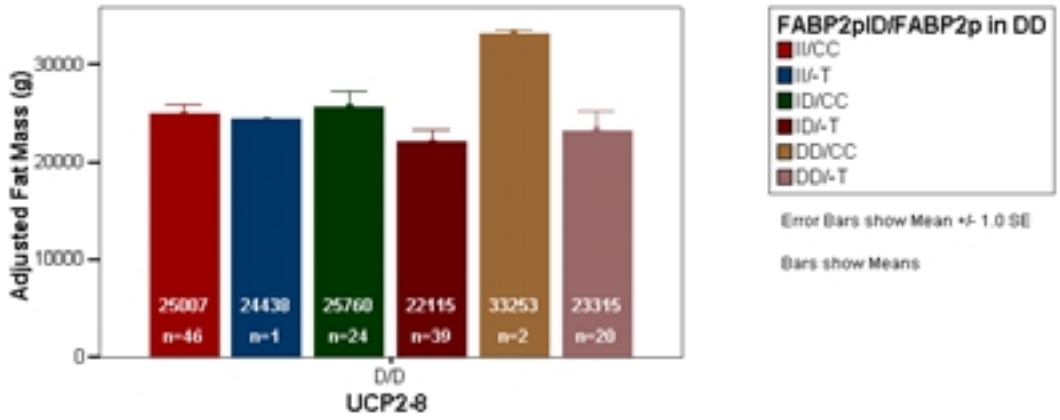


Figure 17: UCP2-8 DD modification by FABP2 promoter genotypes for adjusted fat mass in males

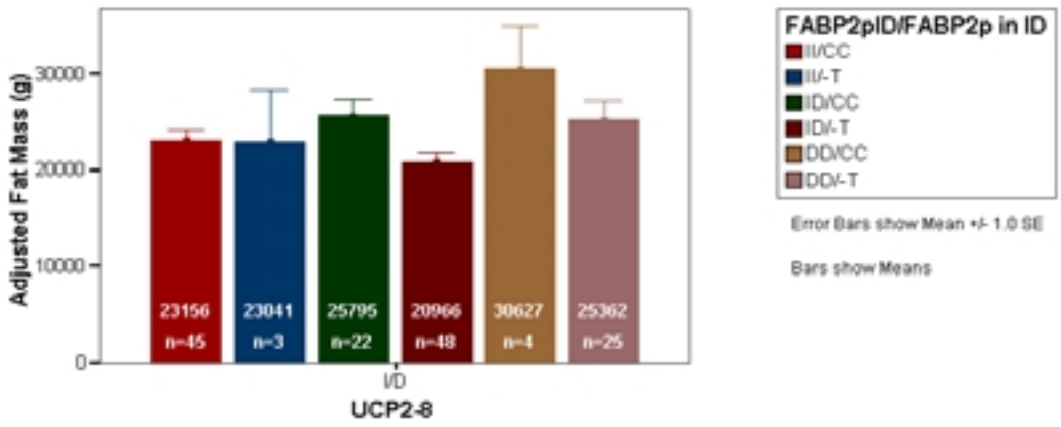


Figure 18: UCP2-8 DI modification by FABP2 promoter genotypes for adjusted fat mass in males

The overall model for lean mass in males was also a combination of terms from the adipose and intestinal models (see Table 45). The adipose term, an interaction between FABP4-2bd and UCP2-4, shows that individuals with either the II/TT, -D/CC or –D/CT multi-locus genotypes (FABP4-2bd/UCP2-4) have an increase of 4kg in their lean mass compared to the II/CC or II/CT subjects. Surprisingly, homozygosity for both of the less common alleles reduces lean mass by ~3kg instead of the expected increase of ~8kg if the effect were additive (see Figure 19). In addition to an interaction between PPARb6d and UCP2-4, the intestinal model also adds an effect of FABP2p-834 that is modified by both UCP2-4 and UCP2-8. In individuals with the CC genotype for FABP2p-834, the T allele appears to reduce lean mass in an additive manner and then UCP2-8 modifies this effect somewhat (see Figure 20).

Table 45: Overall Tissue Specific Regression Model for Lean Mass in Males

Term	β	p-value
Constant	47026	0.000
PPARb6d*UCP2-4v1	-5890	0.001
UCP2-4v2*FABP4-2bd	-2503	0.046
UCP2-4v1*FABP2p-834v1	6235	0.005
UCP2-8v1*FABP2p-834v1	-3416	0.033
UCP2-8v2*FABP2p-834v1	-2855	0.039

r^2=0.074
p-value=0.001

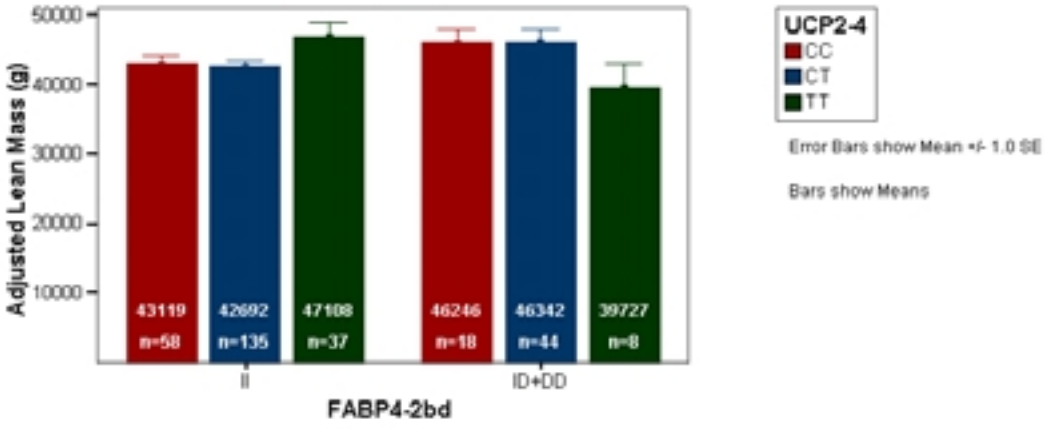


Figure 19: UCP2-4*FABP4-2b interaction for adjusted lean mass in males

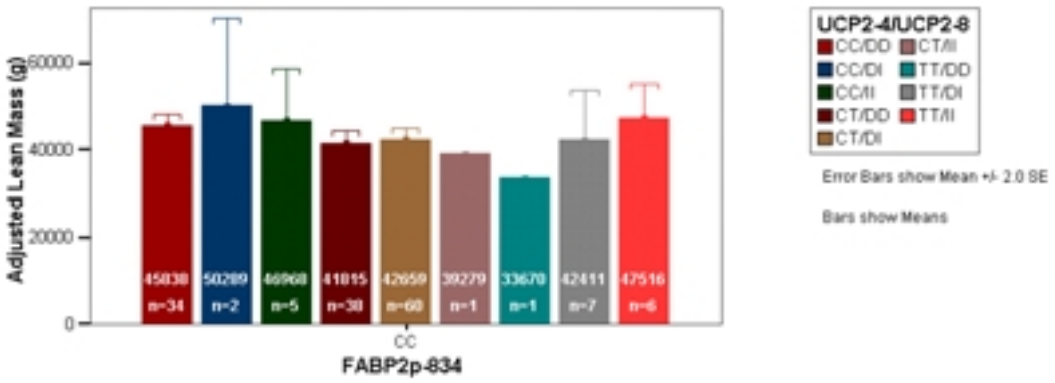


Figure 20: FABP2p CC modification by UCP2-4/UCP2-8 genotypes for adjusted lean mass in males

The overall model for lean mass in females again showed the additive contribution of both the adipose and intestinal tissues to explain a total of 15.5% of the adjusted trait variation. This complex model included a single locus effect by FABP4p-376 and three interactions (UCP1-2d*UCP2-8, UCP1-2*FABP4p-376 and UCP2-4*UCP2-8) from the adipose tissue as well intestinal effects by UCP2-4, FABP2p-834, PPARb6d*UCP2-8 and UCP2-4*FABP2p-834 (see Table 46). The major effect from the adipose tissue seemed to be due to FABP4p-376 although this effect was compensated for in UCP1-2 GG subjects (see Figure 21). UCP2-4 and FABP2p-834 appear to be the most important site from the intestinal polymorphisms. The UCP2-4 terms predict a difference of ~27kg between the two homozygous groups while FABP2p-834 would show an 18kg difference. Interactions between the different genotypes at these two sites, however, compensate for these differences in many individuals. Individuals with the TT/CC genotypes (UCP2-4/FABP2p-834) do show a mean lean mass 12kg lower than TT/TT subjects (see Figure 22).

Table 46: Overall Tissue Specific Regression Model for Lean Mass in Females

Term	β	p-value
Constant	54127	0.000
UCP2-4v1	-9599	0.017
UCP2-4v2	-18252	<0.001
FABP2p-834v1	-12933	0.002
FABP2p-834v2	-6702	0.045
FABP4p-376d	7537	<0.001
PPARb6d*UCP2-8v2	-8079	<0.001
UCP1-2d*UCP2-8v2	8151	<0.001
UCP1-2d*FABP4p-376d	-7462	<0.001
UCP2-4v1*UCP2-8v1	7735	0.021
UCP2-4v1*FABP2p-834v1	6726	0.047
UCP2-4v2*FABP2p-834v1	21008	<0.001
UCP2-4v2*FABP2p-834v2	15384	<0.001

r^2=0.155
p-value<0.001

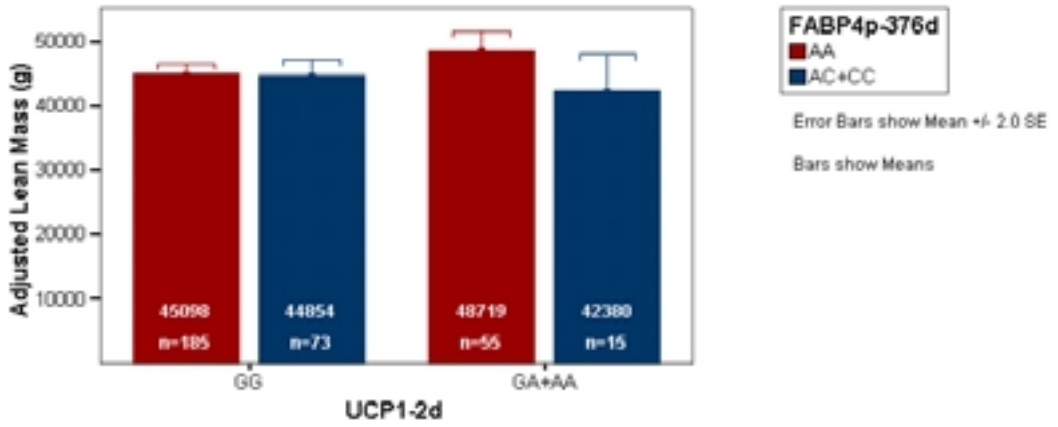


Figure 21: UCP1-2d*FABP4p-376 interaction for adjusted lean mass in females

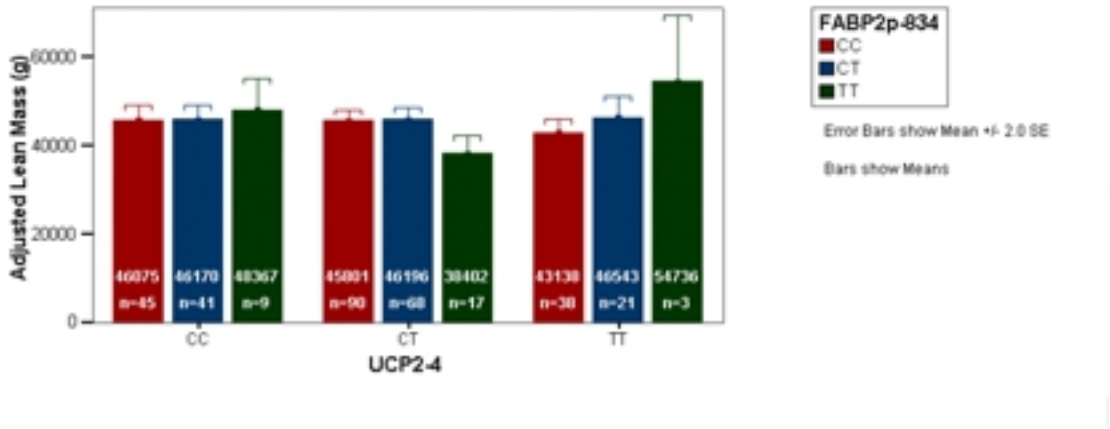


Figure 22: UCP2-4*FABP2p-834 interaction for adjusted lean mass in females

D. Other Analyses

1. PPARb6

Further analysis of the PPARb6 variant was preformed to explore its effect on fasting glucose, fasting insulin and HOMA IR. Allele frequencies for PPARb6 in the two ethnic groups were similar (q=0.137 in H and q=0.127 in NH) and conformed to the expectations of Hardy-Weinburg equilibrium. Analysis of variance showed that PPARb6 was significantly associated with fasting glucose (p=0.013), fasting insulin (p=0.034) and HOMA IR (p=0.013) (see Figure 23, Figure 24, and Figure 25). Individuals homozygous for the less common allele of PPARb6 had on average 11 mg/dL lower fasting glucose, 3 mg/dL lower fasting insulin and 8 units lower HOMA IR when

compared to the common homozygote. Regression analysis showed that PPARb6, sex, and skin reflectance were all significant predictors of ln(fasting glucose) ($p<0.001$) while PPARb6 and skin reflectance were both significant predictors of ln(fasting insulin) ($p=0.001$) and ln(HOMA IR) ($p<0.001$) (see Table 47, Table 48, and Table 49).

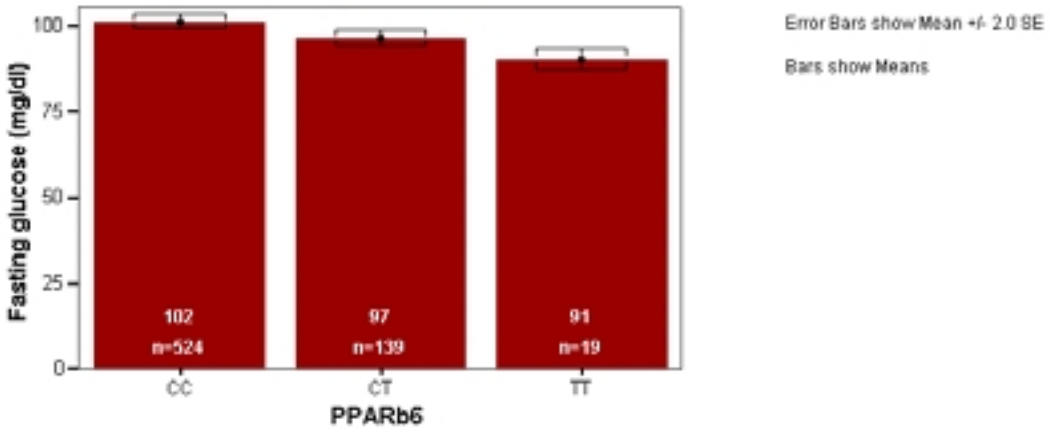


Figure 23: PPARb6 vs. fasting glucose in SLVDS sample

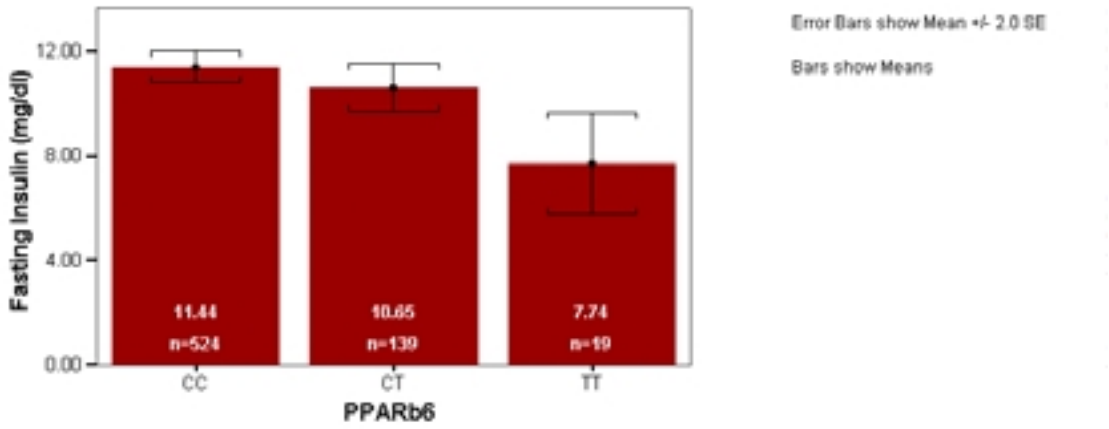


Figure 24: PPARb6 vs. fasting insulin in SLVDS sample

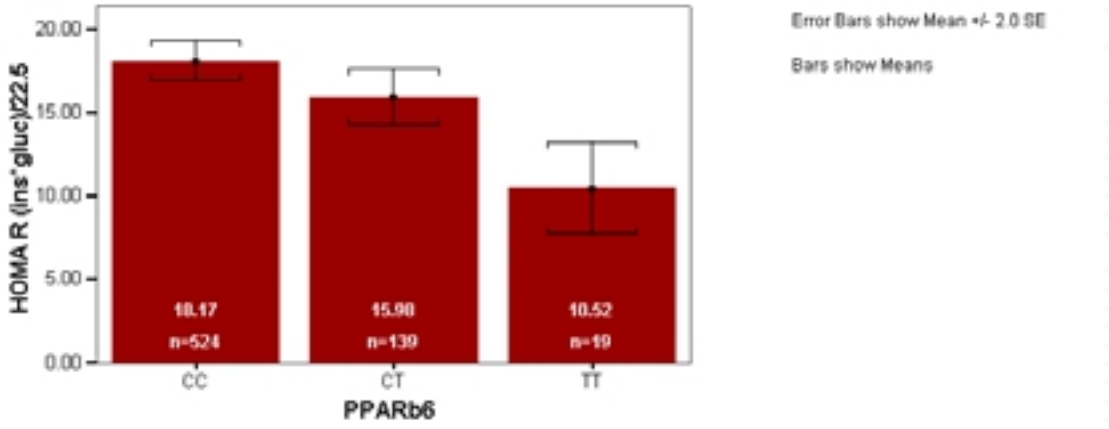


Figure 25: PPARb6 vs. HOMA IR in SLVDS sample

Table 47: Regression Model for ln(Fasting Glucose) in the SLVDS

Term	β	p-value
Constant	4.82	<0.001
Skin reflectance	-0.0035	0.039
Sex	-0.068	<0.001
PPARb6	-0.043	0.001

r^2=0.056
p>0.0001

Table 48: Regression Model for ln(Fasting Insulin) in the SLVDS

Term	β	p-value
Constant	2.77	<0.001
Skin reflectance	-0.014	0.004
PPARb6	-0.088	0.023

r^2=0.019
p=0.001

Table 49: Regression Model for ln(HOMA IR) in the SLVDS

Term	β	p-value
Constant	3.29	<0.001
Skin reflectance	-0.0018	0.002
PPARb6	-0.129	0.006

r^2=0.024
p=0.0003

2. Analysis of PPARγ Polymorphisms

Linkage disequilibrium was calculated for the two polymorphisms in PPARγ, PPARg2 and PPARg6, which were found to be in high LD (D'=0.767). In order to determine the relative contribution of each of the polymorphisms in the PPARγ gene, PPARg2d and PPARg6d were tested for associations between several outcome variables that had been adjusted for skin reflectance (fasting glucose, fasting insulin, HOMA IR, fasting free fatty acids, percent body fat, and fat mass). No significant associations between these variables and either of the polymorphisms were found for males; however, females showed significantly different genotypic means for PPARg6d in fasting insulin and HOMA IR (see Table 50). Presence of the PPARg6 T allele reduced fasting insulin levels by 2.2mg/dL in females CC homozygotes (p=0.008). Similarly, mean HOMA IR was reduced by almost 5 units in carriers of the PPARg6 T allele (p=0.007). After correcting for age, smoking, fat mass, and skin reflectance, linear regression showed that PPARg6d was a significant predictor of fasting insulin and HOMA IR while PPARg2d was not. Models were created for each polymorphism alone and then with both sites plus the interaction between them. The PPARg2d models were not significant and only explained 0.02% (HOMA IR) to 0.04% (fasting insulin) of the trait variation while both models with only PPARg6d accounted for 1.5% of the variation in fasting insulin (p=0.031) or HOMA IR (p=0.028). No significant interactions between the two sites were observed for either fasting insulin or HOMA IR (see Table 51 and Table 52).

Table 50: Genotype Means (±SD) for PPARg2d and PPARg6d vs. outcome variables adjusted for skin reflectance in females

	PPARg2d		PPARg6d	
	CC	CG or GG	CC	CT or TT
Fasting Glucose (mg/dL)	89±19	89±14	90±20	86±10
Fasting Insulin (mg/dL)	11.5±7.3	10.0±5.7	**11.7±7.4**	**9.5±5.0**
HOMA IR	17.9±14.9	15.3±11.5	**18.4±15.3**	**13.8±8.6**
Free Fatty Acids (μmol/L)	662±245	661±236	663±237	653±253
Percent Fat (%)	42.1±7.2	41.3±7.6	42.2±7.1	41.0±8.0
Fat Mass (kg)	29.2±9.7	29.0±10.1	29.4±9.8	27.8±10.0
N	271	84	244	86

Values in **bold** represent significantly different means between the genotype groups ($p<0.05$).

Table 51: Regression Models for ln(Fasting Insulin) (mg/dL) vs. PPARg2d and PPARg6d

Models	**β**	**p-value**	**Constant**	**r^2**
PPARg2d	-0.065	0.268	2.224	0.004
PPARg6d	-0.123	0.031	2.240	0.015
PPARg2d	0.114	0.347	2.233	0.018
PPARg6d	-0.109	0.285		
g2d*g6d	-0.122	0.461		

Table 52: Regression Models for ln(HOMA IR) vs. PPARg2d and PPARg6d

Models	**β**	**p-value**	**Constant**	**r^2**
PPARg2d	-0.057	0.418	2.577	0.002
PPARg6d	-0.150	0.028	2.602	0.015
PPARg2d	0.188	0.193	2.590	0.021
PPARg6d	-0.161	0.188		
g2d*g6d	-0.158	0.426		

V. Discussion

A. Screening and Identification of Variation

The coding regions of exons 2 through 8 in the PPARβ gene were screened using SSCP and direct sequencing resulting in identification of one new polymorphism in exon 6. This substitution of a T for a C (PPARb6) does not alter an amino acid or restriction enzyme site, but can be detected through an engineered *Nde*I site. An estimated allele frequency of 0.3125 was calculated for the C allele from the eight screening samples with reliable genotypes. Approximately 1kb of the region 5' to exon 1 was also screened for variation although no polymorphisms were identified. One kilobase of the promoter region for the PPARγ2 isoform and the putative promoter region (5' of exon 1) for PPARα were also screened for genetic variation. No polymorphisms were identified in any of these segments. However, further screening of the PPARα, PPARβ and PPARγ genes may be warranted since the only newly identified polymorphism is a silent substitution and other potentially functional regions of the genes remain unscreened. This study only looked at the γ2 promoter for the PPARγ gene, but there could be variation in the γ1, γ3 or γ4 promoters that would alter gene transcription levels. In addition, given that PPARγ has four functional promoter regions as well as four separate transcripts, it is possible that there are also multiple promoters for PPARα and PPARβ. Functional characterization of the 5' putative promoter regions for these genes is necessary to delineate the regulatory elements for the genes and to aid in identification of polymorphisms that could alter expression.

The coding region of RXRα was also screened using SSCP and direct sequencing in sixteen individuals although no variations were identified. Recently, another group screened RXRα for genetic variation and was able to identify three polymorphisms in the gene (Hegele, 2001). Two of these sites are intronic, an A to G substitution in intron 7 at +69bp and a G to A substitution at –25bp in intron 9. The third site, a C to T substitution, is found in the 3' untranslated region at 1470bp. The intronic variations were at a frequency of q=0.28 for the A allele of intron 7 +69A/G and q=0.32 for the G allele in intron 9 –25G/A in 30 Caucasian individuals. The T allele for the 3'-UTR 1470C/T polymorphism was only found at a frequency of q=0.05 in 20 Caucasians (Hegele, 2001). None of these variations are within the regions screened in this project and the report of their existence was published after the screening and genotyping phase of the project was complete thus they were not included in any analyses.

B. Genotyping

Seven polymorphisms in the PPARα, PPARβ, PPARγ and RXRβ genes were identified either from the literature or from screening by SSCP and sequencing. These polymorphisms were typed in 764 individuals from the SLVDS population and were all found to conform to the expectations of Hardy-Weinburg equilibrium in each of the ethnic groups. Most of these sites showed similar frequencies in the two ethnic subsets of the SLVDS although the rare alleles for PPARa6 and RXRb10 showed somewhat higher frequencies in the Hispanic individuals than in the Non-Hispanic individuals

(q=0.035 vs. q=0.009 for PPARa6 and q=0.20 vs. q=0.129 for RXRb10). The allele frequencies for PPARa5, PPARg2 and PPARg6 found in this study were similar to previously reported frequencies for these sites in similar populations (Flavell, 2000; Vohl, 200; Cole, 200; Yen, 1997; Beamer, 1998).

C. Multi-Locus Analysis

1. Descriptive Statistics

Descriptive statistics were calculated for all variables used in the analysis. The SLVDS study population was comprised of 42% individuals of Hispanic descent, similar to the frequency of Hispanic individuals found in the San Luis Valley (43.6%)(Hamman, 1989). There were similar proportions of males and females in the two ethnic groups (48% NH male and 45% H male). Participants were between 34 and 88 years of age with a mean age of 63 years. There was a marginally significant difference in the mean age between Hispanics (62 years) and Non-Hispanics (64 years) although this effect should not have altered our results since the ethnic groups were either treated separately or age was adjusted for in the regression model. Hispanic individuals did show higher mean values for fasting free fatty acids, triglycerides, fasting glucose, fasting insulin, and HOMA IR and a lower mean for skin reflectance, as expected. Interestingly, Non-Hispanics showed increased caloric intake (NH=2016, H=1810; p=0.001) and fat intake (NH=79, H=71; p=0.015) when compared to Hispanic individuals (see Table 15).

2. Univariate Analysis of Outcome and Predictor Variables

Analysis of variance was used to test for associations between the outcome and continuous predictor variables and the genotypic data. Two of the original outcomes, BMI and fat mass, did not show any significant associations with the PPAR or RXR polymorphisms. Fasting free fatty acid levels, on the other hand, showed an additive effect of PPARb6 in the unsplit sample as well as in the Hispanics. In the Hispanic group, each T allele decreased FFA levels by ~92μmol/L with a total difference of 185μmol/L between the CC and TT homozygotes. While not significant, the genotypic means for PPARb6 were likewise decreasing with addition of each T allele in the Non-Hispanic group. It is not surprising that a genotypic effect on FFA would be more pronounced in the Hispanic group since they have significantly higher mean FFA levels compared to the Non-Hispanics. The PPARb6 polymorphism was also found to be associated with decreased fasting glucose levels in the unsplit sample supporting the idea that the T allele is in linkage disequilibrium with a protective allele at another site.

The PPARa6 polymorphism was found to have varying effects for different traits. Heterozygosity for PPARa6 was associated with increased fasting glucose levels in Non-Hispanics (TT=97, TC=119; $p<0.001$) but decreased cholesterol levels in Hispanics (TT=201, TC=185; 0.045). While the effect on fasting glucose is stronger, there are only 8 Non-Hispanic heterozygous individuals compared to 20 Hispanic heterozygotes. When the analysis was preformed for each of the ethnic/gender groups, Non-Hispanic males heterozygous for PPARa6 showed an even greater increase in fasting glucose levels compared to the TT homozygous subjects (TT=100, TC=128; $p<0.001$). On the

other hand, the effect on cholesterol in Hispanic females was similar yet only marginally significant (TT=207, TC=189; 0.052). Without functional studies of the PPARa6 variation, it is difficult to determine if the C (Ala) allele is detrimental, protective or actually has different effects in the ethnic groups although the current study leans toward a detrimental role for the C allele.

In the Hispanic sub-group, the T allele of RXRb+140 was found to have a dominant effect on cholesterol levels with an increase of ~12 mg/dL over the AA individuals. This effect was even more pronounced when only the males were analyzed showing an increase of ~20mg/dL with the presence of the T allele. Because this site is intragenic and has no known effect on RXRβ function, it is possibly in linkage disequilibruim with another variant in RXRβ or a neighboring gene that has different frequencies in the two ethnic groups.

Gene-gene interactions were initially examined by two-way analysis of variance. All pair-wise combinations of the seven polymorphisms were tested against each of the outcome variables in the seven subject groups. The Non-Hispanic subset showed a significant interaction between PPARa5d and PPARb6 for fasting free fatty acids which could indicate that PPARb6 is important in both ethnic groups, but the effect in Non-Hispanics is not as strong so that two PPAR variations are required for the same reduction in FFA levels. The PPARa5d*PPARb6d interaction was also significantly associated with cholesterol in the Hispanic individuals. Several interactions between the PPARα sites and the PPARγ sites were found for fasting glucose. PPARa6 had

significant interactions with both variants in PPARγ for Non-Hispanics while the Hispanic group showed a significant interaction between PPARa5d and PPARg2d. The presence of multiple PPAR interactions could be due to the small effect size of each polymorphism so that significant alterations in a trait only occur when an individual has more than one allele with a similar effect (i.e. protective or deleterious).

3. Regression Models

a) PPAR and RXR Models

Regression models were tested with the potentially interesting polymorphisms determined by analysis of variance in either the PPARs or RXRβ. Most of the final models were reduced to one or two significant terms, either single locus effects or interactions, which explained between 1% and 4% of the variation in the unadjusted outcome variables. The Non-Hispanic female model for fasting glucose is a good example of these simple models. Individuals homozygous for the common alleles of PPARb6 and PPARg6 were found to have 6mg/dL higher fasting glucose levels compared to subjects with at least one of the less common alleles for either site (see Figure 8). This model again illustrates the protective nature of the PPARb6 T allele as well as the T allele for PPARg6 that is in linkage disequilibrium with the G (Ala) allele of PPARg2. Other studies have found evidence that the PPARg2 G allele protects against susceptibility to type-2 diabetes, an association that should be closely related to reduced fasting glucose levels (Altshuler, 2000).

Another example of a single term effect was the Hispanic male model for total cholesterol. This model predicts that the T allele of RXRb+140d increases total cholesterol levels by almost 20mg/dL. The mean cholesterol levels for the AT and TT individuals were very similar in this group (AT=204mg/dL, TT=206mg/dL vs. AA=186mg/dL) suggesting that the T allele acts in a dominant manner (see Figure 9). This single polymorphism, surprisingly, was able to account for over 7% of the variation in total cholesterol levels in the Hispanic male subjects. Similarly, RXRb+140d was also a significant predictor of fasting glucose levels in Hispanic males, along with PPARa5, PPARg2 and PPARg6. Various interactions between these four sites were able to explain 17% of the variation in fasting glucose for that group (see Table 31). Each of these variants was found in an interaction with at least two of the other sites possibly suggesting the presence of higher order interactions that were not tested for in this analysis.

The model constructed for fasting free fatty acids in the Hispanic females was also able to account for a large portion of the trait variation (r^2=0.110) with only two terms as significant predictors. The PPARb6d common homozygote again showed an increase of 156μmol/L over the T allele carriers although this effect was modified by homozygosity for the common alleles of both PPARa5d and RXRb+140d that reduced FFA by 109μmol/L (see Figure 10).

b) Combined Regression Models

The regression models constructed for fasting free fatty acids and cholesterol were expanded by adding eleven polymorphisms from the UCPs and FABPs to the analysis. A similar method was used by Coleen Damcott to construct models for each of the outcome variables using the eleven UCP/FABP sites (Damcott, 2002). These models combined with the PPAR/RXR models described previously became the basis for the combined model testing. Interaction terms between the PPAR/RXR sites and the UCP/FABP sites were also calculated to look for other potentially interesting terms to include in the model building.

The Hispanic male model for cholesterol again showed a significant effect of the RXRb+140d variation as well as an effect by UCP1-2 that was modified by UCP2-8. Inidividuals with the GG genotype for UCP1-2 and II genotype for UCP2-8 had mean cholesterol levels of 243mg/dL, an increase of ~53mg/dL over the other genotypes (see Figure 13). When the effect of the RXRb+140d T allele is added to this, the GG/II/-T multi-locus genotype is predicted to have cholesterol levels of 257mg/dL. These three polymorphisms were able to account for almost 20% of the variation in cholesterol for Hispanic males. It is interesting to note that RXRb+140d and UCP2-8 were both found to be single locus predictors of cholesterol levels in the separate analyses, but that UCP1-2 was not in the previous model (see Table 32 and Table 71).

The model for fasting free fatty acids in Hispanic females was also found to explain a large portion of the trait variation, 23.4%, and included six terms. When considered alone, the A allele for UCP1-2 increases FFA levels by ~90μmol/L over the GG homozygotes (see Figure 14). However, three other polymorphisms, FABP3pID, UCP3p-55 and RXRb+140d, alter the UCP1-2 GG FFA levels to give a range of values from 839μmol/L in the twelve GG subjects with none of the protective modifying genotypes to 525μmol/L in the thirteen individuals with all three protective genotypes (see Figure 15). The UCP3p-55d*PPARb6d interaction was also a significant predictor of FFA where individuals homozygous for both alleles were predicted to have FFA levels 132μmol/L higher than the other genotypes. This effect appears very similar to the PPARb6d effect seen previously in the PPAR/RXR models for Hispanic females and may not actually represent a modification of the PPARb6 effect by UCP3p-55. Instead, the PPARb6d*UCP3p-55 interaction could be indicating a higher order interaction between PPARb6d, UCP3p-55, and UCP1-2 which was not tested.

c) Tissue Specific Regression Analysis

A third set of models was constructed using the polymorphisms in the PPARs, UCPs and FABPs using fat and lean body mass as outcomes. These models, unlike the previous ones, were built around the known expression patterns of the proteins in three tissue types: muscle, adipose and intestine. By choosing the isoforms with the highest expression for each of the tissues and then testing for all possible interactions

between these sites for a given tissue, a more biologically meaningful final model may have been achieved. This analysis plan allowed for detection of multiple interactions that are interdependent and not necessarily significant as seen in a two-way analysis of variance. Another advantage of this method was that, for this study, it was less time consuming since the initial determination of potential loci to include in the regression analysis was unnecessary.

Three of the four overall final models constructed (lean mass in males, lean mass in females and fat mass in males) were combinations of the adipose and intestinal final models. The female overall model for fat mass only included a single term from the muscle specific model since the adipose and intestinal models did not retain any significant terms. In this case, other genes seem to be having a larger effect on fat mass in females since no major effect was noted here although higher order interactions between the tested polymorphisms is a possibility. The male fat mass model, a combination of the adipose and intestinal models, was able to explain 9.5% of the variation in adjusted fat mass. The contributions from adipose and intestinal models to the overall model each illustrate multi-locus gene effects. The adipose terms show an effect by FABP4-2bd that is modified by both UCP1-2d and PPARg2d (see Figure 16). UCP2-8 from the intestinal model was also a major predictor of fat mass in males with a modified effect from the FABP2 promoter polymorphisms (see Figure 17 and Figure 18). It is interesting to note that the two variants in the FABP2 promoter had opposing effects on fat mass and thus would cancel each other out in many haplotypes.

The overall tissue model for lean mass in males also showed a significant interaction between UCP2-8 and FABP2p-834 although this was counteracted by a UCP2-4*FABP2p-834 interaction instead of UCP2-8*FABP2pID (see Figure 20). In addition, UCP2-4 modified the adipose FABP4-2b effect (see Figure 19). The complex model constructed for lean mass in females again showed an interaction between UCP2-4 and FABP2p-834, the two terms that showed the greatest effect in the final model (see Figure 22). The adipose model terms in the final overall model included interactions between UCP1-2 and FABP4p-376, UCP1-2 and UCP2-8 as well as UCP2-4*UCP2-8 (see Figure 21).

The most interesting observation from these models is that combinations of adipose and intestinal genes seem to be significantly influencing lean body mass. In fact, no significant predictors of lean mass were found in the list of sites tested for muscle in females. Likewise, all of the terms were removed from the female adipose model for fat mass. Given that there is overlap in gene expression, the other genes designated as being primarily in another tissue could still be present in either the muscle or fat at levels capable of altering lean or fat mass. In addition, variations in PPAR, RXR, UCP or FABP function in any tissue could potentially alter whole body homeostasis and thereby alter traits in seemingly distant tissues. For example, mice that overexpress UCP3 in their muscles show, in addition to reduced lean mass, reductions in adipose mass, insulin, glucose and cholesterol levels (Clapham, 2000).

D. Other Analyses

1. PPARb6

Further analysis of the PPARb6 variant found that it is associated with fasting levels of glucose and insulin as well as insulin sensitivity measured by HOMA IR. Specifically, the T allele corresponded to lower glucose and insulin levels and therefore greater insulin sensitivity in normoglycemic individuals. Knowing that this population is of mixed sex and ethnic background, regression models were constructed to include sex and skin reflectance as covariates. Regression analysis confirmed that PPARb6 is a significant predictor of phenotypic variation even when sex and skin reflectance are taken into account. These results repeat the PPARb6 T allele effect seen in the regression analysis (see Sections C.2 and C.3.a) lending support to the idea that the T allele is protective. The results from this study overall suggest a role for PPARβ in glucose regulation and insulin metabolism independent of sex and ethnicity in this population. A more exhaustive screening of the PPARβ gene would be useful in order to determine if there are other functional polymorphisms in linkage disequilibrium with the PPARb6 variant.

2. Analysis of PPARγ Polymorphisms

Knowing that PPARg2 and PPARg6 are in high linkage disequilibrium (D'=0.767 for this study) and that PPARg6 is a silent substitution and likely non-functional, it has been assumed by many that PPARg6 is merely a marker for PPARg2 and is useful

because of it's increased frequencies in some populations. However, the multi-locus regression analysis results from this study found PPARg6 present in eight different final models while PPARg2 only appeared in three models, two of which contained the PPARg2d*PPARg6d interaction. As the functional polymorphism, PPARg2 should have been a better predictor of any trait that it is influencing, especially since the frequencies were similar between the two ethnic groups. Another study also found evidence of an interaction between these sites by observing that there was a greater difference in BMI and fat mass between the common and rare double homozygous individuals compared to the difference in homozygotes when the polymorphisms were considered separately (Valve, 1999). The results from the current study in combination with the findings from other groups suggested the possibility that PPARg6 is actually marking another functional variant in the PPARγ gene region. Further analysis of these two polymorphisms revealed that the T allele of PPARg6 is associated with a significant reduction in fasting insulin levels and HOMA IR in females (see Table 50, Table 51, and Table 52). This effect was small, 2.2mg/dL for FI and 4.6 units for HOMA IR, but was highly significant (p=0.008 and p=0.007, respectively). This association was still present after adjustment for age, smoking, fat mass and skin reflectance, but was not as strong (p=0.031 for FI and p=0.028 for HOMA IR). However, PPARg6d did explain a greater amount of variation in these traits compared to PPARg2d (r^2=0.015 for g6d vs. $r^2 \approx$0.003 for g2d). No significant interactions between the two sites were observed for these traits.

In this population, PPARg6d appears to be a better predictor than PPARg2d of fasting insulin levels and insulin resistance although the effect of this variant is small. This supports the hypothesis that PPARg6 may be in linkage disequilibrium with another functional variant other than PPARg2. If true, this could help to explain some of the inconsistencies in the PPARγ association studies since another functional polymorphism in the region could be at a different frequency or level of linkage disequilibrium with PPARg2 for various populations. This possibility has already been proposed by Hegele, *et al,* who found that the G (ala) allele for PPARg2d was associated with increased susceptibility to type-2 diabetes in a Canadian Indian population which conflicted with the findings of many other studies (Hegele, 2000).

E. Conclusions

The PPARb6 polymorphism identified in this study was found to be associated with multiple obesity and diabetes related traits. The less common T allele showed a protective effect on fasting free fatty acids, fasting glucose, fasting insulin and HOMA IR in many different analyses. The magnitude and frequency of the PPARb6 effect in this population strongly suggests that the T allele is in linkage disequilibrium with another functional variant with increased activity that provides this protective effect. The PPARg6 T allele was also found to have a protective effect on several traits, although not as strong as the PPARb6 effect. The reduction in insulin and HOMA IR seen in the PPARg6 TT individuals is not likely to be caused by the potentially protective nature of

the PPARg2 G allele since g2 itself was not an important predictor for these traits. If PPARg2 were actually influencing these traits, it should show a greater effect than the non-functional PPARg6 because of lower noise. It is interesting to note that the less common alleles in all three of these sites appear to be associated with lower risk phenotypes for obesity and type-2 diabetes related traits.

Comparison of the PPAR/RXR models with the combined models including the FABPs and UCPs shows that consideration of more polymorphisms for a regression model leads to stronger models that explain more variation. The PPAR/RXR model for cholesterol in Hispanic males predicted that the T allele of RXRb+140 increases cholesterol levels by approximately 20mg/dL while the UCP/FABP model showed an effect by only UCP2-8. The overall model replicated these results but added another polymorphism, UCP1-2, so that together, these three sites were able to explain almost 20% of the variation in cholesterol. Similarly, the Hispanic female model for fasting free fatty acid repeated some of the terms from the previous models, but, by adding interactions between the PPARs, RXRs and UCPs, was able to explain 23.4% of the trait variation.

These models exemplify the effect of multi-locus genotypes on quantitative traits, but are they consistent with previous single gene studies? No association studies have been published for three of the sites seen in these two models (PPARb6, RXRb+140 and FABP3pID), but their effects have been mostly consistent within this study when they were seen as a single gene effect. Hamann et al (1998) found that the A allele of

UCP1-2 was more prevalent in obese children compared to lean subjects possibly linking it to increases in body fat. The UCP1-2 term is a significant predictor in the Hispanic male model for cholesterol although its effect only appears in conjunction with UCP2-8 (see Figure 13). In the Hispanic female model for fasting free fatty acids, the UCP1-2 A allele is associated with increased FFA levels which is consistent with increased adiposity (see Figure 14). The I allele of UCP2-8 has been associated with increased BMI in several studies which is consistent with it's predicted effect in the Hispanic male model for cholesterol (Cassell, 1999; Walder, 1998) (see Figure 13). In the case of UCP3p-55, skeletal muscle from non-diabetic Pima Indians showed increased mRNA levels in individuals with the T allele of UCP3p-55 (Schrauwen, 1999). Increased UCP3 mRNA levels should correlate with increased UCP activity that could lead to lower fasting free fatty acids. However, the CC genotype appears to be lowering fasting free fatty acids in the UCP1-2 GG individuals in the Hispanic female model for FFA. Since UCP3p-55 is only seen in interactions in this model, its direct effect may be difficult to separate from the other polymorphisms.

F. Implications and Future Studies

This study tested several regression modeling methods for determining multi-locus genotype predictors of quantitative traits related to obesity and type-2 diabetes. Because this was an exploratory analysis, no correction for multiple testing was used in the analysis. Therefore, the results need to be interpreted with caution. The conclusions from this study are primarily based on repeated, consistent results from the different analyses. Still, this study does provide a foundation for further research into multi-locus genotype analysis and suggests a role for the PPAR pathway in obesity and diabetes.

There are many potential studies that would be useful in confirming the results of this study. A major priority would be to re-screen the genes encoding PPARβ and RXRβ in an attempt to identify functional variation. Further screening in individuals homozygous for the less common allele at either PPARb6 or RXRb+140 would increase the chances of finding a functional polymorphism in linkage disequilibrium with the sites used in this analysis. This approach would be more effective than screening random samples because it would increase the number of carriers of an unidentified polymorphism in high linkage disequilibrium with the previously identified sites.

Replication of parts of the analysis in other populations would help to confirm the effects of the different polymorphisms either alone or in combination. For example, it would be interesting to test the Hispanic male model for cholesterol that included effects from RXRb+140, UCP1-2, and UCP2-8 in another larger, unrelated, Hispanic

population. Analysis of a larger population would allow for comparison of more individuals in each of the genotype groups. This would help to clarify whether the apparent sex specific effect is real or is undetectable in the small number of Hispanic females in this study. Given that the size of the Hispanic female group is slightly larger than the Hispanic male group, it is possible that both of these options are true. If the effect is reduced in females as compared to males, then it could be undetectable in our population and apparent in another larger study. Several of the other models would be worth further analysis as well, including the Hispanic female model for fasting free fatty acids from the combined analysis and the overall models from the tissue specific analysis.

Another potential direction for this study would be to use other multi-locus genotype analysis techniques such as CPM or MDR on the SLVDS population. Principal component analysis would be another interesting approach to analyzing this data given the collinearity within the genetic data. Re-analysis of this data by different techniques would be interesting to determine the consistencies, strengths and weaknesses of the various methods. Side by side comparison with other analysis methods would also be useful in developing and refining the regression model approach used in this study.

G. Summary

This study illustrates the importance of examining multiple polymorphisms in genes with inter-related functions in order to understand their full contribution to a given trait. Multi-locus genotypes were almost always the best predictor of a trait and were often able to explain large portions of the variation. In the long run, analysis techniques developed specifically to test multi-locus genotype will be a powerful tool in determining the genetic influences on complex traits and disorders, but for now, modifications of more traditional methods that allow for multi-locus effects are likely to provide a better understanding of disease related polymorphisms than simple single locus analysis.

APPENDICES

Appendix A

Single Stranded Nuceotide Polymorphism (SSCP) Protocol

1. Clean and label the gels before attaching them to the gel box. Fill behind the gels with 1 x TBE buffer.

2. Combine 7.5μL of amplified DNA with 3.75μL of denaturing solution then incubate at 42^{o}C for 10 minutes.

3. Put samples on ice immediately when finished with the 42^{o}C incubation then add 3.75μL of formamide dye to each reaction.

4. Load all 15μL of the denatured/stained sample into a well using a fine pipette tip.

5. Run the gel at 150 volts until the dye is run off the gel or until the bands are sufficiently separated.

6. Remove the gels from the casing and stain with SYBR Green II RNA Gel Stain for 20 to 30 minutes depending on the freshness of the reagent. Gels and stain should be kept in the dark and mildly agitated.

7. Rinse the gels with dH_2O then photograph gel using UV fluorescence to visualize the bands.

Solutions Used in SSCP

SYBR Green II RNA Gel Stain (Molecular Probes Inc., Catalog #S-7586)

Dilute 1:10,000 by adding 4μL to SYBR Green stain to 50μL of 1xTBE

Keep solution in the dark

Stain can be used 2 to 3 times

Formamide Dye

10ml formamide

5mg Xylene Cyanol (0.05%)

5mg Bromophenol Blue (0.05%)

200μL EDTA (0.5M)

store at –20^{o}C

Denaturing Solution (NaOH/EDTA)

200μL EDTA (0.5M)

5ml NaOH (1M)

4.8ml dH_2O

store at –20^{o}C

Appendix B

Oligonucleotide Ligation Assay (OLA) Protocol

1. Dilute PCR samples with 0.1% triton water. (1:1 or 50:20 water to sample)

2. Get out strepavidin plates to warm and make 0.5% BSA in PBS
   0.25g BSA in 50mls of PBS (50mls is enough for 2 OLA plates)

3. Make up OLA reaction mix, one for each allele.

# PCR trays (# OLA)	1 tray (2)
NAD	240 ul
10X Ligase Buffer	240 ul
0.1% triton water	690 ul
1M KCl	30 ul
unique oligo	4 ul
common oligo	4 ul
ampligase	4 ul

   Oligos should be kept at a stock conc of 50uM and diluted to 5uM each day.

4. Place 10 ul of reaction mix for allele A into the odd rows of a 96-well plate and 10 ul of allele B into the even rows. Two 96-well plates are needed for each PCR tray in a 2 allele system.

5. Place 10 ul of diluted PCR product into the OLA plates. Row 1 of the PCR tray goes into rows 1 and 2 of the OLA plate. Row 2 of the PCR goes into rows 3 and 4 of the OLA plate. Overlay with oil if necessary.

6. Ligation reaction – 10 cycles of 93˚C for 30s and 58˚C for 2 min.

7. After ligation reaction is started, block the strepavadin plates. Empty plate in sink then dry by tapping on paper towels. Add 200 ul/ well of 0.5% BSA in PBS. Let sit at RT for at least 30 min.

8. To stop the ligation reation, add 10 ul of 0.1M EDTA and 0.1% triton water to each well. Add as soon as possible after the ligation reaction has finished cycling to avoid non-specific ligation.

9. Wash blocked strepavadin plates once with Tris Wash.

10. Capture phase - Transfer all of the ligation reaction, including the oil, to the strepavadin plates and allow to sit at RT for at least 30 min and up to 3 hours.

11. About 15 min before the end of the capture phase get out ELISA reagents and dilute the antibody. 4 mls of antibody soltion are needed for each OLA plate. 1ul of antibody is diluted into 1 ml of 0.5% BSA in PBS.

12. Remove the reaction mix from the strepavadin plates with the plate washer then wash the OLA plates 2X with NaOH wash and 2X with Tris wash. Approximately 40 mls of NaOH wash are needed per OLA plate.

13. Add 40 ul of anitbody solution to each well and let sit at RT for at least 25 min but no more than 30 min.

14. Prepare substrate and amplifier solutions by adding the vial of liquid to the vial with powder in it. Each vial will do about 4 OLA plates.

15. Wash the OLA plates 6X with Tris wash.

16. Add 25 ul of substrate to each well and let sit at RT for about 15 min.

17. Add 25 ul of amplifier to each well. Color change should begin within a few minutes.

18. When the color has reached a good intensity, add 25 ul of 0.3M H_2SO_4 to stop the reaction before the background builds up too much.

Solutions Used

100mM NAD
0.663g NAD into 10mls of dH_2O

store at –20˚C in 1ml aliquots

1M Tris-HCl at pH 8.0
60.55g Tris
upto 500 mls with dH_2O
pH to 8.0 with HCl

10X Ligase Buffer
10 mls of 1M Tris HCl (8.0)
1.02g $MgCl_2$
0.134g DTT
upto 50mls with dH_2O

store at –20˚C in 1ml aliquots

0.1% Triton Water
50uls of triton-X 100 in 50mls of dH_2O

Phosphate Buffered Saline (PBS)
8g NaCl
0.2g KCl
1.44g Na_2HPO_4
0.24g KH_2PO_4
upto 1L with dH_2O
filter before use

1M KCl

3.725g KCl
upto 50mls with dH_2O

NaOH Wash

25mls 1M NaOH
125ul Tween 20
upto 250mls with dH_2O

10X Tris Wash

121.1g Tris (to 1M)
87.6g NaCl
upto 1 L with dH_2O

1X Tris Wash

200mls 10X Tris Wash
1ml Tween 20
upto 2 L with dH_2O

Stop Solution

50mls 0.5M EDTA
250mls Triton-X 100
upto 250mls with dH_2O

Strepavidin Plates

1.25mls of 1mg/ml strepavidin
upto 50 mls with 1X PBS

add 50ul of diluted strepavidin to each well
store at 4˚C for upto 6 months
(use Falcon plates 3075)

Appendix C

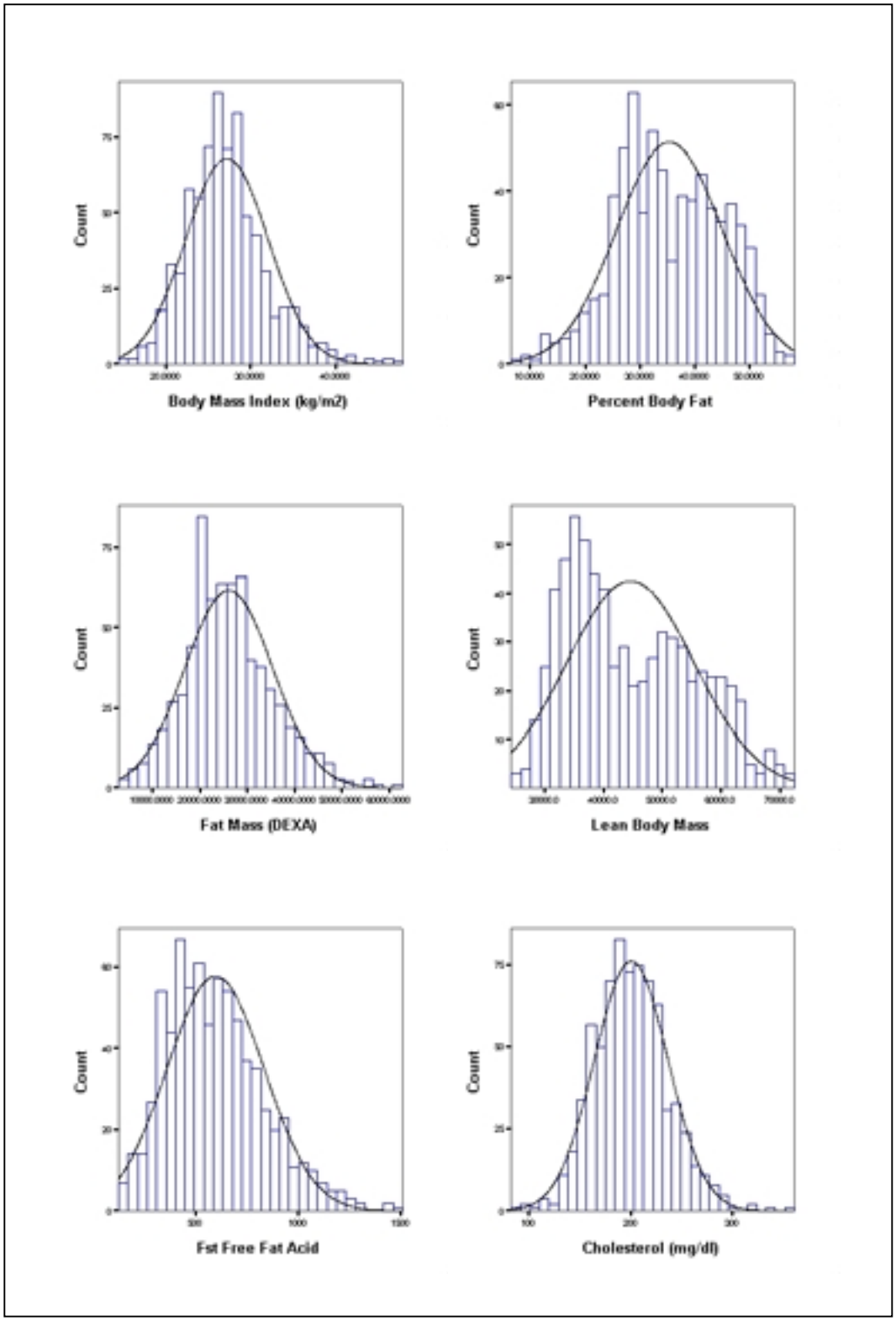
Descriptive Statistics

Table 53: Frequencies of categorical variables in SLVDS population including outliers

	Category	Frequency
Smoking Status	Non-smoker	49.9
	Current smoker	14.1
	Ex-smoker	36.0
Sex	Male	45.5
	Female	54.5
Ethnicity	Non-Hispanic	57.8
	Hispanic	42.2

Table 54: Descriptive statistics of continuous variables including outliers

Variables	Mean±SD	Skewness	Kurtosis	N
BMI (kg/m^2)	27.3±4.9	0.743	1.299	763
Fat Mass (kg)	26.2±9.3	0.455	0.416	712
% Body Fat (%)	35.4±9.6	-0.112	-0.537	712
Lean Mass (kg)	44.7±10.8	0.427	-0.855	712
Fasting FFA (μmol/L)	603±244	0.777	0.936	762
Cholesterol (mg/dl)	201±36	0.371	0.615	762
Triglycerides (mg/dl)	181±104	2.958	16.679	762
Fasting Glucose (mg/dl)	101±27	5.550	47.801	762
Fasting Insulin (mg/dl)	11.2±6.6	1.980	5.060	762
HOMA IR	17.7±14.0	3.142	16.681	762
Systolic BP (mm)	127±18	1.115	2.344	762
Diastolic BP (mm)	74±9	0.320	0.736	762
Caloric Intake (cal)	1957±940	2.292	13.887	729
Fat Intake (g)	76.9±49.3	4.219	46.768	729
Physical Activity (kCal/kg/hr)	285±68	2.288	6.572	741
Age (years)	63±12	-0.156	-0.782	763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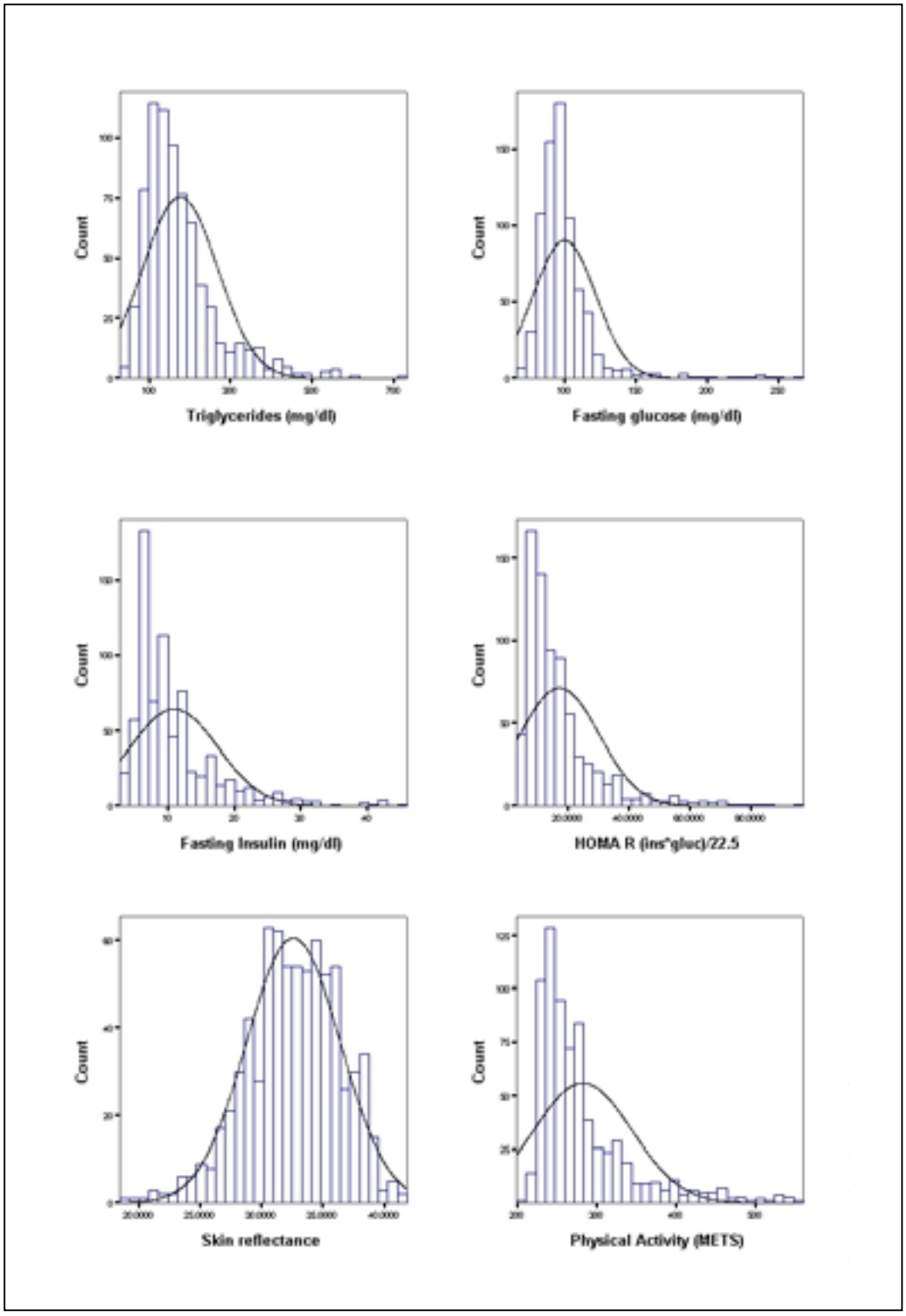
Body Mass Index (kg/m2)
Count
Percent Body Fat
Count
Fat Mass (DEXA)
Count
Lean Body Mass
Count
Fst Free Fat Acid
Count
Cholesterol (mg/dl)


Count
Triglycerides (mg/dl)
Count
Fasting glucose (mg/dl)
Count
Fasting Insulin (mg/dl)
Count
HOMA R (ins*gluc)/22.5
Count
Skin reflectance
Count
Physical Activity (METS)

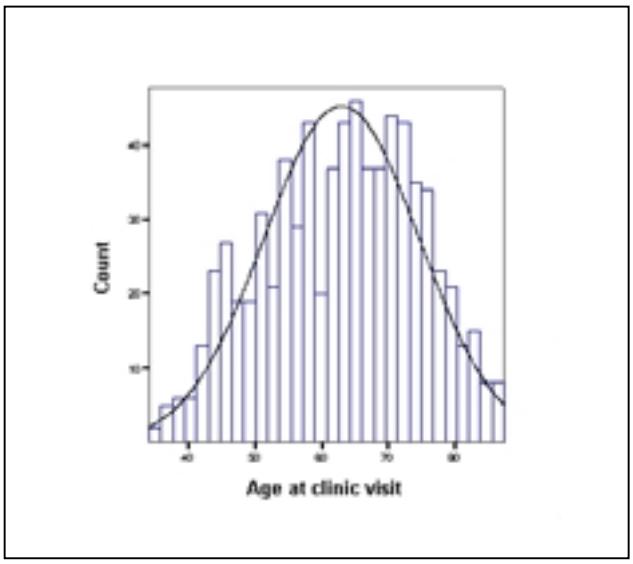


Figure 26: Histograms for continuous variables without outliers

Appendix D

Predictor Correlations

Table 55: Correlation coefficients for outcome and predictor variables

	BMI	PFT	FM	LM	FFA	CHL	TRG	FG	FI	HIR	SKR	PA	AGE	SMK
BMI	-	.563	.862	.296	.105	-0.7	.183	.225	.499	.457	.011	.011	-.11	.016
PFT	.563	-	.824	-.51	.323	.124	.119	.027	.327	.273	.046	-.28	-.03	-.14
FM	.862	.824	-	.025	.190	.001	.166	.149	.452	.400	.082	-.13	-.12	-.04
LM	.296	-.51	.025	-	-.32	-.18	.076	.179	.085	.103	.054	.306	-.15	.206
FFA	.105	.323	.190	-.32	-	.131	.176	.184	.170	.198	-.06	-.16	.206	-.01
CHL	-.07	.124	.001	-.18	.131	-	.261	-.02	-.06	-.06	-.03	-.07	.032	-.02
TRG	.183	.119	.166	.076	.176	.261	-	.269	.298	.309	-.08	.020	-.08	.051
FG	.225	.027	.149	.179	.184	-.02	.269	-	.349	.618	-.07	.057	.071	.075
FI	.499	.327	.452	.085	.170	-.06	.298	.349	-	.934	-.10	-.11	-.01	.019
HIR	.457	.273	.400	.103	.198	-.06	.309	.618	.934	-	-.10	-.07	.011	.029
SKR	.011	.046	.082	.054	-.06	-.03	-.08	-.07	-.10	-.10	-	.037	.102	-.08
PA	.011	-.28	-.13	.306	-.16	-.07	.020	.057	-.11	-.07	.037	-	-.21	.047
AGE	-.11	-.03	-.12	-.15	.206	.032	-.08	.071	-.01	.011	.102	-.21	-	.023
SMK	.016	-.14	-.04	.206	-.01	-.02	.051	.075	.019	.029	-.08	.047	.023	-

Abbreviations: BMI – body mass index (kg/m^2); PFT – percent body fat (%); FM – fat mass (g); LM – lean mass (g); FFA – fasting free fatty acids (μmol/L); CHL – cholesterol mg/dL); TRG – triglycerides (mg/dL); FG – fasting glucose (mg/dL); FI - fasting insulin (mg/dL); HIR – HOMA IR; SKR – skin reflectance (%); PA – physical activity (kCal/kg/hr); AGE – age at phase IV visit (years); SMK – smoking.

Table 56: Polymorphism vs. polymorphism significance level for Pearson X^2 test

	PPARa5	PPARa6	PPARb6	PPARg2	PPARg6	RXRb10	RXRb+140
PPARa5	-	0.295	0.090	0.378	0.918	0.007	0.332
PPARa6	0.295	-	0.331	0.210	0.311	0.696	0.507
PPARb6	0.090	0.331	-	0.171	0.008	0.864	0.504
PPARg2	0.378	0.210	0.171	-	<0.001	0.061	0.054
PPARg6	0.918	0.311	0.008	<0.001	-	0.015	0.812
RXRb10	0.007	0.696	0.864	0.061	0.015	-	<0.001
RXRb+140	0.332	0.507	0.504	0.054	0.812	<0.001	-

Appendix E

Final Regression Models for PPAR/RXR Analysis

Table 57: Final regression model for fasting free fatty acids (μmol/L) in the unsplit sample

Term	β	p-value
Constant	497	0.000
PPARb6v1	111	0.042
PPARb6v2	58	0.311

r^2=0.013
p-value=0.011

Table 58: Final regression model for fasting free fatty acids (μmol/L) in Non-Hispanics

Term	β	p-value
Constant	563	0.000
RXRb+140v2	104	0.031
RXRb10d*RXRb+140v2	-119	0.019

r^2=0.016
p-value=0.061

Table 59: Final regression model for fasting free fatty acids (μmol/L) in Hispanics

Term	β	p-value
Constant	609	0.000
PPARb6d	108	0.004
PPARa6*PPARg6d	-70	0.028

r^2=0.052
p-value=0.001

Table 60: Final regression model for fasting free fatty acids (μmol/L) in Non-Hispanic males

Term	β	p-value
Constant	468	0.000
PPARa5d*PPARb6d	53	0.066

r^2=0.018
p-value=0.066

Table 61: Final regression model for ln(fasting glucose) (mg/dL) in the unsplit sample

Term	β	p-value
Constant	4.372	0.000
PPARa6	0.158	0.008
PPARb6d	0.050	0.002
PPARg6d	0.188	0.018
PPARa6*PPARg6d	-0.163	0.029

r^2=0.026
p-value=0.002

Table 62: Final regression model for ln(fasting glucose) (mg/dL) in Non-Hispanics

Term	β	p-value
Constant	4.422	0.000
PPARa6	0.112	0.038
PPARa6*PPARb6d	0.038	0.029

r^2=0.030
p-value=0.003

Table 63: Final regression model for ln(fasting glucose) (mg/dL) in Hispanics

Term	β	p-value
Constant	4.603	0.000
RXRb10d*RXRb+140v2	0.069	0.024

r^2=0.021
p-value=0.024

Table 64: Final regression model for ln(fasting glucose) (mg/dL) in Non-Hispanic males

Term	β	p-value
Constant	4.561	0.000
PPARb6d	0.058	0.024

r^2=0.027
p-value=0.024

Table 65: Final regression model for cholesterol (mg/dL) in Non-Hispanics

Term	β	p-value
Constant	192.5	0.000
PPARa5d*PPARa6	10.3	0.022

r^2=0.012
p-value=0.022

Table 66: Final regression model for cholesterol (mg/dL) in Hispanics

Term	β	p-value
Constant	206.9	0.000
PPARa6*RXRb+140d	-12.6	0.002

r^2=0.040
p-value=0.002

Table 67: Final regression model for cholesterol (mg/dL) in Non-Hispanic Females

Term	β	p-value
Constant	209.2	0.000
RXRb10d*RXRb+140d	-12.7	0.034

r^2=0.026
p-value=0.034

Appendix F

Regression Model Summary for UCPs and FABPs

Table 68: Regression Model Summary for Non-Hispanic Females

Outcome Variable	Genotype Terms	p-value	r^2
Fat Mass	UCP2-4*UCP3-5	<0.001	0.133
Fasting Free Fatty Acids	UCP2-4*UCP3-5	0.002	0.058
ln(Triglycerides)	UCP3p-55*FABP3p-ID	0.001	0.053
Cholesterol	UCP3p-55 FABP2p-ID UCP3p-55*FABP3p-ID	0.002	0.075
ln(HOMA IR)	UCP3-5*FABP3p-313 UCP3p-55*FABP3p-313	0.003	0.056

Table 69: Regression Model Summary for Non-Hispanic Males

Outcome Variable	Genotype Terms	p-value	r^2
Fat Mass	FABP2p-ID FABP3p-ID UCP1-2*FABP3p-ID UCP2-8*FABP3p-ID FABP2p-ID*FABP4p-376 FABP3p-ID*FABP4p-376	<0.001	0.226
Fasting Free Fatty Acids	FABP3p-ID	0.031	0.023
ln(Triglycerides)	UCP3p-55*FABP3p-ID UCP3-5*FABP2p-ID	<0.001	0.076
Cholesterol	FABP3p-313	0.050	0.019
ln(HOMA IR)	FABP2p-ID FABP3p-ID FABP2p-ID*FABP3p-ID	0.044	0.057

Table 70: Regression Model Summary for Hispanic Females

Outcome Variable	Genotype Terms	p-value	r^2
Fat Mass	UCP1-2 UCP3-5 UCP1-2*UCP3-5	<0.001	0.122
Fasting Free Fatty Acids	UCP1-2*UCP3p-55 UCP1-2*FABP3p-ID UCP3p-55*FABP3p-ID	<0.001	0.117
ln(Triglycerides)	UCP1-2 UCP1-2*UCP2-8	0.002	0.074
Cholesterol	UCP3-5*FABP3p-ID	0.001	0.071
ln(HOMA IR)	UCP2-8 UCP3p-55*FABP2p-834	0.061	0.034

Table 71: Regression Model Summary for Hispanic Males

Outcome Variable	Genotype Terms	p-value	r^2
Fat Mass	UCP1-2*FABP2-2	0.009	0.054
Fasting Free Fatty Acids	UCP2-4 UCP2-8	0.001	0.096
ln(Triglycerides)	UCP3-5*FABP3p-313	0.010	0.049
Cholesterol	UCP2-8	0.018	0.058
ln(HOMA IR)	No Model		

Appendix G

Final Regression Models for Combined Analysis

Table 72: Final combined regression model for fasting free fatty acids (μmol/L) in Non-Hispanic males

Term	β	p-value
Constant	466.2	0.000
PPARa5d*PPARb6d	78.2	0.035
PPARb6d*FABP3pIDv1	-76.5	0.070
PPARb6d*FABP3pIDv2	6.2	0.876

r^2=0.054
p-value=0.018

Table 73: Final combined regression model for fasting free fatty acids (μmol/L) in Non-Hispanic females

Term	β	p-value
Constant	627.8	0.000
PPARg6d	170.6	0.038
PPARa5d*FABP3pIDv1	-35.6	0.547
PPARa5d*FABP3pIDv2	-98.3	0.039
PPARg6d*UCP3-5v1	34.2	0.509
PPARg6d*UCP3-5v2	-128.0	0.012
PPARg6d*FABP3pIDv1	-154.9	0.068
PPARg6d*FABP3pIDv2	-5.4	0.941

r^2=0.139
p-value>0.001

Table 74: Final combined regression model for fasting free fatty acids (μmol/L) in Hispanic males

Term	β	p-value
Constant	559.0	0.000
UCP2-4v1	-43.2	0.561
UCP2-4v2	154.2	0.009
UCP2-8v1	-27.3	0.774
UCP2-8v2	-136.1	0.142

r^2=0.101
p-value=0.008

Table 75: Final combined regression model for cholesterol in Non-Hispanic males

Term	β	p-value
Constant	216.2	0.000
PPARb6d	-27.9	0.014
PPARg6d	-33.4	0.009
PPARg6d*RXRb10d	23.8	0.033
PPARb6d*UCP1-2	34.2	0.001
PPARg6d*FABP2p-834v1	18.7	0.039
PPARg6d*FABP2p-834v2	5.5	0.531
RXRb10d*UCP1-2	-26.3	0.005

r^2=0.0.094
p-value=0.025

Table 76: Final combined regression model for cholesterol in Non-Hispanic females

Term	β	p-value
Constant	197.8	0.000
FABP2pIDv1	5.2	0.487
FABP2pIDv2	-7.6	0.271
PPARa5d*UCP3-5v1	15.9	0.010
PPARa5d*UCP3-5v2	12.5	0.039

r^2=0.0.064
p-value=0.009

Table 77: Final combined regression model for cholesterol in Hispanic females

Term	β	p-value
Constant	182.9	0.000
UCP2-8v1	24.5	0.068
UCP2-8v2	-2.9	0.820
UCP3-5v1*FABP3pIDv1	7.8	0.391
UCP3-5v1*FABP3pIDv2	34.4	0.000
UCP3-5v2*FABP3pIDv1	9.4	0.309
UCP3-5v2*FABP3pIDv2	0.7	0.920
PPARg6d*UCP2-8v1	-9.0	0.383
PPARg6d*UCP2-8v2	22.4	0.019

r^2=0.157
p-value=0.002

Appendix H

Regression Model Summary for Lipid Variables in Tissue Specific Analysis

Table 78: Cholesterol Tissue Specific Regression Model Summary for Males

Tissue	Genotype Terms	p-value	r^2
Muscle	FABP3p-313	0.025	0.015
Adipose	UCP2-8*FABP4p-376	0.031	0.014
Intestine	No Model		
Overall	UCP2-8*FABP4p-376	0.034	0.014

Table 79: Cholesterol Tissue Specific Regression Model Summary for Females

Tissue	Genotype Terms	p-value	r^2
Muscle	FABP3p-313 PPARa5*UCP3-5 FABP3p-ID*FABP3p-313	0.006	0.036
Adipose	No Model		
Intestine	UCP2-8 UCP2-4*UCP2-8 UCP2-4*FABP2p-834	0.039	0.030
Overall	UCP2-8 FABP3p-313 UCP2-4*UCP2-8 UCP2-4*FABP2p-834 FABP3p-ID*FABP3p-313	0.002	0.061

Table 80: Triglycerides Tissue Specific Regression Model Summary for Males

Tissue	Genotype Terms	p-value	R^2
Muscle	FABP3p-313 UCP3-5*UCP3p-55	0.005	0.033
Adipose	FABP4-2b UCP2-4*FABP4-2b UCP2-4*FABP4p-376 UCP2-8*FABP4-2b UCP2-8*FABP4p-376	0.005	0.065
Intestine	UCP2-4*FABP2p-ID	0.012	0.020
Overall	UCP2-4 FABP4-2b UCP2-4*FABP2p-ID UCP2-4*FABP4-2b UCP2-4*FABP4p-376 UCP2-8*FABP4-2b UCP2-8*FABP4p-376	0.003	0.075

Table 81: Triglycerides Tissue Specific Regression Model Summary for Females

Tissue	Genotype Terms	p-value	r^2
Muscle	No Model		
Adipose	UCP2-4 PPARg2*UCP2-4 PPARg2*UCP2-8 UCP1-2*UCP2-4 UCP1-2*UCP2-8	<0.001	0.080
Intestine	UCP2-4	0.007	0.020
Overall	UCP2-4 PPARg2*UCP2-4 PPARg2*UCP2-8 UCP1-2*UCP2-4 UCP1-2*UCP2-8	<0.001	0.080

Table 82: Fasting Free Fatty Acids Tissue Specific Regression Model Summary for Males

Tissue	Genotype Terms	p-value	r^2
Muscle	No Model		
Adipose	UCP1-2 UCP2-4 UCP2-8 UCP1-2*UCP2-8 UCP2-4*FABP4-2b UCP2-8*FABP4-2b	0.003	0.074
Intestine	UCP2-4 FABP2p-834 PPARb6*FABP2p-834 UCP2-4*UCP2-8 UCP2-8*FABP2p-834 UCP2-8*FABP2p-ID	0.006	0.065
Overall	UCP1-2 UCP2-4 UCP2-8 UCP1-2*UCP2-8 UCP2-4*FABP4-2b UCP2-8*FABP4-2b	0.003	0.074

Table 83: Fasting Free Fatty Acids Tissue Specific Regression Model Summary for Females

Tissue	Genotype Terms	p-value	R^2
Muscle	PPARa5*UCP3-5	0.010	0.018
Adipose	UCP1-2 FABP4-2b PPARg2*UCP2-8 PPARg6*UCP1-2 UCP1-2*UCP2-8 UCP2-4*FABP4-2b	0.008	0.058
Intestine	FABP2p-ID FABP2p-834 UCP2-4*FABP2p-ID UCP2-4*FABP2p-834 UCP2-8*FABP2p-ID UCP2-8*FABP2p-834	0.001	0.090
Overall	UCP1-2 FABP2p-ID PPARa5*UCP3-5 UCP1-2*UCP2-8 UCP2-4*FABP2p-ID UCP2-4*FABP2p-834 UCP2-8*FABP2p-ID UCP2-8*FABP2p-834	<0.001	0.113

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