

Genetic Markers Associated with an Intermediate Phenotype of the Metabolic Syndrome: Insulin Resistance and Hypertension

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Boston College

William F. Connell School of Nursing

Genetic Markers Associated with an Intermediate Phenotype of the Metabolic Syndrome:

Insulin Resistance and Hypertension

a dissertation

by

Patricia C. Underwood

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2010

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Insulin Resistance and Hypertension

Patricia C. Underwood

Dissertation Chair: Catherine Y. Read, PhD, RN

Abstract

Background and Significance: The metabolic syndrome is a heterogeneous disorder leading to increased morbidity and mortality. Components of the metabolic syndrome are known to be inherited, however efforts to identify genomic markers in humans have been unsuccessful and a candidate-gene/intermediate phenotype approach may be useful. Evidence supports a relationship between altered metabolic function and three candidate genes, caveolin-1 (CAV1), peroxisome proliferator receptor-activated gamma (PPAR γ), and angiotensinogen (AGT). These genes may serve as markers for the co-aggregation of insulin resistance and hypertension.

Research Question: To examine whether single nucleotide polymorphisms (SNPs) in the CAV1, PPAR γ and AGT genes are associated with the co-aggregation of insulin resistance and hypertension.

Methods: Three gene association studies were conducted in a Caucasian hypertensive cohort (HyperPATH). The homeostasis assessment model (HOMA-IR), hyperinsulinemic euglycemic clamp, and salt sensitive blood pressure were determined in each subject. Statistical analyses were conducted using a general linear model accounting for relatedness and adjusting for the following covariates: age, gender, body mass index, study site. Replication was assessed in a hypertensive Mexican-American cohort (HTN-IR) for the CAV1 gene and a hypertensive African American cohort (HyperPATH) for the PPAR γ gene.

Results: SNPs of the CAV1 gene were significantly associated with insulin resistance in Caucasians from HyperPATH. These results were replicated in the HTN-IR cohort. A SNP of the PPAR γ gene was associated with salt sensitive blood pressure and increased plasma renin levels in Caucasians and African Americans from HyperPATH. SNPs of the AGT gene were associated with insulin sensitivity in Caucasians from HyperPATH.

Conclusion: CAV1 and AGT are genomic markers for the co-aggregation of insulin resistance and hypertension. The PPAR γ gene is a potential genomic marker for vascular dysfunction in hypertension.

Clinical Perspective: Genomic markers for insulin resistance exist in human populations with hypertension. These markers explain the inter-individual variability of insulin resistance and hypertension and help identify potential underlying mechanisms. Use of these bio-markers in clinical practice may improve individualized prevention and treatment strategies, decreasing the incidence of and improving outcomes for this chronic disease. Promoting health through individualized care makes the incorporation of genomic markers into nursing practice essential.

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Chapter 1

Statement of the Problem

Introduction.

The metabolic syndrome leads to long term health complications, including cardiovascular disease and type 2 diabetes mellitus (T2DM) (Lorenzo et al., 2003; Lorenzo, Williams, Hunt, & Haffner, 2007; Obunai, Jani, & Dangas, 2007). In the United States, this complex syndrome is on the rise, affecting approximately 20-30% of individuals (Park et al., 2003; Davila et al., 2010), creating a strain on the health care system and increasing total health care costs (Clouse, Zitter, & Herman, 2002; Cornier et al., 2008; Elliott, 2003). Ill-defined definitions and the heterogeneous nature of the metabolic syndrome have led to a poor understanding of its cause, hindering the development of effective prevention and treatment strategies. An improved understanding of the syndrome may further efforts to improve individualized management of the metabolic syndrome, decreasing related morbidity and mortality.

Definition of metabolic syndrome: A heterogeneous disorder.

Originally defined by G.M. Reaven and termed syndrome X, the metabolic syndrome was characterized for clinicians to identify individuals at risk for increased cardiovascular disease (Reaven, 2005; Reaven, 1988). Over the past twenty years, multiple definitions of the metabolic syndrome have ensued (Cornier et al., 2008) (see table 1 below). All definitions include a variation of four components: hypertension, dyslipidemia, abdominal obesity and insulin resistance (IR). However, the number of components required to be present in an individual and the characterization of IR and hypertension differ among definitions (Alberti & Zimmet, 1998; Alberti, Zimmet, & Shaw, 2006; Balkau & Charles, 1999; Bloomgarden, 2003).

For example, IR is defined as an elevated fasting insulin level (hyperinsulinemia), impaired fasting glucose (IFG) (fasting serum glucose >100mg/dl), or impaired glucose tolerance (IGT) (serum glucose >200mg/dl 120 minutes after 75gm glucose load) (Alberti & Zimmet, 1998; Balkau & Charles, 1999; Bloomgarden, 2003; Lorenzo, Williams, Hunt, & Haffner, 2007) in different definitions of the metabolic syndrome. The heterogeneous nature of these definitions results in the diagnosis of the metabolic syndrome in an ill-defined group of individuals (Cornier et al., 2008).

Table 1. Definitions of the Metabolic Syndrome. WHO: World Health Organization, EGIR: European Group for the study of Insulin Resistance, NCEP ATPIII: National Cholesterol Education Program Adult Treatment Panel III, AACE: American Academy of Clinical Endocrinologists, IDF: International Diabetes Federation, WC=waist circumference, WHR=waist hip ratio, BP= blood pressure.

WHO 1998	EGIR 1999	NCEP ATPIII 2005	AACE 2003	IDF 2006
Hyperinsulinemia: (impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) and two of the following: abdominal obesity: waist hip ratio (WHR)>0.9, BMI \geq 30kg/m ² , waist circumference (WC)>37 inches Dyslipidemia: triglycerides > 150mg/dl, HDL-C<35mg/dl Hypertension: BP>140/90mmHG	Hyperinsulinemia: top 25% of fasting insulin values among non-diabetic individuals and two of the following: abdominal obesity: WC \geq 94cm for men, >80cm for women Dyslipidemia: triglycerides \geq 2.0mmol/L and HDL-C <1.0mg/dl Hypertension: BP=140/90mmHG or antihypertensive medications Impaired Fasting Glucose: glucose \geq 6.1mmol/L	Three or more of the following: abdominal obesity: WC \geq 40 in. for men, >35in. For women Dyslipidemia: triglycerides \geq 150mg/dl HDL-C <40mg/dl for men, <50mg/dl for women (if both count for 2) Hypertension: BP \geq 130/85mmHg Impaired Fasting Glucose: \geq 100mg/dl	Impaired glucose tolerance: glucose > 200mg/dl at 180min after 75g oral glucose tolerance test and two or more of the following Triglycerides \geq 150mg/dl HDL-C <40mg/dl for men, <50mg/dl for women Hypertension: BP \geq 130/85mmHg	central obesity: WC Triglycerides \geq 150mg/dl HDL-C <40mg/dl for men, <50mg/dl for women Impaired Fasting Glucose: >100mg/dl

Metabolic syndrome: unclear etiology.

The second problem hindering the development of effective prevention and treatment strategies is an unclear understanding of the cause of the metabolic syndrome. While it is known that hypertension, hyperinsulinemia, dyslipidemia, and IR often co-aggregate in individuals (Chen, Jeng, Hollenbeck, Wu, & Reaven, 1988; Ferrannini et al., 1987; Manicardi, Camellini, Bellodi, Coscelli, & Ferrannini, 1986), the underlying mechanism of this co-aggregation remains elusive. Insulin resistance was originally proposed as the primary mechanism for the co-aggregation of these metabolic conditions (Reaven, 1988). However, some have questioned this tenet, citing an inability to definitively relate IR to all components of the metabolic syndrome (Kahn et al., 2005) and recognizing the emerging role of adiposity and inflammation on the development of the metabolic syndrome (Ferrannini et al., 2007; Nishimura, Manabe, & Nagai, 2009). It is possible that the heterogenic nature of the metabolic syndrome is contributing to the controversy surrounding the syndrome's etiology. Clarifying the definition would support research efforts to address this problem and improve patient outcomes.

Proposed solution: identifying genomic biomarkers in homogeneous subsets.

Recent evidence supports the concept that many illnesses are not diseases but a collection of syndromes with a common final presentation, e.g., an elevated blood pressure for hypertension (Williams, 1994). Thus, improved outcomes may result by first identifying homogeneous subsets of heterogeneous disorders and treating these subsets, rather than the final presenting symptom (Williams, 1994; Williams & Fisher, 1997). This approach is similar to a culture and sensitivity test, where clinicians identify subcategories of bacteria within a larger heterogeneous condition (fever) and individualize treatment based on the bacterial culture (Williams, 2009). Currently, homogeneous subsets of chronic disease are identified through

phenotype analysis, a cumbersome and costly task requiring stringent environmental control within a research setting. Fortunately, another, simpler method of identifying homogeneous subsets exists: genotyping.

Specifically, genetic biomarkers associated with well defined intermediate phenotypes of the metabolic syndrome will identify homogeneous subsets of the disease facilitating an improved understanding of disease etiology and identifying those most at risk. A genetic approach is reasonable to pursue since the co-aggregation of hypertension, IR, abdominal obesity, and dyslipidemia occurs more frequently in related individuals than non-related individuals (Raji, Williams, Hopkins, Simonson, & Williams, 2006; Tang et al., 2006) demonstrating familial aggregation. Using this approach, genetic variants of candidate genes were examined to determine whether they are associated with the homogeneous intermediate phenotype of the metabolic syndrome, IR and hypertension.

Significance

The prevalence of the metabolic syndrome, particularly the sub-phenotype IR and hypertension, is on the rise, creating a strain on the health care system and increasing total health care costs (Elliott, 2003; McKeown et al., 2004). Poor treatment strategies lead to long term health complications, as well as increased morbidity and mortality (Brown, Pedula, & Bakst, 1999; Clouse et al., 2002; Collins, 2002). For example, patients with a cluster of metabolic abnormalities, including IR and hypertension, are more likely to have polycystic ovary syndrome, fatty liver, cholesterol gallstones, asthma, sleep disturbances, and some forms of cancer (Grundy, 2004). Clearly, the presence of poorly treated IR and hypertension contributes to the onset of many long term health complications with devastating effects.

The identification of genomic biomarkers associated with a homogeneous subset of the metabolic syndrome, IR and hypertension, may support the development of individualized treatment approaches. The results of this study contribute to the following:

- *A priori* identification of patients at risk for IR and hypertension, facilitating individualized health promotion and disease prevention strategies, decreasing the incidence of the disease
- An improved understanding of the pathophysiology of the disease, providing information for future individualized pharmacologic, physiologic, and behavioral treatment strategies and recommendations for individualized disease management (exercise, diet, other health behavior strategies), decreasing the long term complications of the illness, increasing overall health and quality of life.

The significance of identifying genomic information for the prevention and treatment of chronic illnesses is recognized by nurse scientists. Frazier et al. (2004) and Conley & Tinkle (2007) describe the importance of using genetic information to support individualized prevention and treatment strategies in chronic illness. Further, the National Institute of Nursing Research (NINR) recognizes the need “to identify susceptibility genes for at-risk individuals for the design of interventions to moderate risk” (National Institute of Nursing Research, 2006). Thus, to nurses, whose profession is directly involved in identifying individuals at risk for chronic disease and providing education and support for prevention and treatment, knowledge of genetic markers for IR and hypertension is important and will facilitate steps to improve both nursing practice and patient care.

Definition of Terms

The outlined genomic approach provides a framework to improve the identification and treatment of the co-aggregation of IR and hypertension. The following definitions clarify important terms related to the approach and implementation of this study.

Candidate Gene Study: A research methodology used to identify genomic biomarkers. Genes are chosen based on their known role in a specific physiologic pathway. This is a hypothesis-based, functional cloning approach (Amos, 2007).

Caveolin-1 (CAV1) gene: located at chromosome 7q31 and the first candidate gene of this study. This gene is one of three genes that make up caveolae, plasma membrane lipid domains involved in the sequestering and organization of cell signaling proteins. CAV1 is known to be involved in insulin signaling (Kabayama et al., 2007; Yamamoto et al., 1998), insulin mediated glucose uptake (Penumathsa et al., 2008; Yuan, Hong, Yao, & Liao, 2007), and vascular tone (Lam et al., 2006).

Peroxisome Proliferator-Activated Receptor gamma (PPAR γ) gene: located on chromosome 3p25 and the second candidate gene of this study. This gene encodes PPAR γ , a ligand activated transcription factor, involved in adipogenesis and glucose metabolism and recently found to be involved with regulation of renin gene transcription (Todorov, Desch, Schmitt-Nilson, Todorova, & Kurtz, 2007; Weatherford, Itani, Keen, & Sigmund, 2007).

Angiotensinogen (AGT) gene: located at 1q31, the AGT gene encodes angiotensinogen.

Angiotensinogen is the first component of the renin-angiotensin-aldosterone system (RAAS), is produced and released into the circulation by the liver, and is a precursor to both Angiotensin I (AngI) and AngII(AngII) (Basso & Terrango, 2001). The RAAS is known to directly affect the insulin signaling pathway (Velloso et al., 1996; Velloso, Folli, Perego, & Saad, 2006) and is

involved in the development of hypertension (Williams, 1982) making the first peptide of this system an important variable of study.

Single nucleotide polymorphisms (SNPS): Common, but minute, variations that occur in human DNA at a frequency of one every 1,000 bases. These variations can be used to track inheritance in families (Amos, 2007).

Allele: One of the possible mutational states of a gene, distinguished from other alleles by phenotypic effects (Klug & Cummings, 1997).

Minor Allele: The less frequent allele present in a population (Klug & Cummings, 1997).

Major Allele: The more frequent allele present in a population (Klug & Cummings, 1997).

Intermediate Phenotype: a study design that is driven by the concept that the “disease” (distant phenotype) consists of several homogeneous subsets each sharing a common mechanism manifest by a shared intermediate phenotype, and each resulting in a common distant phenotype—e.g., hypertension and/or T2DM (Williams, 1994).

Insulin Resistance: fasting glucose between 110-125mg/dl or a two hour glucose challenge glucose between 140 and 200mg/dl as defined by the American Academy of Clinical Endocrinologists (AACE) (Einhorn et al., 2003).

Hypertension: an elevated blood pressure $> 135/85$ mmHG on no anti-hypertensive medications, (Raji, 2001).

Salt Sensitive Blood Pressure: An increased in blood pressure greater than 10mmHg in response to salt loading (systolic, diastolic, mean arterial pressure). This variable was examined as a measurement of vascular dysfunction in the hypertensive population.

Purpose

The purpose of this study was to identify genomic biomarkers associated with the homogeneous intermediate phenotype of the metabolic syndrome, insulin resistance and hypertension. This study was conducted using a secondary analysis from the HyperPATH dataset, a dataset of individuals with and without hypertension collected from Boston, MA, Salt Lake City, UT, Nashville, TN, Paris, France, and Italy.

The specific aims of this study are:

1. **To determine if polymorphisms in three candidate genes (CAV1, PPAR γ , and AGT) are associated with insulin resistance in individuals with hypertension.**
2. **To determine if the underlying physiology of the significant gene associations is related to vascular dysfunction, measured by salt sensitivity.**

Research questions.

Q.1a. Is there a relationship between polymorphisms in the CAV1 gene and insulin resistance in individuals with hypertension?

H.01a. Individuals who are homozygous minor allele carriers for SNPs in CAV1 will not be more insulin resistant than heterozygote or homozygous major allele carriers.

H.1a. Individuals who are homozygous minor allele carriers for SNPs in CAV1 will be more insulin resistant than heterozygote or homozygous major allele carriers.

Q.1b. Is there a relationship between polymorphisms in the CAV1 gene and salt sensitivity in individuals with hypertension?

H.01b. Individuals who are homozygous minor allele carriers for SNPs in CAV1 will not be more salt sensitive than heterozygote or homozygous major allele carriers.

H.1b. Individuals who are homozygous minor allele carriers for SNPs in CAV1 will be more salt sensitive than heterozygote or homozygous major allele carriers.

Q.2a. Is there a relationship between polymorphisms in the PPAR γ gene and insulin resistance in individuals with hypertension?

H.02a. Individuals who are homozygous minor allele carriers for SNPs in, PPAR γ will not be more insulin resistant than heterozygote or homozygous major allele carriers.

H.2a. Individuals who are homozygous minor allele carriers for SNPs in the PPAR γ gene will be more insulin resistant than heterozygote or homozygous major allele carriers.

Q.2b. Is there a relationship between polymorphisms in the PPAR γ gene and salt sensitivity in individuals with hypertension?

H.02b. Individuals who are homozygous minor allele carriers for SNPs in the PPAR γ gene will not be more salt sensitive than heterozygote or homozygous major allele carriers.

H.2b. Individuals who are homozygous minor allele carriers for SNPs in the PPAR γ gene will be more salt sensitive than heterozygote or homozygous major allele carriers.

Q.3a. Is there a relationship between polymorphisms in the AGT gene and insulin resistance in individuals with hypertension?

H.03a. Individuals who are homozygous minor allele carriers for SNPs in AGT gene will not be more insulin resistant than heterozygote or homozygous major allele carriers.

H.3a. Individuals who are homozygous minor allele carriers for SNPs in the AGT gene will be more insulin resistant than heterozygote or homozygous major allele carriers.

Q.3b. Is there a relationship between polymorphisms in the AGT gene and salt sensitivity in individuals with hypertension?

H.03a. Individuals who are homozygous minor allele carriers for SNPs in AGT gene will not be more salt sensitive than heterozygote or homozygous major allele carriers.

H.3b. Individuals who are homozygous minor allele carriers for SNPs in the AGT gene will be more salt sensitive than heterozygote or homozygous major allele carriers.

Assumptions

The first assumption of this study is that the intermediate phenotype/candidate gene method is a more specific approach to identifying true positive gene associations with disease. Alternate approaches for identifying genomic markers for chronic disease exist, including the genome wide association study approach and the rare variant approach (Amos, 2007; Bodmer & Bonilla, 2008); however these results have not been successful in the search for genes related to the metabolic syndrome (Lusis, Attie, & Reue, 2008). Alternatively, the intermediate phenotype approach has been successful in identifying genomic markers for other complex disease, including hypertension, and therefore, may be successful in the search for genomic markers of an intermediate phenotype of the metabolic syndrome.

A second assumption is that the genotype assigned by the genotyping platform represents the actual nucleotide harbored by an individual at a particular locus. Quality control metrics, as described in the statistics section, were tested to identify SNPs genotyped incorrectly (Ryckman & Williams, 2008).

A third assumption is that the demographic and clinical information recorded in the HyperPATH dataset is accurate. Quality control metrics including assessing each variable's numeric range and removing influential outliers were conducted to ensure the data used is clean and appropriate for analysis.

Chapter 2

Theory

Conceptual framework.

Historical perspective.

Scientists have recognized the difficulty of studying genetics in heterogeneous phenotypes for some time. In fact, Gregor Mendel, often cited as the father of genetics, commented on this very problem in 1856, writing:

The value and utility of any experiment are determined by the fitness of the material to the purpose for which it is used, and thus in the case before us it can not be immaterial what plants are subjected to what experiment and in what manner such experiment is conducted (p.2). Some characters do not permit sharp and certain separations since the differences of the more or less nature are often difficult to define (Mendel, 1856). (p.4.)

This quote reveals Mendel's struggle that some characteristics do not permit clear phenotypic definitions, making it difficult to conduct well defined genetic experiments and interpret the results.

Intermediate phenotype-candidate gene framework.

The intermediate phenotype- candidate gene approach for the identification of biomarkers in complex disease proposed by Dr. Gordon Williams (Williams, 1994; Williams, Hollenberg, Hopkins, & Jeunemaitre, 1996) provides a solution to the dilemma outlined by Mendel and is the conceptual framework used for this study. The hallmark of the intermediate phenotype framework is a clearly defined phenotype, studied within a tightly controlled environmental setting. By clarifying the phenotype of interest, the search for genes within known physiologic pathways is easier, increasing the likelihood of identifying genomic biomarkers of disease.

In the case of the metabolic syndrome, multiple disease processes including dyslipidemia, IR, hypertension, older age, and obesity contribute to the syndrome's onset. However, by clarifying the physiologic process and analyzing the particular intermediate phenotype of the metabolic syndrome, a clearer picture emerges. Further, by controlling for extraneous variables that may be contributing to the disease outcome (i.e., diet, medication, activity level); one can more clearly delineate the relationship between genes and physiologic processes that contribute to the particular intermediate phenotype.

Previous success.

The identification of genomic markers using well-defined intermediate phenotypes has been successful in other cases of complex illness, particularly in the case of hypertension (Agarwal, Williams, & Fisher, 2005; Fisher et al., 2002; Pojoga et al., 2006; Williams, 1994). Non-modulating hypertension is one intermediate phenotype that occurs in 25% of all individuals with hypertension (Hollenberg, Moore, Shoback, Redgrave, Rabinowe, & Williams, 1986; Williams et al., 1992; Williams, Hollenberg, Hopkins, & Jeunemaitre, 1996). Research participants with non-modulating hypertension have alterations in the response of the RAAS exhibiting a blunted aldosterone response to angiotensin II (AngII) on a low salt diet and altered renal blood flow on a high salt diet (Hollenberg, et al. 1986; G. H. Williams, Hollenberg, Hopkins, & Jeunemaitre, 1996). The angiotensin converting enzyme (ACE) gene, angiotensinogen (AGT) gene, and the aldosterone synthase gene (CYP11B2) have been found to be associated with non-modulating hypertension (Kosachunhanun et al., 2003). A separate intermediate phenotype of hypertension, low renin hypertension, is defined as individuals with hypertension and a low plasma renin activity (PRA) level ($<2.5\text{mU/ml}$) in response to standing for 45 minutes (upright posture study) (Williams, Williams, Jeunemaitre, Hopkins, & Conlin,

2005). The adducin gene and beta-2 adrenergic gene have been associated with low-renin hypertension (Agarwal, Williams, & Fisher, 2005; Fisher et al., 2002; Pojoga et al., 2006). The successful use of these two intermediate phenotypes supports this framework as a model for future studies investigating the genomics of complex disease.

Premise

The theory underlying the current study is the thrifty genotype theory (Neel, 1962). The theory purports that a human trait enabling the efficient storage of food as energy has been passed on through thousands of generations. Due to the advantageous nature of the trait during times of famine and starvation, it has been conserved throughout human evolution. However, in recent times of food abundance (i.e., modern day United States), this inherited trait results in excessive fat storage, obesity and metabolic dysfunction.

The theory guides the current studies in three ways. First, the theory supports the notion that metabolic dysfunction is inherited and caused by evolutionary conserved genetic variants, supporting a search for genomic markers of the metabolic syndrome. Second, the theory proposes that metabolic dysfunction occurs frequently in society, due to its conservation throughout evolution. This is true today where a high incidence of the metabolic syndrome, hypertension, and cardiovascular disease occurs (Park et al., 2003). Third, the theory supports the notion that the disease-causing genomic variants associated with metabolic dysfunction occur frequently in the population and are not rare variants. For this reason, the candidate gene approach used in this study analyzed SNPs that occur relatively frequently in the population (i.e., minor allele frequency (MAF) greater than 10%).

Many scientists support the thrifty genotype theory as an appropriate premise for the search for genomic markers of complex disease (Joffe & Zimmet, 1998; Sharma, 1998).

Research findings identifying genomic markers of T2DM support this theory. For example, Altshuler et al (2000) found that the ancestral allele of the PPAR γ SNP Pro12Ala (a proline [Pro] to alanine [Ala] change) increases a person's risk for T2DM (and altered glucose metabolism) while the less common variant, Ala is protective (Altshuler, et al. 2000).

Literature Review

Introduction.

This literature review details the current state of the science related to the co-aggregation of IR and hypertension in humans. First, research analyzing the effects of metabolic dysfunction, specifically hyperinsulinemia, on various tissues is reviewed to demonstrate that hyperinsulinemia and genes associated with this state may contribute to the onset of IR and hypertension. Second, the inheritance pattern of the metabolic syndrome and the co-aggregation of IR and hypertension is reviewed indicating that the search for genomic markers is reasonable. Third, a detailed explanation of each candidate gene and its known role in the physiological pathways of insulin signaling and endothelial function will be reviewed to support the decision to analyze these genes as genomic markers for the co-aggregation of IR and hypertension.

Definition of insulin resistance: Altered glucose uptake.

Insulin stimulated glucose uptake in both the adipose and muscle tissue is necessary for whole body glucose homeostasis (Kahn, 1996). Insulin resistance can result from alterations with either 1) intracellular insulin signaling or 2) altered insulin stimulated glucose uptake in the muscle or adipose tissue by the intra-cellular transporter glut-4 (Kahn, Hull, & Utzschneider, 2006). The insulin signaling pathway has been studied extensively demonstrating the strong interplay between different signaling proteins and sub-pathways including PI-3 kinase, AKT, and glycogen synthase kinase 3 (GSK3) (White, 1997; White, 1998). Interference with any one of

these interactions can result in altered insulin signaling and subsequent IR (Boura-Halfon & Zick, 2009). Additionally, alterations in the transport of glut-4, an intracellular transport molecule required for insulin stimulated glucose uptake, to the plasma membrane can cause IR in humans. Individuals with T2DM demonstrate both decreased glut-4 levels in adipose and muscle tissue (Garvey, Maianu, Hancock, Golichowski, & Baron, 1992; Giacchetti et al., 1994) as well as alterations in intracellular insulin signaling pathway (Boura-Halfon & Zick, 2009), highlighting the importance of these two pathways in the development of IR.

Definition of insulin resistance: beta-cell failure.

Alterations in insulin signaling and/or insulin stimulated glucose uptake are not the sole mechanisms for insulin resistance. Damage to pancreatic beta cells may also contribute to insulin resistance since they are the source of insulin in the body. Individuals with T2DM have decreased beta cell mass with impaired insulin secretion (Talchai, Lin, Kitamura, & Accili, 2009). The mechanism underlying the decrease in beta cell mass is yet to be determined, but is believed to be a slow, progressive loss after an initial phase of hypertrophy with hyperinsulinemia (Talchai et al., 2009). This loss of beta cell mass results in less insulin secretion, lowering the availability of insulin in the body and resulting in hyperglycemia and IR (Kahn, 1996). Multiple events seem to be causing beta cell mass depletion including inflammation, increased free fatty acids, and systemic hyperinsulinemia secondary to an initial insulin resistant state (Muoio & Newgard, 2008). Through these processes IR progresses to T2DM resulting in poor cardiovascular outcomes.

Potential mechanism: Hyperinsulinism.

It is clear that hyperinsulinemia results in response to insulin resistance, enabling the body to compensate for altered glucose metabolism. Interestingly, hyperinsulinism has been

implicated in the co-aggregation of IR and hypertension in both animal and human studies and may be the pathway linking these two conditions (Chen et al., 1988; Munoz, Giani, Dominici, Turyn, & Toblli, 2009; Reaven, 1988; Reaven, Hollenbeck, Jeng, Wu, & Chen, 1988).

Specifically, hyperinsulinism has been shown to alter endothelial reactivity leading to vascular damage and changes in endothelial function (Cersosimo & DeFronzo, 2006). This damage leads to altered glucose metabolism worsening insulin mediated glucose uptake and causing IR (Cersosimo & DeFronzo, 2006). Further, even when other systems, including the sympathetic nervous system (SNS) and RAAS, were associated with the development of IR in humans, hyperinsulinism was seen as an intermediary effect, highlighting the vast influence of hyperinsulinism on the onset of metabolic dysfunction in humans (Engeli et al., 2003; Engeli et al., 2005; Kopf et al., 2001; Sarzani, Salvi, Dessi-Fulgheri, & Rappelli, 2008; Yanai et al., 2008). Because of this, the effect of hyperinsulinism on various tissues is an important process to study when understanding the mechanism underlying the co-aggregation of insulin resistance and hypertension.

Hyperinsulinism: effects on the liver and muscle.

In healthy people, insulin regulates many aspects of hepatic glucose and lipid homeostasis including gluconeogenesis, lipogenesis, and lipolysis (Shulman, 2000). In the hyperinsulinemic state of IR this homeostasis is altered. Chronic hyperinsulinism leads to a decrease in hepatic insulin sensitivity and a lack of insulin induced suppression of hepatic gluconeogenesis leading to hyperglycemia. Further, while insulin stimulated lipogenesis continues, lipolysis is inhibited resulting in hypertriglyceridemia (Eckel, Grundy, & Zimmet, 2005; Muoio & Newgard, 2008). Hepatic steatosis also ensues with alterations in fatty acid metabolism resulting in the accumulation of Long-chain acyl-CoA esters (LC-CoAs), 1,2-

Diacylglycerol (DAG), ceramides, and triglycerides in the liver. These fatty acid metabolites further inhibit the insulin signaling pathway, leading to further IR, hyperinsulinism, and hyperglycemia (Muoio & Newgard, 2008).

Alterations in fatty acid metabolism, lipid accumulation, and insulin desensitization also contribute to IR in the muscle (Shulman, 2000). Increased free fatty acids leads to increased acetyl CoA/CoA and NADH/NAD ratios resulting in the inactivation of pyruvate dehydrogenase and hexokinase within the cell (Shulman, 2000). This inactivation causes increased intracellular glucose levels which inhibits insulin stimulated glucose uptake in the cell resulting in hyperglycemia. Further, disruptions in AMP kinase (AMPK) and glut-4 translocation in the muscle also lead to alterations in glucose uptake furthering IR in the muscle (Witczak, Sharoff, & Goodyear, 2008).

Hyperinsulinism: effects on the vasculature.

Hyperinsulinism also affects the vasculature and as a result, a relationship between increased insulin levels and increased blood pressure exists. Approximately 30 percent of individuals with hypertension are insulin resistant and hyperinsulinemic (Mozaffarian, Kamineni, Prineas, & Siscovick, 2008). Further, obesity and IR can predict the development of hypertension (Takase et al., 2008), supporting a link between hyperinsulinemia and hypertension. The mechanism underlying the link between hyperinsulinism and hypertension is unknown; however, the co-aggregation of the two conditions may be related to alterations in the RAAS and increased sympathetic activity (Savoia, Volpe, Alonzo, Rossi, & Rubattu, 2009). Individuals with hypertension lose the vasodilatory effects of insulin while maintaining insulin's increased effect on sodium re-absorption in the kidney (Eckel et al., 2005). Further, insulin

increases sympathetic activity resulting in increased vascular tone (Engeli et al., 2003; Engeli et al., 2005).

Hyperinsulinism also affects components of the RAAS, often activating it, which may affect vascular tone and volume retention, leading to hypertension. Specifically, hyperinsulinemic euglycemic clamps in healthy men, where participants are subjected to a constant hyperinsulinemic infusion, demonstrated an increase in plasma renin levels (PRA) and renal plasma flow, markers of an activated RAAS (Perlstein, Gerhard-Herman, Hollenberg, Williams, & Thomas, 2007). Further, individuals with T2DM, known to be insulin resistant, have elevated PRA levels during upright posture and on a high salt diet when compared to healthy controls (Price, De'Oliveira, Fisher, Williams, & Hollenberg, 1999). Recently, elevated aldosterone levels, known to exist with hypertension (Williams, 1982), have also been linked to obesity, cardiovascular damage, and T2DM (Bentley-Lewis et al., 2007; Lastra-Lastra, Sowers, Restrepo-Erazo, Manrique-Acevedo, & Lastra-Gonzalez, 2009; Zennaro, Caprio, & Fève, 2009). It is hypothesized that increased aldosterone secreted by adipose tissue contributes to IR by increasing levels of reactive oxygen species through mechanisms related to the mineralocorticoid receptor (MR) (Zennaro et al., 2009). While much more work is necessary, it is clear that hyperinsulinism does affect the vascular tissue and volume status, both of which likely contribute to the development of hypertension in the co-aggregation of IR and hypertension.

Genetics.

Familial inheritance of the metabolic syndrome.

Evidence supports the heritability of components of the metabolic syndrome via sibling-pair concordance studies and familial aggregation studies. Specifically, the clustering of cardiovascular risk factors in hypertension, including IR, is heritable (Raji et al., 2006). Sibling

concordance rates were significantly higher ($p < 0.001$) in individuals scoring in the highest quartile range for the homeostatic assessment model (HOMA-IR) (a measurement of insulin resistance), triglycerides, and low density lipoprotein cholesterol (LDL-C) indicating related individuals had more IR and higher lipid levels than unrelated individuals (Raji et al., 2001). Further, Tang et al. (2006) found that the clustering of increased body mass index (BMI), increased waist circumference, lower HDL cholesterol, higher triglycerides, and elevated insulin occurred more frequently in families ($p < 0.05$) indicating that these conditions may be inherited together. Additional studies support this finding, demonstrating that the clustering of both cardiovascular and metabolic components of the metabolic syndrome occur more frequently in families (Lee, Klein, & Klein, 2003; Pollex & Hegele, 2006). Together, these studies demonstrate the inheritance of IR and hypertension supporting a search for genomic biomarkers associated with this intermediate phenotype of the metabolic syndrome.

Methods for identifying genomic markers of complex disease: genome-wide association study vs. candidate gene approach.

Two approaches have been used to identify the genetic underpinnings of the metabolic syndrome. First and most commonly used is the genome wide association study (GWAS) (Hattersley & McCarthy, 2005; Manolio et al., 2009). This approach scans the entire genome for hundreds of thousands of SNPs to determine which ones associate with disease (Amos, 2007). One advantage of this method is its unbiased nature, which can potentially lead to the discovery of novel pathways in disease pathophysiology. However, the disadvantage of GWAS is that extremely large sample sizes are required with highly significant p values (5×10^{-8}) (Dahlman et al., 2002) to insure that a type I error does not occur. GWAS have failed to identify genomic markers for both the metabolic syndrome and the intermediate phenotype IR and hypertension.

A recent review in *Nature Medicine* (Lusis et al., 2008) highlighted the failure of GWAS to find positive gene associations for either the metabolic syndrome or hypertension. Other components of the metabolic syndrome including BMI (Frayling, 2007; Loos et al., 2008), fasting glucose (Chambers et al., 2008; Prokopenko et al., 2009), and plasma triglycerides (Kathiresan et al., 2009; Kooner et al., 2008) have been found to have positive gene associations using GWAS; however, many of these studies have yet to be replicated.

The second approach, the candidate gene association approach, is a hypothesis based approach that does not require the large sample size or small p values necessary in the GWAS approach. Genes of the RAAS pathway, angiotensin-converting enzyme (ACE) and angiotensinogen (AGT) have been associated with both the metabolic syndrome (as described by WHO & NCEP III) and components of the metabolic syndrome, including IR and hypertension using the candidate gene approach (Bonnet et al., 2008; Guo et al., 2005; Lee et al., 2003; Perticone et al., 2007; Pollex et al., 2006). However, results are conflicting. While Perticone et al (2007) found a significant association between ACE genotypes and IR in participants with hypertension, the results have not been replicated. Further, additional studies analyzing associations between the ACE gene and IR in hypertension were not successful (Jeng, Harn, Jeng, Yueh, & Shieh, 1997), with some questioning the association of the ACE gene to hypertension entirely (Jeng et al., 1997; Phillips & Kimura, 2005). It is clear that additional research is needed to determine the genetic underpinning of hypertension, particularly the co-aggregation of IR and hypertension. Heterogeneous populations were used in many of the articles demonstrating negative associations. Using both the intermediate phenotype framework with a candidate gene approach may improve success rate for finding positive gene associations with the co-aggregation of IR and hypertension.

Candidate genes for IR and hypertension.

Since hyperinsulinism has been identified as a pathway involved in the development of IR and hypertension, it is important to identify potential genes within this pathway. Three genes were studied for this project. Recent studies indicate that CAV1 is involved in both insulin signaling (via insulin receptor stabilization) and the RAAS (via an interaction with aldosterone) and may be the link between IR and hypertension (Pojoga et al., 2010; Kabayama et al., 2007). Further, recent work analyzing the role of PPAR γ gene in glucose metabolism and vascular function highlight this gene as a candidate gene for IR and hypertension (Duan et al., 2007). Finally, the AGT gene of the RAAS, known to be involved in the development of hypertension, may also be involved in insulin sensitivity (Guo et al., 2005) making it a strong candidate gene for the co-aggregation of IR and hypertension.

Caveolin -1.

Caveolae are plasma membrane lipid domains that are involved in organizing and sequestering signaling molecules (Cohen, Combs, Scherer, & Lisanti, 2003; Cohen, Hnasko, Schubert, & Lisanti, 2004). Evidence supports the role of caveolae as regulators of many proteins and cell signaling pathways including endothelial nitric oxide synthase (eNOS) and p42/p44 MAP Kinase pathway (Schubert et al., 2002). Secondary to this function, caveolae receive attention for their possible contribution to cancer, atherosclerosis, vasculoproliferative diseases, cardiac hypertrophy, heart failure, and T2DM (Cohen et al., 2004; Mercier et al., 2009).

Evidence supports the involvement of CAV1 in many aspects of insulin signaling and glucose uptake, including stabilization of the insulin receptor, localization of the glut-4 transporter, and activation of the insulin signaling pathway (Hnasko & Lisanti, 2003; Razani, Woodman, & Lisanti, 2002). Disruptions in the CAV1 gene may lead to problems in the normal

insulin pathway as demonstrated by CAV1 knockout mice. Loss of CAV1 in these mice results in post-prandial hyperglycemia on a high fat diet, increased circulating free fatty acids (FFA), increased triglycerides, and decreased glucose uptake with an insulin tolerance test (Razani et al., 2002). While loss of the CAV1 gene does not cause T2DM by itself, it does result in a problematic insulin response (Capozza et al., 2005), similar to that seen in participants with IR, supporting the investigation of this gene in the development of IR in hypertension.

Research in human participants also supports the role of CAV1 in insulin signaling and insulin sensitivity. Obese participants with and without type 2 diabetes had increased CAV1 mRNA expression levels when compared to lean, normo-glycemic participants, indicating a role for CAV1 in obesity, a common initiator of IR (Catalan et al., 2008). Further, Grilo et al (2006) found a significant association with SNPs of the CAV1 gene and normotensive, normo-glycemia suggesting a protective effect of this particular polymorphism against metabolic syndrome and hypertension. Two rare mutations in the CAV1 gene cause lipodystrophy, a disease of abnormal fat distribution and severe IR, in humans (Cao, Alston, Ruschman, & Hegele, 2008). Finally, the CAV1 gene has been implicated in the stabilization of the insulin receptor with mutations in the insulin receptor gene at the CAV1 docking domain causing severe IR in humans (Cohen, Razani et al., 2003; Iwanishi et al., 1993; Moller, Yokota, White, Pazianos, & Flier, 1990). Together, these studies highlight the role of CAV1 in the activation of insulin signaling and subsequent glucose metabolism in humans.

CAV1 and vascular function.

CAV1 is abundant in endothelial cells and is a negative regulator of the vasodilator molecule endothelial nitric oxide synthase (eNOS), making CAV1 a possible mediator of vascular dysfunction (Maniatis, Shinin, Schraufnagel, Okada, Vogel, Malik et al., 2008). CAV1

knockout mice exhibit increased levels of eNOS and nitric oxide (NO) however, increased NO levels seems to be balanced by changes in the vascular media and increased vascular resistance (Rahman & Sward, 2008; Schubert et al., 2002). Further, these mice show evidence of endothelial dysfunction including cardiac hyperproliferation and vascular abnormalities (Maniatis, Shinin, Schraufnagel, Okada, Vogel, Malik, et al., 2008).

Peroxisome proliferator activated receptor gamma (PPAR γ).

The peroxisome proliferator-activated receptors (PPAR_a, PPAR_{b/d}, PPAR _{γ}) are well known ligand activated transcription factors and members of the nuclear hormone receptor subfamily (Picard & Auwerx, 2002). Pharmacological agonists targeting these receptors contribute greatly to the understanding of the receptors' role in a variety of metabolic systems, particularly in the case of PPAR γ . The realization that PPAR γ agonists improved insulin sensitivity while decreasing hepatic glucose production led to the understanding of PPAR γ as regulator of glucose metabolism (Rubenstrunk, Hanf, Hum, Fruchart, & Staels, 2007). Subsequently, PPAR γ agonists are used frequently to improve insulin sensitivity in individuals with T2DM (Nathan et al., 2009; Robinson, 2008).

SNPs in the PPAR γ gene are associated with altered glucose metabolism in numerous human studies. Florez et al (2007) demonstrated that the SNP Pro12Ala predicted onset of T2DM. Individuals homozygous for the Proline/Proline (P/P) allele had a 20 percent increase risk for T2DM while the Alanine/Alanine (A/A) carriers are protected against the onset of T2DM. The association of this SNP with IR has been replicated in numerous studies (Altshuler et al. 2000). However, a recent meta-analysis demonstrated no association between the Pro12Ala SNPs in PPAR γ gene and IR in non-diabetic individuals highlighting the importance of study population in interpreting the results (Tonjes, Scholz, Loeffler, & Stumvoll, 2006).

Polymorphisms in the PPAR γ gene are associated with BMI with the A/A allele predicting lower BMI, a contributor to insulin sensitivity (Mohamed et al., 2007). Further, in an extensive review, Picard and Auwerx (2002) provide evidence that polymorphisms (P115G, P467L, and V290M) in the PPAR γ gene contribute to alterations in insulin sensitivity. All of these studies demonstrate the important link between the PPAR γ gene and insulin sensitivity in humans.

Similar to studies with the CAV1 gene, animal studies of the PPAR γ gene demonstrate a link between IR and hypertension. Duan and colleagues (2007) created a generalized PPAR γ knockout mouse exhibiting lipodystrophy, IR, and lower blood pressure with PPAR γ deficiency. Further, mice heterozygous for the L466A mutation of the PPAR γ gene demonstrate IR and reduced glucose uptake in skeletal muscle (Freedman, Lee, Park, & Jameson, 2005). Finally, PPAR γ agonists demonstrate increased vascular endothelial growth factor (a vascular permeability factor) and improved insulin sensitivity in mice (Rubenstrunk et al., 2007). Together, these studies provide an association between PPAR γ , IR, and hypertension indicating the importance of further investigation.

Angiotensinogen (AGT).

Angiotensinogen (AGT) is the initial component of the renin-angiotensin-aldosterone system (RAAS) and a precursor to both angiotensin I (AngI) and AngII(AngII). Variants of the AGT gene are associated with plasma angiotensinogen levels, hypertension, and adrenal and renal blood flow (Hopkins et al., 1996; Hopkins et al., 2002; Watkins et al., 2010) likely through the downstream effects of AGT on AngII. Unfortunately, a role for AGT and AngII in the development of altered glucose metabolism is not as clear. Pharmacologic blockade of the RAAS has been shown to decrease the incidence of new onset type 2 diabetes in some studies (Abuissa,

Jones, Marso, O'Keefe, 2005; The Navigator Group, 2010), but not others (Dream Trial Group, 2006). Further, gene association studies report both positive and negative associations for genes of the RAAS, including AGT, with both insulin resistance (IR) and insulin sensitivity (Bonnet et al., 2006; Pollex et al., 2005; Guo et al., 2005; Sheu et al., 1998). It is possible that studies analyzing components of RAAS with glucose metabolism conflict due to the heterogeneity of the populations studied and the inconsistent measurements of IR, blood pressure, and T2DM.

AGT: Cross-talk RAAS and insulin signaling: animal models.

Interestingly, interplay between the RAAS and the insulin signaling pathway has been shown in both animal and cellular models (Velloso et al., 1996; Velloso et al., 2006). In rat heart muscle, AngII infusion has been shown to induce insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation and decrease phosphatidylinositol 3-kinase (PI3K) activity through its effects on the angiotensinogen receptor 1 (ATR1) (Velloso et al., 1996). It has been proposed that these mechanisms decrease insulin sensitivity (Ogihara et al., 2002; Velloso et al., 2006); however, this hypothesis is yet to be tested. Additional evidence supports crosstalk between AngII and insulin signaling in animal models however, their effects on tissue specific insulin sensitivity are conflicting. Ogihara et al (2002) demonstrated that chronic AngII infusion (12 days 100ng/kg/min) in rats resulted in IR in liver, skeletal muscle, and adipocytes demonstrating both altered glucose uptake and decreased glut-4 translocation in skeletal muscle and adipocytes and altered glycogen synthase activation in the liver. Conversely, Juan et al (2005) found that acute injection of AngII (2ug/100g body weight) increased insulin stimulated glucose uptake in rat adipocytes. Further, when adipocytes were removed and incubated in AngII, stimulated tyrosine phosphorylation of the insulin receptor increased, AKT phosphorylation increased and glut-4

transport increased demonstrating a role for AngII in whole body glucose homeostasis (Juan et al., 2005).

AGT: human studies.

Numerous studies analyzing the effects of AngII infusion in humans have also found conflicting results. First, Townsend and colleagues (1993) show no differences in insulin stimulated glucose uptake measured by euglycemic insulin clamp before and after subpressor doses of AngII (0.3ng/kg/min and 1ng/kg/min) in normotensive men. This result was confirmed in individuals with well-controlled, recently diagnosed T2DM (HgbA1c=6, mean age=46) receiving either 2ng/kg/min of AngII or normal saline (sham infusion) with insulin sensitivity measured by insulin clamp (Fliser, Arnold, Kohl, Hartung, & Ritz, 1993). Alternatively, other studies show an improvement in insulin sensitivity after infusion with AngII. Morris et al (1994) studied normotensive individuals with T2DM demonstrating subpressor (1ng/kg/min) and pressor (5ng/kg/min) doses of AngII improve insulin sensitivity measured by euglycemic insulin clamp. Further, this effect was seen even with the subpressor dose of AngII, without an increase in blood pressure, demonstrating that hemodynamic alterations are not the sole mechanism for improved insulin sensitivity. Two additional studies in normotensive men confirmed an increase in insulin sensitivity with AngII infusion (Fliser et al., 1993; Widgren, Urbanavicius, Wikstrand, Attvall, & Persson, 1993). Buchanan et al (1993) conducted additional mechanistic studies during the AngII infusion demonstrating an improvement in skeletal muscle blood flow and decrease in renal blood flow with AngII infusion. These studies demonstrate conflicting results on the role of AngII in IR.

Few studies have examined other aspects of the RAAS system on IR and hypertension including renin and aldosterone. However, renin has been implicated with T2DM in participants

with hypertension demonstrating that these individuals have inappropriately activated RAAS (Price et al., 1999). Further, aldosterone level after AngII infusion predicted HOMA values with higher aldosterone levels associating with increased HOMA (and IR) in normotensive, obese individuals (Bentley-Lewis et al., 2007).

AGT: human genetics studies.

Human genetic studies of genes in the RAAS to date fail to clarify the relationship between AGT and insulin sensitivity. A well known SNP of the angiotensinogen gene (AGT), M235T, has been associated with increased plasma angiotensinogen levels, hypertension, and increased risk for cardiac hypertrophy (Kosachunhanun et al., 2003; Niemiec, Zak, & Wita, 2008; Watkins et al., 2010). Few groups have studied this SNP's role in IR and hypertension; however, Guo and colleagues (2005) have shown an association between SNP AGT M235T and insulin sensitivity. Three studies found associations with another gene of the RAAS, ACE, and IR in populations of T2DM, healthy participants, and participants with hypertension. However, a separate study refuted this finding (Jeng et al., 1997). These conflicting results are likely the result of the heterogeneous populations that were studied, differing definitions of the metabolic syndrome and insulin sensitivity, and small sample sizes. Analyzing variants of genes in a more homogeneous subset of individuals (i.e., an intermediate phenotype) may provide clarification on the role of RAAS gene in the development of IR and hypertension.

Summary

A clear association exists between IR and hypertension, two key components of the metabolic syndrome. This association is supported by data indicating that the two conditions often co-aggregate and are inherited together. Previous studies attempting to find genes associated with the metabolic syndrome have been unsuccessful. Of importance, the intermediate

phenotype approach has not been used. Further, these specific candidate genes, CAV1, PPAR γ , and AGT, have not been examined.

Recent research in animal models and humans support the role of the CAV1, PPAR γ , and AGT genes in both IR and hypertension. Therefore, this study aimed to analyze whether polymorphic changes in the CAV1, PPAR γ , and AGT genes contribute to the development of IR and hypertension in humans using an intermediate phenotype approach. An understanding of the association between CAV1, PPAR γ , and AGT genes with IR and hypertension will contribute to the development of more specific prevention and treatment strategies, contributing to individualized care, decreasing the morbidity associated with both of these disorders

Chapter 3

Study Design

This research is a gene association study analyzing the effect of genotype on insulin sensitivity in individuals with hypertension. In gene association studies, confirming the results from the primary population in a second population is important to validate the potential generalizability of the conclusions. For two of the three genes analyzed, a validation study was performed. Where two populations were studied a meta-analysis, described below, was also performed. In the primary association studies, the dependent variable is insulin sensitivity, a continuous variable, measured by two methods: euglycemic insulin clamp and HOMA-IR. This study controlled environmental factors (i.e., diet and medication) that may influence the gene effects on the outcome variable. The resources of the Human Research Center (HRC) at Brigham and Women's Hospital (BWH) were used and an analysis of data collected through the HyperPATH protocol was conducted.

Study Variables: Operational Definition and Measurement.

Hypertension: Baseline systolic, diastolic, and mean arterial blood pressures was measured as the mean of three consecutive readings (by Dinamap; Critikon, Tampa, Fl.) separated by 5 minutes each, taken after at least 15 minutes of rest (Chamarthi et al., 2007). Hypertension is defined as previously described in the HyperPATH Protocol ((Pojoga et al., 2006; Raji, Williams, Jeunemaitre, Hopkins, Hunt, Hollenberg, & Seely, 2001) as blood pressure > 135/85mmHG on no anti-hypertensive medications, blood pressure > 130/80 on one medication, or patient taking two or more anti-hypertension medications.

Dependent variables.

Insulin Sensitivity: Two techniques were used to measure insulin sensitivity in the HyperPATH database 1) Homeostasis assessment of insulin resistance (HOMA-IR) and 2) euglycemic insulin clamp (DeFronzo, Tobin, & Andres, 1979; Matsuda & DeFronzo, 1999; Muniyappa, Lee, Chen, & Quon, 2008; Trout et al., 2007; Trout, Homko, & Tkacs, 2007; Wallace, Levy, & Matthews, 2004). HOMA-IR calculates insulin sensitivity using the following equation: fasting glucose mmol/L X fasting insulin in mU/mL)/22.5 (Matthews, 1985). A high HOMA-IR score indicates insulin resistance. The M value of the euglycemic clamp was also used to determine insulin sensitivity. The amount of glucose infused in milligrams per square meter per minute during the last 120 min of the clamp is used as an index of insulin sensitivity and termed the M value (DeFronzo et al., 1979).

Vascular Dysfunction and Salt Sensitivity: Salt sensitive blood pressure (response of systolic, diastolic, and mean arterial blood pressure to salt loading) was examined as a measurement of vascular dysfunction in the hypertensive population. Salt sensitive blood pressure was measured by subtracting blood pressure measurements obtained during low salt diet (10 mmol sodium/day) from blood pressure measurements obtained from the same individual on a high salt diet (200 mmol sodium/day).

Independent variables.

SNPs of CAV1 gene: 1=homozygote major allele carrier for SNP (AA), 2=heterozygote (Aa), 3=homozygote minor allele carrier (aa).

SNPs of the PPAR γ gene: 1=homozygote major allele carrier for SNP (AA), 2=heterozygote (Aa), 3=homozygote minor allele carrier (aa).

SNPs of the AGT gene: 1=homozygote major allele carrier for SNP (AA), 2=heterozygote (Aa), 3=homozygote minor allele carrier (aa).

Table 2. SNPs of CAV1 gene: 11 SNPs were tested, rs numbers from dbSNP, Maj=major allele, Min=minor allele.

11 Genotyped SNPs		
Polymorphism	Location: chr.7	Maj/Min
rs2215448	115951188	G/A
rs926198	115954444	T/C
rs1543293	115965784	G/C
rs959173	115969290	T/C
rs3807989	115973477	G/A
rs12668226	115974926	A/C
rs3815412	115977929	T/C
rs1022436	115980467	C/G
rs3757732	115980941 intronic	C/A
rs729949	115982141	G/A
rs1049337	115987823	C/T

Table 3. SNPs of the PPAR γ gene: 11 SNPs were tested, rs numbers from dbSNP, Maj=major allele, Min=minor allele.

11 Genotyped SNPs		
Polymorphism	Location: chr.3	Maj/Min
rs17036242	intron 12324490	G/A
rs10510418	intron 12363563	A/C
rs7649970	intron 12367272	G/A
rs1373641	intron 12377474	A/G
rs10510419	intron 12401936	C/A
rs2959272	intron 12417833	A/C
rs4135275	intron 12418844	A/G
rs13099634	intron 12443463	G/A
rs1797912	intron 12445239	A/C
rs3856806	coding- synonymous 12450557	G/A
rs1152003	intron 12452055	C/G

Table 4. SNPs of the AGT gene: 16 SNPs were tested.

Polymorphism	Location:chr1	Maj/Min
rs7536290	228903325	A/G
rs7079	228904954	C/A
rs11568045	228906106	A/A
rs3789670	228910337	G/A
rs3789671	228910423	C/A
rs2478545	228910744	G/A
rs6687360	228911615	G/A
rs11122576	228913302	A/G
rs11568026	228914146	A/A
rs2004776	228915325	G/A
rs1078499	228915719	A/G
rs7539020	228915813	G/A
rs2493134	228915982	T/C
rs3789678	228916105	G/A
rs5050	228916509	A/C
rs2493137	228918739	A/G

Covariates.

Age: Age of individuals in years and obtained by self report or date of birth.

Gender: a dichotomous variable (female/male) obtained by participant self report. Gender has been known to affect the metabolic syndrome (Ahonen et al., 2009) as well as hypertension (Liu et al., 2003) and IR (Geer & Shen, 2009), therefore should be included as a covariate

BMI: Body mass index (BMI) is a measure of body fat based on height and weight. Calculation: $\text{Weight (kg)} / (\text{Height (m)} \times \text{Height (m)})$ (Duncan, 2010).

Sibling status: individuals sharing the same biological parents. Status obtained by participant self report and coded by study subject number.

Sample

The HyperPATH dataset, initiated in 1994, consists of approximately 1300 subjects, over 500 of them with hypertension from Boston, Utah, Nashville, Paris, and Italy. Participants with hypertension within this dataset met the following criteria: between the ages of 18-70 years, without renal insufficiency or untreated thyroid disease, not taking medications other than thyroid replacement, and had no forms of secondary hypertension (Chamarthi et al., 2007; Raji et al., 2006).

This study sample consists of Caucasian participants with hypertension from the HyperPATH dataset previously genotyped and having data for either HOMA-IR or the euglycemic insulin clamp. In addition, individuals without hypertension were studied to determine if the genotyped SNPs meet adequate quality control parameters (detailed later). An African American sample of individuals with hypertension (approximately 60) from the dataset was used to attempt to replicate positive findings for associations with the PPAR γ gene.

Study inclusion criteria.

Participants between the ages of 18-70 years with a BMI $\leq 40 \text{kg/m}^2$ were included. A BMI $< 41 \text{kg/m}^2$ insures that morbid obesity, a cause of IR (Kim, Wei, & Sowers, 2008), does not influence the insulin sensitivity measurements.

Study exclusion criteria.

Participants with fasting plasma glucose (FPG) greater than 126 mg/dL or a random plasma glucose greater than 200mg/dL were excluded to rule out T2DM (Executive, 2008). Participants with T2DM are all known to be IR and this diagnosis would confound any measurements of insulin sensitivity (Hoerger & Ahmann, 2008).

The HyperPATH study had the following exclusion criteria (data already collected): Participants with secondary hypertension, renal disease (with the exception of microalbuminemia, $< 100 \text{mg albumin/g creatinine}$), known coronary artery, peripheral vascular disease, or cerebral vascular disease, recent myocardial infarction, cerebral vascular attack, or active malignancy, systolic blood pressure $> 160 \text{mmHG}$, diastolic blood pressure $> 100 \text{mmHG}$, spot urine shows $\text{Na} > 30 \text{mmol}$ after low salt diet, pregnancy, current excessive alcohol use ($> 12 \text{ oz/ETOH/week}$), participants using recreational drugs, smokers unwilling to refrain from smoking for 72 hours prior to inpatient study, abnormal labs related to renal, liver, hematologic, or immune function, subjects with $> 50\%$ renal artery stenosis.

Detailed Description of HyperPATH Protocol

Details of the HyperPATH protocol have been described elsewhere (Hopkins et al., 2002; Chamarthi et al., 2007; Pojoga et al., 2007). In brief, all participants of the HyperPATH protocol received an alcohol and caffeine free isocaloric low salt diet (10 mmol/d sodium, 100 mmol/d potassium, 1000 mmol/d calcium) for 7 days and a high salt diet for 5 days on an outpatient

basis. On the final day of the diets, participants were admitted to the Human Research Center (HRC). Subjects in sodium balance (≤ 30 mmol sodium/d for low salt and >150 mmol sodium/day for high salt) by measurement of sodium and creatinine excretion in a 24-hour urine collection were studied. As previously described, on the morning of low salt admission day one, plasma renin activity (PRA) and aldosterone levels were measured after participants assumed and maintained upright posture for 90 minutes (Chamarthi et al., 2007). On the evening of the seventh low salt day and fifth high salt day, participants stayed in the HRC overnight, remaining fasting and supine after midnight. The AngII (3ng/kg/min for 60 minutes) infusion and additional blood draws for laboratory assessment were conducted the following morning. Plasma renin activity (PRA) and aldosterone were measured using standardized methods as previously described (Pojoga et al., 2007). Baseline systolic and diastolic blood pressures were taken as the mean of three consecutive readings (by Dinamap; Critikon, Tampa, Fl.) separated by 5 minutes each, 30 minutes before the initiation of the AngII infusion.

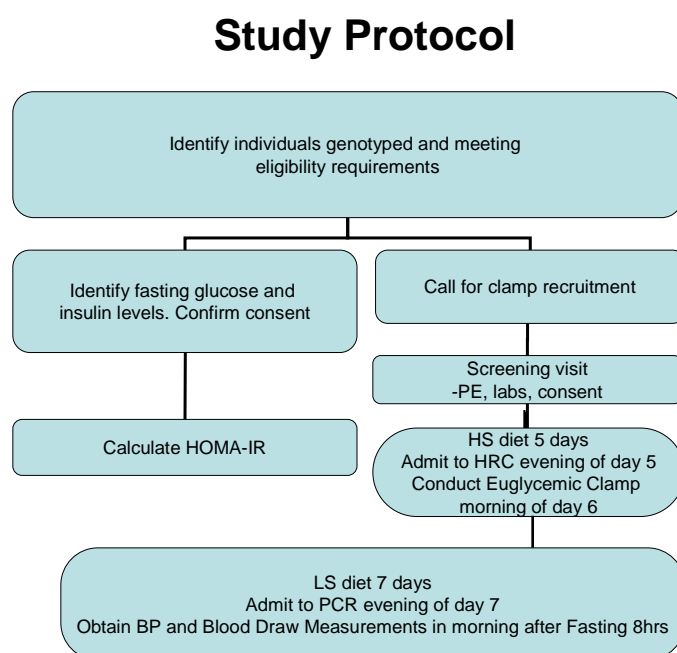
Detailed Description of HTN-IR Protocol: Replication of CAV1 Analysis

The Mexican-American Hypertension-Insulin Resistance (HTN-IR) cohort consists of Hispanic families (939 individuals from 160 pedigrees) ascertained via a hypertensive proband (essential hypertension defined as sitting blood pressure $\geq 140/90$ mm Hg off medication). Probands were recruited through the Hypertension Clinic at Los Angeles County, University of Southern California Medical Center or the General Clinical Research Center at the University of California at Los Angeles. Participating offspring were aged at least 16 years, and spouses of the proband were aged 18 to 65 years. Hypertensive individuals were studied after discontinuing antihypertensive medications for 2 weeks if permissible. No dietary intervention or medication changes were made by investigators during the study. For this study, only individuals with

hypertension were included. Phenotypic characteristics including blood pressure (BP), fasting glucose, and insulin were obtained and measured as previously described (Xiang et al. 2002). Hyperinsulinemic euglycemic clamps were performed on individuals with fasting serum glucose concentrations <140 mg/dL.

Study Procedures Necessary to Obtain Data for Study

Figure 1. Study schema.



Recruitment, Informed Consent, IRB Approval.

This study was reviewed by and conducted in compliance with the Brigham and Women’s Hospital (BWH) institutional review board (IRB) and Boston College IRB through an IRB authorization agreement (see **Appendix 1**). Insulin resistance was measured in individuals by either HOMA-IR or euglycemic insulin clamp. The difference in study protocol for the two measurements is outlined in Figure 1. The PI and team study coordinators re-contacted the HyperPATH participants that met the appropriate genotyping criteria (were successfully

genotyped for AGT SNPs for ongoing R01 project) by telephone to determine if they were interested in participating in the euglycemic clamp study. If participants agreed, they were consented, and completed the euglycemic clamp protocol shown in Figure 2. All data related to the HOMA index was collected previously during prior HyperPATH protocols.

Admission screen.

If the participants agreed, they came in for an ambulatory screening interview and physical exam (PE). After a detailed explanation (by the PI) of the study procedures, including all possible risks, the participant signed a consent form. Each participant was informed that he/she was free to discontinue participation in the experiment at any time and that the investigators reserved the right to discontinue the research protocol at any time. A copy of the signed informed consent form was given to each participant. After the consent was signed, the participant received a complete PE from the PI, height and weight measurements were obtained, and blood was drawn for additional DNA extraction. Urine and serum were collected for basic tests (complete blood count, complete metabolic panel, urinalysis) to assess kidney, liver, hematologic, and immune function. Three blood pressures were obtained (to identify mean blood pressure measurement) and the participant was scheduled for the euglycemic hyperinsulinemic insulin clamp protocol (see below) in the inpatient HRC.

If participants were on ACE inhibitors (ACEI) or angiotensin receptor blockers (ARBs), the participant was switched off these medications three months prior to study. If needed to maintain BP <135/85 mm Hg, calcium channel blockers and hydrochlorothiazide were prescribed by the nurse practitioner and supervising physician. ACE-inhibitor, ARB medications, and beta-blockers were avoided in this study since these medications affect the RAAS and insulin sensitivity and may confound the results of the study. All medications were removed 2

weeks prior to the study to insure study variables were not affected by medications. Participants were sent home with a blood pressure cuff and educated about how to take daily, home blood pressure measurements by the PI. The participants reported blood pressures to study staff twice a week.

Laboratory Measurements

Plasma glucose, serum insulin, and lipids levels were measured after an eight hour fast and collected between 08:00 and 09:00. Serum insulin, glucose, and lipids were measured as previously described (Raji et al., 2001).

Inpatient Study for Hyperinsulinemic Euglycemic Clamp.

After screening, an inpatient study was conducted to obtain additional blood for measurements of insulin sensitivity via the euglycemic hyperinsulinemic clamp technique. Upon admission, blood pressure was obtained (x3), random blood glucose was measured, and the inpatient protocol was reviewed with the participant.

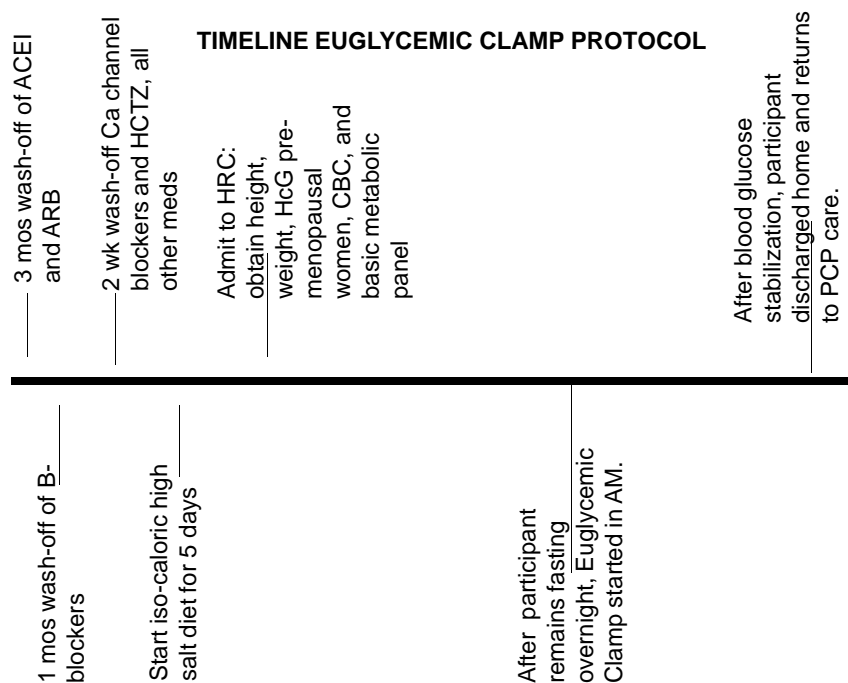
Euglycemic hyperinsulinemic clamp measurement.

The euglycemic insulin clamp was performed over 3 hours. After an overnight fast, participants remained supine and two intravenous catheters were placed. The first IV catheter was placed in an antecubital vein with a three way stop cock for the insulin and glucose infusions. A second catheter was placed retrograde in the cephalic vein of the opposite hand and warmed at 180 degrees Fahrenheit for blood sampling. After the collection of baseline samples, a continuous infusion of crystalline insulin was administered at a rate of 80 mU/m²/min. The plasma glucose was measured at 5-min intervals and maintained at 90 mg/dl by a variable infusion of 20% dextrose. Continuous monitoring of the participant including symptom surveys was conducted throughout the protocol.

After the euglycemic insulin clamp, participants were monitored for at least 2 hours ensuring normal blood glucose levels are maintained. Finger stick blood glucoses were monitored every 5 minutes after the completion of the clamp for 30 minutes and then every ½ hour for 2 hours to insure a stable normal glucose level. The patient was discharged home after receiving a carbohydrate rich meal and maintaining blood glucose levels >90mg/dl at least 3 hours after the completion of the clamp.

The amount of glucose infused in milligrams per square meter per minute during the last 120 min of the clamp is used as an index of insulin sensitivity (M value) (DeFronzo et al., 1979). The euglycemic clamp is highly valued technique to measure insulin sensitivity with known validity and reliability (Matsuda & DeFronzo, 1999; Sarafidis et al., 2007).

Figure 2. Timeline of Euglycemic Hyperinsulinemic Insulin Clamp Protocol.



HOMA-IR index.

The HOMA-IR was used as a second measure of insulin sensitivity. Insulin resistance is measured using the following formula [(fasting plasma glucose (mmol/L) X fasting plasma insulin (mU/L)]/ (22.5) (Trout et al., 2007). Plasma glucose levels and plasma insulin were obtained in the morning of the in-patient visit, after an overnight fast.

Genotyping:

Genotyping was performed in the Harvard Partners Center for Genetics and Genomics. Blood samples were chilled on ice immediately after being drawn and centrifuged at 4 degree Celsius within one hour to separate the cellular component from the plasma. EDTA anticoagulated blood samples were used for DNA extraction within three days of collection. Two identifiers were used to label DNA samples: 1) participant # and 2) Patient Research Center number. The names of the participants were not linked to the blood samples for storage or processing purposes. The genotyping for PPAR γ and AGT was carried out in 384, 768, and 1536 plex formats using the Illumina Bead Station GoldenGate assay system. The system analyzes polymorphic changes using microbead technology assembled into 96 sample arrays. The bead arrays are manufactured based on the custom SNP sets selected and configured onto the array surface. Incubation of the processed genomic DNA on the bead array allows hybridization to the appropriate probe on the bead enabling identification of a particular SNP. The HyperPATH CAV1 SNPs were genotyped using a Sequenom platform. The two CAV1 SNPs analyzed in the HTN-IR were genotyped using a 7600-SNP Illumina iSelect platform. Ten percent of samples are duplicated to act as Qualitative Control. The genotype duplications must agree at least 98% of the time, ensuring validity. Further, previous genotype analysis run on 730 SNPs using the Illumina system demonstrated reproducibility rates of greater than 99%.

Gene characterization.

Each gene was characterized by using the Tagging SNPs method from the HAP MAP database (HAPMAP Project, 2008). Tagging SNP analysis allows researchers to narrow a genetic region of interest and increase the precision of localization without having to genotype the entire gene. This method significantly decreases cost and effort without minimizing outcome (Iles, 2006; Maniatis, Collins, & Morton, 2007). The pairwise method of tagging SNP selection was used for tagging SNP identification. Linkage disequilibrium (LD) was used to identify tagging SNPs with the following parameters: An $R^2 > 0.8$ (i.e., the SNP will “tag” all other SNPS with an $R^2 > 0.80$) was chosen with a minor allele frequency (MAF) cutoff of 10% . These recommendations match literature analyzing the best methods for choosing tagging SNPs without requiring a large sample size and without decreasing power (de Bakker et al., 2005). In addition, de Bakker (2005) demonstrated that relaxing the threshold for perfect correlation to .80 when choosing tagging SNPs does not affect power.

Study Analyses

Data quality control.

Before using the SNPs in the analysis, the following quality control measurements were conducted. First, MAF of all SNPs were analyzed in the normotensive, healthy control population. If the MAF was less than 10% or the SNP was not in Hardy-Weinberg equilibrium (HWE), the SNP was removed from the analysis because low MAF or SNPs not in HWE in healthy controls may indicate a poorly defined SNP (Ryckman & Williams, 2008). Second, genotype completion rates were analyzed in the entire population and SNPs with completion rates less than 95% were removed from the analysis insuring missing data did not influence the statistical results. Third, LD charts were created and SNPS with an R-squared of greater than

95% rate with another SNP in the analysis were removed since these SNPs are in complete LD with one another, provide similar information, and by removing them the study power was increased. SNPs with an R squared of greater than 95% provide similar information. By decreasing the total number of SNPs tested, the analysis decreased the number of multiple tests that need to be accounted for and improved the power of the analysis. After the SNPs were removed, haploview was used to determine the percent coverage of the entire gene.

Sample size: Insulin resistance.

Analysis 1. The sample size calculation was done using G power statistical software (Faul, Erdfelder, Lang, & Buchner, 2007). Since it is hypothesized that single SNPs have a small effect on the outcome of complex disease, a small effect size of SNP influence on insulin sensitivity was used in the calculation. A sample size of 264 was calculated, with a power 0.80 and an alpha of 0.05.

Analysis 2. Since this project is the first time euglycemic clamps have been used to analyze the SNPs of interest, this is a pilot study using 5 participants in each group. This pilot study will inform future work with a larger sample size. Other studies have found significant differences in M values between groups using a total sample size of eight, indicating this pilot sample size may be sufficient to identify differences by genotype using this sensitive measurement of insulin resistance (Donovan, Solomon, Seely, Williams, & Simonson, 1993).

Sample size: Salt Sensitive Blood Pressure.

Analysis 1. The sample size calculation was done using G power statistical software (Faul, Erdfelder, Lang, & Buchner, 2007). Since it is hypothesized that single SNPs have a small effect on the outcome of complex disease, a small effect size of SNP influence on salt

sensitive blood pressure was used in the calculation. A sample size of 264 was calculated, with a power 0.80 and an alpha of 0.05.

Statistical analysis.

Statistical analyses were performed using SAS 9.1 (SAS Institute; Cary N.C.). HWE testing was performed for each SNP using a chi-square test. Pairwise linkage (D' and R^2) was estimated using Haploview. A mixed effect linear regression was conducted for each of the three genes to analyze whether differences in HOMA-IR or salt sensitive blood pressure exist by genotype. Sibling relatedness was accounted for and improved the power of the analysis due to the enrichment of disease alleles in the sample (Li, Boehnke, & Abecasis, 2006). The linear mixed effects method accounted for sibling relatedness by analyzing siblings as one individual measured over 2 time points (Fisher, 1912). The linear mixed effect regression analysis further increases power by distinguishing within-subject variation (sibling measurements are considered random effects and subject specific) from between-subject sources of variation (fixed effects are considered population effects) (Finucane, 2007). Further, age, gender, and BMI were included as co-variates in the analysis since these variables are known to affect insulin resistance and blood pressure measurements. The equation for the regression model is as follows (Der & Everitt, 2009):

$$y = B_0 + B_{(\text{SNP sibling1})} + B_{(\text{SNP sibling2})} + B_{(\text{age})} + B_{(\text{gender})} + B_{(\text{BMI})} + u_{(\text{random effect=sibling})} + v_{i(\text{sibling})} + e_{(\text{fixed effects=site})}.$$

B_0 = population's average intercept

$B_{(\text{SNP sibling1})}$ = fixed effects regression parameter for SNP time 1 (baseline)

$B_{(\text{SNP sibling2})}$ = fixed effects regression parameter for SNP time 2 (sibling)

$B_{(\text{age})}$, $B_{(\text{gender})}$, $B_{(\text{BMI})}$ = regression parameter for each covariate

u (random effect=sibling)= deviation of the individual (random effect) from the population intercept B_0

V_i (sibling)= represents the deviation of the individual person's slope from the population average $B_{(SNP\ sibling1)}$.

e (fixed effects=site)= fixed effects error

Since dominant genetic models were analyzed for the euglycemic clamp, Student's t-test was used for each of the three genes to analyze differences in the M value by genotype. Sibling relatedness was not accounted for in these analyses since siblings were not studied in the clamp protocol.

Since mixed effects linear regression was used to analyze the HOMA-IR and salt sensitive blood pressure data, all assumptions of regression statistics remain. The assumptions include: 1) linearity (the dependent variable [Y], is in a linear relationship with the independent variables [X_x], non-correlation (the independent variables are not correlated, and the error terms are not correlated with any of the independent variables), measurement (all independent variables are measured as interval, ratio, or dichotomous and the dependent variable is continuous and unbounded), and homoscedasticity (the variance of the error term is the same or constant for all values of the independent variable) (Menard, 2002; Fox, 1991).

Normal distribution of the dependent variable was tested using the Shapiro Wilk test (Munro, 2005). Where necessary, variables were natural log transformed to meet the normality assumptions of the regression model.

Haplotype analysis.

Previous studies highlight the importance of haplotype analysis when examining the AGT gene (Watkins et al., 2010; Watkins, Hunt, et al. 2010). Thus, for the AGT analysis,

haplotypes were constructed using the Haploview program and an association of each haplotype with HOMA-IR was assessed using PLINK (Purcell et al., 2009). Plink estimates haplotype frequencies via the expectation-maximization (EM) algorithm, computing global and haplotype-specific score statistics for tests of association between a trait and haplotype weighted by their posterior possibility. Since PLINK is unable to account for relatedness, the haplotype analysis was conducted in unrelated individuals only. All statistical tests were 2 sided. Nominal significance is indicated for $p < 0.05$. A Bonferroni correction for multiple comparisons is conservative due to the linkage disequilibrium (LD) between SNPS of the gene (Watkins et al., 2010). However, significance at the Bonferroni-corrected level ($0.05/\#$ SNPs tested) is indicated when it exists.

Regression diagnostics.

Regression diagnostics were conducted to insure assumptions of the regression model were met for each significant model found. Linear relationships between independent variables are termed “collinearity” and can result in an imprecise estimate of beta values (Fox, 2008). The test for collinearity is the variance-inflation factor (VIF) and was determined by the equation $1/(1-R^2)$ for all significant regression models tested. The linearity assumption was tested through an analysis of partial residual plots using SAS (plots of the independent variables plotted against residuals) (Ayyengar, 2007). The residuals of a regression model must be uncorrelated and independent of each other (Pedhazur, 1997). The Durbin Watson statistic would be used to test autocorrelation between residuals (Ayyengar, 2007). However, when conducting a longitudinal study (mixed effects regression), independence of residuals may not always be met (Finucane, Samet, & Horton, 2007; Fitzmaurice, Laird, & Ware, 2004). In the dataset, the relatedness of

siblings will result in correlated data and may, at times, result in a significant Durbin-Watson's statistic. Thus, this statistic was not examined.

Limitations of study design: causation, bias, random error.

It is important to note that due to the observational nature of the study, causation cannot be proven from this study or any gene association study. However, associations can be validated by eliminating alternative explanations (Ashengrau & Seage, 2005), mainly bias, confounding factors, and random error. Bias is eliminated by insuring that no systematic error in subject recruitment exists. Bias due to population stratification (Newton-Cheh & Hirschhorn, 2005) was controlled for by using an ethnically homogeneous population of Caucasians. Replication was attempted in an African American population when available. Confounding factors were controlled for specifically, BMI, age, and gender. Random error, the probability that results are due to chance (Ashengrau & Seage, 2005), was controlled by ensuring the study design has adequate power (0.80) and a strong enough p value (0.05 with appropriate Bonferroni correction) to control for Type I and Type II statistical errors (Munro, 2005).

Meta-analysis.

Meta-analysis is a statistical method that enables scientists to determine an overall effect of one variable on another through the combination of multiple studies analyzing similar hypotheses. In genetics, meta-analysis is often used to clarify discrepancies observed between multiple gene association analyses and overcomes a major flaw of gene association studies: lack of power (Munafo & Flint, 2004). Where appropriate, meta-analysis was used to determine the overall effect of a SNP on a specific phenotype in two different cohorts. Two techniques of meta-analysis were used for the two gene association studies with replication: 1) Fisher's combined p value PPAR γ 2) weighted Z score analysis (CAV1). In the PPAR γ analysis, Fisher's

combined p values were also calculated to determine the overall significance of the observed independent findings (Fisher, 1948). The weighted z-score method of meta-analysis was conducted using the freely available METAL software package (<http://www.sph.umich.edu/csg/abecasis/metal/>). This approach accounts for the direction of association relative to a chosen reference allele and the sample size of each cohort. First, p values from each study are converted to z scores. A weighted sum of z scores is calculated where each statistic is weighted by the square root of the sample size for each study. The resulting sum is divided by the square root of the total sample size to obtain an overall z statistic (Willer, Li, & Abecasis, 2010)

Presentation of Study Results and Discussion.

Results for the analyses from the described study are presented in the following chapters 4 through 6. Each chapter describes the results for an individual gene (chapter 4: CAV1, chapter 5: PPAR γ , chapter 6: AGT). Descriptive tables and figures for the data are represented in the appropriate chapters. A discussion of the findings for each respective gene is detailed at the end of each results chapter.

Chapter 4

Study Results: The Association of Single Nucleotide Polymorphisms of the Caveolin-1 Gene with Insulin Resistance and Vascular Dysfunction in Humans

Group Characteristics.

Three hundred and twenty four Caucasian individuals with hypertension and available CAV1 genotype were analyzed from the HyperPATH Cohort (**Table 5**). The African American hypertensive sample (N=54) had similar blood pressure and cholesterol values compared with the Caucasian group with hypertension. The Caucasian group without hypertension (N=143), used for evaluating the effects of hypertension on any significant results, had lower blood pressure values and a better overall metabolic profile. The replication sample (HTN-IR) demonstrates similar blood pressure and metabolic profile to the Caucasian HyperPATH cohort however; the HTN-IR cohort consists of a higher mean BMI (**Table 5**).

Gene characterization.

Eleven tagging SNPs covering a 36.6 kb region of the CAV1 gene were chosen from the HAPMAP database (The International HAPMAP Consortium, 2005) and analyzed in the HyperPATH cohort (**Table 6**). Tagging SNPs were identified using the CEU population with an R^2 greater than 0.9 and a minor allele frequency (MAF) greater than 10%.

Genotyping was performed using a Sequenom platform. Five SNPs were removed prior to analysis for quality control reasons: rs12668226 was monomorphic, rs2215448 deviated significantly from Hardy-Weinberg equilibrium in both the hypertensive ($p=0.04$) and normotensive ($p=0.0001$) groups and three SNPs (rs959173, rs3815412, and rs729949) were in linkage disequilibrium (LD) with other genotyped variants with an $R^2 > 0.95$ (**Figure 3**). In the replication cohort, SNP rs11773845 is in complete LD with rs3807989 and is used as a surrogate

for rs3807989 since this SNP was not genotyped in the HTN-IR cohort. All genotyped SNPs had a completion rate of greater than 95%. Repeat genotyping for 10% of the SNPs demonstrated concordance with the original genotype call.

Primary phenotype: fasting insulin.

Because CAV1 is known to directly influence the insulin signaling pathway, an additional hypothesis that CAV1's primary effects would be with fasting insulin was devised. Hence, this study analyzed fasting insulin levels as the primary phenotype for the primary CAV1 association analysis. The primary phenotype, fasting insulin, was evaluated for each of the six SNPs. The outcome variable was log transformed to meet the normality assumption of regression. This transformation met normality tests as demonstrated by a non-significant ($p=0.2$) Shapiro Wilk test (Munro, 2005). The total number of individuals analyzed for the fasting insulin analysis (individuals with values for fasting insulin on a high salt diet and CAV1 genotype) was 324.

Using an additive model, rs926198 was found to be significantly associated with fasting insulin levels (TT=7.69 [6.15-9.58] mU/ml, CT=9.02 [7.24-11.25] mU/ml, CC=8.85 [6.82-11.47] mU/ml, $p=0.019$). Based on this result, a dominant model was used for all subsequent analyses (homozygous major allele [TT] vs. minor allele carriers [CT/CC]). Analysis of the 6 SNPs identified significant associations with fasting insulin levels for rs926198 ($p=0.005$) and rs3807989 ($p=0.007$) (**Table 7**). As shown in **Figure 4 part A**, minor allele carriers of rs926198 and rs3807989 had significantly higher fasting insulin levels than individuals homozygous for the respective major allele (**rs926198**: TT=7.69 [6.15-9.58] mU/ml, CT/CC=9.00 [7.23-11.13] mU/ml, $p=0.005$ and **rs3807989**: GG=7.63 [6.10-9.55] mU/ml, AA/AG=8.90 [7.18-11.03]

mU/ml, $p=0.007$). No association was seen for either SNP with fasting glucose levels in the Caucasian-NTN or African American hypertension populations.

These results indicate that the null hypothesis H.01a is rejected and the alternate hypothesis H.1a.01a . (Individuals who are homozygous minor allele carriers for SNPs in CAV1 will be more insulin resistant than heterozygote or homozygous major allele carriers) is accepted. However, these results indicate that all minor allele carriers and not just those homozygous for the minor allele are insulin resistant.

Secondary phenotypes of insulin resistance: HOMA-IR.

Since fasting hyperinsulinemia is a hallmark of insulin resistance (Olesfsky, Farquhar, & Reaven, 1973), this study analyzed whether CAV1 genotypes were associated with measurements of insulin resistance (HOMA-IR, clamp-derived M-value) in the Caucasian HTN cohort. HOMA-IR was naturally log transformed and met a normal distribution (Shapiro Wilk $p=0.2$). Minor allele carriers for both SNPs had significantly higher HOMA-IR values than individuals homozygous for the major allele (**Figure 4 part B**) (mean estimate [95% CI]; **rs926198**: TT= 1.67 [1.42-1.96], CT/CC=1.98 [1.69-2.31] $p=0.005$; **rs3807989**: GG=1.66 [1.42-1.96], AG/AA=1.96 [1.68-2.28] $p=0.008$). No association was seen for either SNP with HOMA-IR in the Caucasian-NTN or African American hypertension populations. Fasting plasma glucose measurements did not significantly differ by genotype for either SNP in any of the populations (data not shown).

Secondary phenotype of insulin resistance: M value of the clamp.

Of the fifteen subjects in the Caucasian-HTN population who underwent the euglycemic hyperinsulinemic clamp, minor allele carriers of both SNPs had significantly lower M values than individuals homozygous for the major allele (mg/kg/min, mean \pm SEM; **rs926198**: TT=

8.35±1.56, CT/CC=5.48± 1.83, p=0.004; **rs3807989**: GG=8.62 ± 2.27, AG/AA=6.17±1.95, p=0.05); consistent with insulin resistance in these individuals (**Figure 4 part C**). The M value for the clamp was normally distributed and thus, was not transformed. Since the clamp study was a pilot study and was not done in the African American sample, replication in a second cohort can not be examined.

Exploratory analysis: effect of BMI on SNP and fasting insulin association.

The effect of BMI on the association of both significant CAV1 SNPs and fasting insulin levels was examined in the Caucasian HTN population. When the cohort was stratified by BMI tertiles (lowest tertile BMI≤25 kg/m², highest tertile BMI≥30 kg/m²), a significant association between genotype and fasting insulin remained only for individuals with the lowest BMI (p=0.03 BMI≤25 kg/m² vs. p=0.08 BMI≥30 kg/m²), suggesting that BMI has an effect on this association

Secondary phenotypes of vascular dysfunction: salt sensitive blood pressure.

Since insulin resistance and salt sensitivity have been frequently associated with one another (Yatabe et al., 2010; Raji, 2001), this study examined the association of CAV1 SNPs with salt sensitive blood pressure. All three salt sensitivity variables were normally distributed. Tests for normality were met using the Shapiro-Wilk test (p=0.1 [systolic blood pressure], p=0.6 [diastolic blood pressure], p=0.3 [mean arterial pressure]). Individuals with complete data for blood pressure measurements on both a high salt diet and low salt diet and CAV1 genotype were included in this analysis. The total number of individuals analyzed was 238 for systolic, diastolic, and mean arterial pressure blood pressure measurements.

Of the six SNPs, rs3807989, demonstrated a non-significant trend for an association with systolic salt sensitive blood pressure (mmHg, mean ± SEM; **rs3807989**: GG=14.7± 2.0,

AG=14.9±1.8 AA=11.4±2.6, p=0.3 additive; p=0.1 recessive model (**Table 8 and Figure 5**).

This association was not replicated in the African American HTN or Caucasian NTN sample (data not shown).

None of the six SNPs demonstrated a significant association with diastolic salt sensitivity (**Table 9**). One SNP, **rs3757732**, demonstrated a non-significant trend with MAP salt sensitive blood pressure (mmHg, mean ± SEM; **rs3757732**: CC=7.05± 2.2, CA=11.2±1.2 AA=10.2±1.0, p=0.6 additive p=0.1 dominant model **Table 10**). Thus, the null hypothesis H.01b. (Individuals who are homozygous minor allele carriers for SNPs in CAV1 will not be more salt sensitive than heterozygote or homozygous major allele carriers) is accepted.

Replication of insulin resistant phenotypes in a Hispanic cohort.

To replicate the findings in an independent hypertensive cohort, this study assessed the association of rs926198 and rs11773845, with fasting insulin levels in a hypertensive, Mexican American Cohort (HTN-IR) (Xiang et al., 2005). Again, minor allele carriers of both SNPs were significantly associated with increased fasting insulin levels (mU/ml; mean estimate [95% CI], **rs926198**: TT= 16.65 [14.97-18.33] CT/CC= 18.09 [15.81-20.38] p=0.005, **rs11773845**: AA= 16.76 [14.85-18.68] AC/CC= 17.48 [15.58-19.38] p=0.02.

Associations between the CAV1 SNPs and HOMA-IR were also tested in the Hispanic HTN-IR cohort and significant associations were found (rs926198 p=0.008; rs11773845 p=0.02). In the HTN-IR cohort, these SNPs manifested trends for reduced M values that did not reach statistical significance.

Regression Diagnostics for Fasting Insulin and HOMA-IR.

Since positive results were found across two cohorts for salt sensitive diastolic blood pressure and low salt PRA levels, regression diagnostics were analyzed to insure the results were

not influenced by outliers, multi-collinear independent variables, or heteroscedasticity. For both models tested, the independent variables (SNP, age, gender, and BMI) had VIF less than 10 indicating that collinearity does not exist. For both models tested, linear relationships existed between the independent variables and the dependent variable as demonstrated by the partial residual plots and correlation plots.

Meta-analysis.

A meta-analysis of the two cohorts (HyperPATH Caucasian HTN and HTN-IR) was carried out for the two most significant phenotypes in the HyperPATH study, fasting insulin and HOMA-IR using the weighted z score approach (Willer, Li, & Abecasis; 2010). As expected, both SNPs demonstrated highly significant associations with increased fasting insulin and increased HOMA-IR measurements using a dominant model (Fasting Insulin: rs926198 [p=0.00008] rs3807989 [p=0.0004]; HOMA-IR: rs926198 [p=0.0001], rs3807989 [p=0.0004]).

Summary of results.

This extensive examination of variants in the CAV1 gene with features of insulin resistance and vascular dysfunction demonstrate a role for CAV1 as a bio-marker for insulin resistance and hypertension in humans. This study found that CAV1 SNPs were associated with two measurements of insulin resistance in a hypertensive population: elevated fasting insulin levels and increased HOMA index. This association was identified first in one hypertensive cohort---Caucasian---and confirmed in an ethnically different cohort---Mexican. Importantly the association of insulin resistance to variants in the CAV1 gene was observed only in hypertensive individuals. While obesity can be an important confounder with insulin resistance, in this study accounting for BMI actually strengthened the phenotype-genotype relationship.

Tables and figures for CAV1.

Table 5. CAV1 Cohort Characteristics. Data represents mean +/- standard deviations.

NTN=normotensive; HTN=Hypertensive; Af. American=African American; HDL=high density lipoprotein; LDL=low density lipoprotein. HTN-IR=Mexican Cohort

Characteristics	POPULATIONS			
	Caucasian NTN	Caucasian HTN	Af.American HTN	HTN-IR
N	143	324	54	192
Age (years)	39.37+/- 11.05	48.44 +/- 8.07	45.82+/-6.98	48.2+/-14.29
Female gender (%)	78(55)	126(39)	29(63)	108(56.25)
Body mass index, kg/m ²	25.17+/- 3.86	28.08+/- 3.99	29.13+/-3.82	30.5+/-5.64
Fasting glucose, mg/dl	84.84+/- 10.68	89.99+/- 10.56	91.09+/-12.09	98.07+/-9.58
Systolic blood pressure, mm Hg	110.36+/- 12.25	145.97+/- 19.68	153.52+/-20.32	138.71+/-22.93
Diastolic blood pressure, mm Hg	66.26+/- 8.11	86.62+/- 11.01	90.35+/-11.26	81.33+/-12.45
Mean arterial pressure, mm Hg	80.96+/- 8.64	106.40+/- 12.97	111.41+/-13.38	100.45+/-14.9
Triglycerides, mg/dl	123.93+/- 93.11	169.11+/- 111.20	101.81+/-46.75	141.29+/-72.87
Total cholesterol, mg/dl	169.22+/- 35.82	197.61+/- 35.82	194.11+/-41.76	187.31+/-32.73
HDL cholesterol, mg/dl	49.29+/- 30.06	39.89+/- 11.94	47.75+/-15.54	47.56+/-13.08
LDL cholesterol, mg/dl	98.01+/- 30.66	123.29+/- 35.06	125.4+/-39.11	111.63+/-29.53

Table 6. Genotyped CAV1 SNPs: SNP location within CAV1 gene. Maj=major allele,

Min=minor allele, MAF=minor allele frequency; HWE= Hardy-Weinberg equilibrium values, p values obtained from chi-square analysis. rs numbers from dbSNP.

11 Genotyped SNPs				
Polymorphism	Location: chr.7	Maj/Min	MAF	HWE p value
rs2215448	115951188	G/A	0.18	.040**
rs926198	115954444	T/C	0.36	0.104
rs1543293	115965784	G/C	0.18	0.576
rs959173	115969290	T/C	0.17	0.453
rs3807989	115973477	G/A	0.40	0.990
rs12668226	115974926	A/C	MONOMORPHIC	
rs3815412	115977929	T/C	0.22	0.370
rs1022436	115980467	C/G	0.16	0.138
rs3757732	115980941 intronic	C/A	0.22	0.359
rs729949	115982141	G/A	0.21	0.303
rs1049337	115987823	C/T	0.30	0.360

Figure 3. Linkage Disequilibrium plot of 11 CAV1 and 1 CAV2 tagging SNPs. Numbers represent R^2 values.

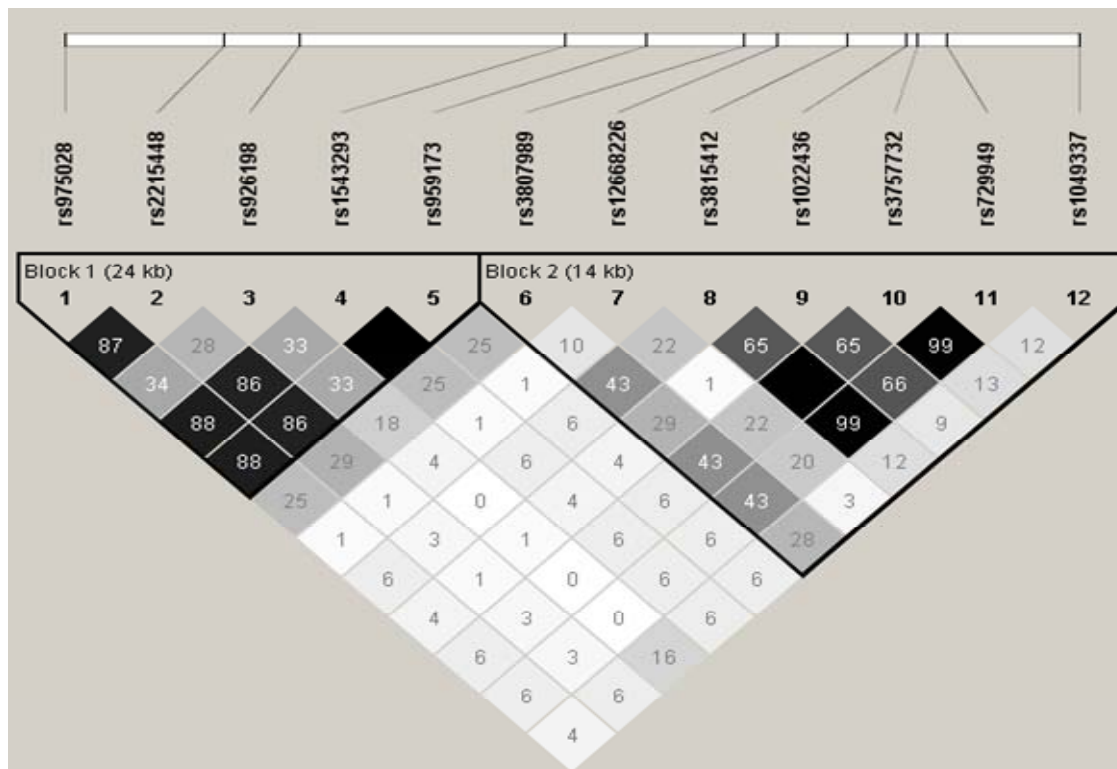


Table 7: Final 6 CAV1 SNPs and their association with fasting insulin (natural log transformed).

MAF=major allele frequency. P-values were obtained from a mixed model regression and accounting for age, gender, study site, and BMI.

6 Genotyped SNPs and Fasting Insulin in HTN			
Polymorphism	Dominant Model	MAF (%)	p value
rs926198	TT vs.CT/TT	36	0.005**
rs1543293	GG vs.CG/CC	17	0.06
rs3807989	GG vs.GA/AA	42	0.007**
rs3757732	CC vs.CA/AA	24	0.400
rs1022436	CC vs.CG/GG	18	0.245
rs1049337	CC vs.CT/TT	30	0.080

Figure 4. CAV1 genotypes associate with fasting insulin (A), HOMA-IR (B) and M-value (C) in the HyperPATH HTN cohort. Point estimates (least-square means), 95%CI, and p values (F test, two sided) were obtained from mixed model regression (A and B). Mean, SD, and p values were obtained from t-test (C).

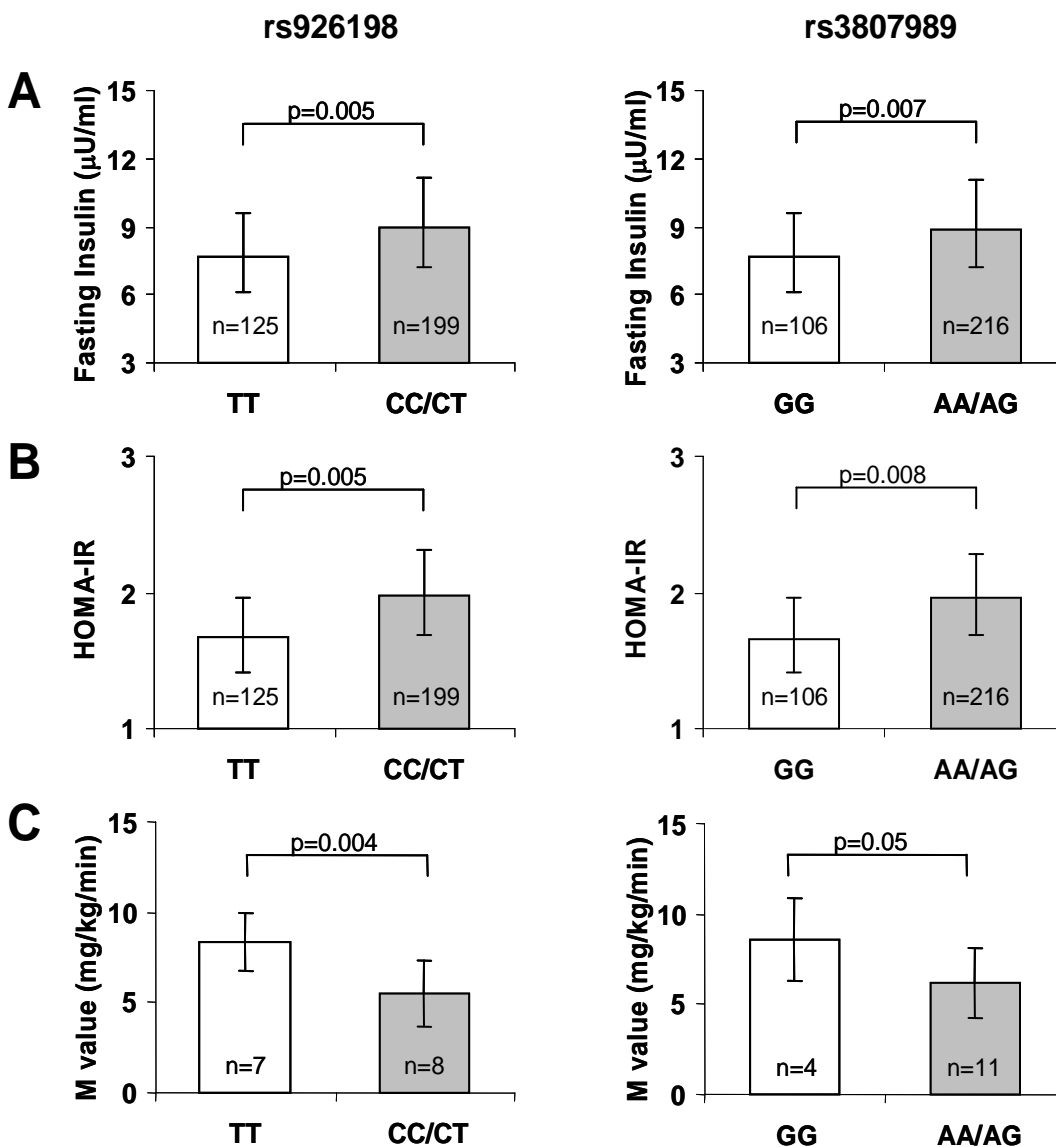


Table 8. The association of 6 CAV1 SNPs with change in Systolic Blood Pressure. P values were obtained from a mixed model linear regression accounting for age, gender, and BMI. Maj=Major allele, Min=minor allele, MAF=major allele frequency.

6 Genotyped SNPs and Systolic Salt Sensitive Blood Pressure in HTN				
Polymorphism	Alleles	MAF (%)	Additive Model p value	Recessive Model p value
rs926198	TT/CT/TT	36	0.3	
rs1543293	GG/CG/CC	17	0.2	
rs3807989	GG/GA/AA	42	0.3	0.1*
rs3757732	CC/CA/AA	24	0.7	
rs1022436	CC/CG/GG	18	0.5	
rs1049337	CC/CT/TT	30	0.7	

*significant if divided by 2 for replication of HTN-IR cohort

Figure 5. Salt sensitive systolic blood pressure and rs3807989. The association of rs3807989 (Recessive Model) with change in systolic blood pressure. P values (p=0.1) were obtained from a mixed model linear regression accounting for age, gender, and BMI. Error bars represent standard deviation.

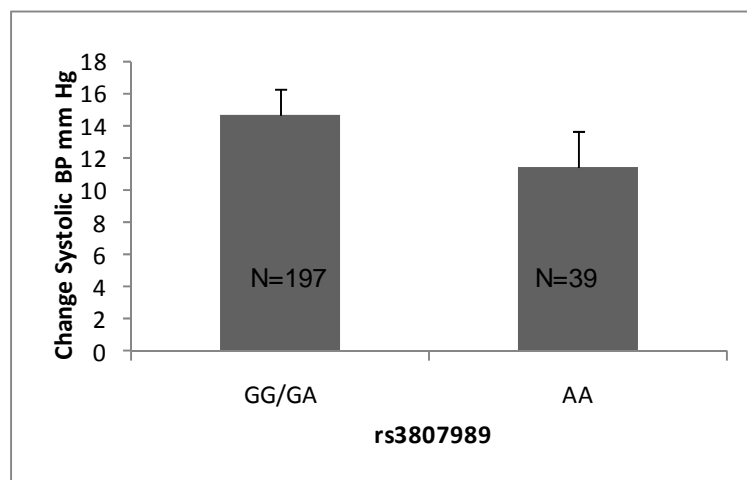


Table 9. The association of 6 CAV1 SNPs with change (High Salt-Low Salt) in Diastolic Blood Pressure. P values were obtained from a mixed model linear regression accounting for age, gender, and BMI. Maj=Major allele, Min=minor allele, MAF=major allele frequency.

6 Genotyped SNPs and Diastolic Salt Sensitive Blood Pressure in HTN			
Polymorphism	Alleles	MAF (%)	Additive Model p value
rs926198	TT/CT/TT	36	0.2
rs1543293	GG/CG/CC	17	0.9
rs3807989	GG/GA/AA	42	0.8
rs3757732	CC/CA/AA	24	0.5
rs1022436	CC/CG/GG	18	0.5
rs1049337	CC/CT/TT	30	0.8

Table 10. The association of 6 CAV1 SNPs with change (HS-LS) in Mean Arterial Blood Pressure. P values were obtained from a mixed model linear regression accounting for age, gender, and BMI. MAF=major allele frequency.

6 Genotyped SNPs and Mean Arterial Salt Sensitive Blood Pressure in HTN			
Polymorphism	Alleles	MAF (%)	Additive Model p value
rs926198	TT/CT/TT	36	0.2
rs1543293	GG/CG/CC	17	0.5
rs3807989	GG/GA/AA	42	0.5
rs3757732	CC/CA/AA	24	0.6
rs1022436	CC/CG/GG	18	0.5
rs1049337	CC/CT/TT	30	0.8

*significant if divided by 2 for replication of HTN-IR cohort

Discussion.

Many gene association studies of insulin resistance have demonstrated inconsistent results (Lusis, Attie, & Reue, 2008). The successful outcome of the present study likely resides in several factors. First, this study used a candidate gene-intermediate phenotype approach. The candidate gene---CAV1---was chosen based on pre-clinical rodent data. The clinical trait was the heritable insulin resistance in hypertensives, i.e., an intermediate phenotype. Second, known environmental factors, including drug therapy, were controlled and/or eliminated, thereby

reducing their confounding effects. Finally, and most importantly, the findings in one population were confirmed in a second population. Thus, these studies identify CAV1 as a genetic marker for metabolic dysfunction and provide insight into a potential mechanism underlying the inter-individual variability of the co-aggregation of insulin resistance and hypertension in humans.

Family studies indicate that the co-occurrence of insulin resistance and hypertension is heritable (Raji, 2001; Xiang, 2001). CAV1 is a known regulator of insulin signaling and insulin receptor stability. Specifically, CAV1 binds directly to the insulin receptor in adipocytes (Cohen et al., 2003) and disruption of this complex (Couet et al., 2001) by ganglioside GM3 (Kabayama et al., 2007) causes altered insulin signaling. Further, depletion of CAV1 results in a ninety percent decrease in adipocyte insulin receptor levels in CAV1 KO mice (Cohen et al., 2003). While the role of CAV1 in insulin mediated glucose uptake is less clear (Cohen et al., 2003), CAV1 has also been shown to be involved in Glut-4 translocation to the plasma membrane in both adipocytes (Karlson et al., 2004) and muscle cells (Oh et al., 2006). It is possible that alterations in the CAV1 gene are affecting one or both of these processes, leading to the hyperinsulinemic state seen in both the human and animal data.

While research in humans is somewhat limited, at least three types of studies support a potential relationship between insulin resistance and CAV1: 1) CAV1 mRNA levels are greater in fat from obese subjects than from lean subjects (Catalan et al., 2008); 2) mutations in the CAV1 gene have been linked to lipodystrophy, a disease of abnormal fat distribution and severe insulin resistance (Cao et al., 2008); and 3) genetic variants within the CAV1 docking domain of the insulin receptor gene cause severe insulin resistance (Cohen et al. 2003; Iwinishi, 1993; Moller et al., 1990). Together, these studies suggest that CAV1 likely is involved in metabolic

regulation and support the findings that CAV1 is a marker for insulin resistance in hypertensive humans.

Of interest, these data demonstrate that the association between CAV1 genotype and fasting insulin levels is strongest in lean individuals. The identification of insulin resistance in lean individuals seems counterintuitive within the current understanding that obesity influences insulin resistance. However, some individuals develop a metabolic phenotype, including insulin resistance and hypertension, without obesity (Wildman et al., 2008; Ferri et al., 1999). These data suggest that alterations in the CAV1 gene may contribute to the development of insulin resistance in lean individuals. Of note, CAV1 KO mice also demonstrate insulin resistance even though they are lean and exhibit resistance to diet induced obesity (Cohen et al., 2003).

Interestingly, SNPs of the CAV1 gene also demonstrated a non-significant trend toward an association with salt sensitivity of blood pressure. Numerous studies link insulin resistance and salt sensitive blood pressure in humans. Raji et al. (2001) demonstrated that the salt sensitive intermediate phenotype of hypertension, non-modulating hypertension, was the most insulin resistant sub-phenotype of hypertension in the HyperPATH cohort. Further, Sharma et al. (2001) found that insulin resistance and salt sensitivity were associated in normotensive individuals and the same relationship was seen in obese hypertensive individuals (Rocchini, 2001). The first CAV1 SNP associated with salt sensitivity is different (rs3757732) than the two SNPs associated with IR, suggesting two different parts of the CAV1 gene are influencing vascular dysfunction and insulin resistance separately. The other SNP (rs3870989) is also associated with IR; however, the minor allele is associated with decreased salt sensitivity and increased insulin resistance suggesting the relationship between CAV1 and these two outcomes is not linked. Alternatively, the CAV1 influence on IR may be protecting individuals from salt sensitivity

through a mechanism currently unknown. Further analyses must be conducted in a larger population to clarify the relationship between the CAV1 gene and salt sensitive hypertension. Importantly, both CAV1 SNP associations with salt sensitivity described were replicated in the HTN-IR cohort (unpublished data).

This study has several limitations. First, CAV1 protein levels were not available to determine whether differences existed by SNP in this human study. Second, the causal alleles at the CAV1 locus remain unknown. The two SNPs identified are located in introns of the CAV1 gene. However, both SNPs are in strong LD ($D' > 0.9$) with CAV1 gene promoter variants (e.g. rs2215448), suggesting that this SNP may be a marker for altered CAV1 gene transcription.

In summary, variants of the CAV1 gene are associated with hyperinsulinemia and insulin resistance in humans with hypertension. These findings have important clinical implications. First, they identify a genetic marker that might aid in identifying individuals at risk for metabolic disease, particularly lean individuals who may not be identified using current risk profiles. Second, this study identifies a novel pathway that contributes to insulin resistance in humans. New therapies targeting this pathway may provide individualized treatment to patients identified to have a defect in the CAV1 gene.

Chapter 5

Results: The Association of Single Nucleotide Polymorphisms of the Peroxisome Proliferator Activated Receptor gamma Gene with Insulin Resistance and Vascular Dysfunction in Humans

Group characteristics.

Three hundred and ninety five Caucasian individuals with hypertension and complete PPAR γ genotype were analyzed from the HyperPATH Cohort (**Table 11**). The group had almost equal numbers of men and women (40% women) and were non-obese (mean BMI=28.25). The African American hypertensive replication sample (N=55) had more females (73%) and had similar blood pressure and cholesterol values compared with the Caucasian group with hypertension. The Caucasian group without hypertension (N=151), used for evaluating the effects of hypertension on any significant results has lower blood pressure values and lower overall cholesterol values.

Gene characterization.

Twenty nine tagging SNPs, identified by the Haploview program, were analyzed in Caucasian individuals with hypertension (The International HAPMAP Consortium, 2005; Barrett, Fry, Maller, & Davy, 2001) (**Figure 6**). Tagging SNPs were identified using the CEU population (HAPMAP Caucasian population) with an R^2 greater than 0.9 and a minor allele frequency (MAF) greater than 10%. The PPAR γ SNP rs1801282 (Pro12Ala) was tagged using SNP rs7649970.

Thirteen SNPs were removed from the analysis since they had an R^2 greater than 0.8 with a second SNP indicating linkage disequilibrium (LD) in this population: rs4684846, rs9817428, rs7620165, rs12636454, rs12493718, rs2067819, rs2881654, 2938395, rs4135263, rs2938392,

rs796290, rs76265806, rs2972162. Four SNPs were removed with a MAF of less than 10 percent: rs3892175, rs12497191, rsrs4135247, rs1175540. One SNP, rs2972164, was removed since it was originally chosen as a tag SNP from the YRI population and is not needed in an analysis of Caucasians. The eleven remaining SNPs captured all 29 SNPs according to the Tagger program of haploview resulting in 100% gene coverage (Barrett, Fry, Maller, & Davy, 2001).

The LD plot for the remaining eleven SNPs is displayed in **Figure 7**. This plot demonstrates that none of the SNPs are in LD with one another indicating duplicate information is not tagged. Quality control metrics are displayed in **Table 12**. All eleven SNPs were in Hardy-Weinberg Equilibrium (HWE) and have MAF greater than 10 percent. All genotyped SNPs had a completion rate of greater than 95%. Repeat genotyping for 10% of the SNPs demonstrated concordance with the original genotype call.

Primary phenotype: insulin resistance.

The primary phenotype, insulin resistance as measured by HOMA-IR, was evaluated for each of the eleven SNPs. The endpoint was log transformed to meet the normality assumption of regression. This transformation met normality tests as demonstrated by a non-significant ($p=0.07$) goodness of fit value for the Cramer-von Mises test (Munro, 2005). The total number of individuals analyzed for the HOMA-IR analysis (individuals with values for HOMA-IR on a high salt diet and PPAR γ genotype) was 338.

None of the eleven SNPs were significantly associated with HOMA-IR levels (**Table 13**) using a mixed effects linear regression accounting for age, gender, BMI, and sibling relatedness. The results demonstrated p values ranging between .2 and .9.

In a subset of the Caucasian hypertensive population (N=15), eleven SNPs were tested for an association with the M value of the hyperinsulinemic euglycemic clamp using an ANOVA with the SNPs categorized in an additive model (1=major allele homozygotes, 2=heterozygotes, 3=minor allele homozygotes). Sibling relatedness was not accounted for since all individuals were unrelated in this subset. Further, BMI, age, and gender were not included as co-variables since these variables did not differ significantly when the population was stratified by SNPs of the PPAR γ gene.

The M value for the clamp was normally distributed and thus, was not transformed. None of the eleven SNPs were significantly associated with insulin sensitivity using an additive model (**Table 14**) however; this analysis demonstrated some trends that were close to significant. When these trends were analyzed further using a dominant or recessive model, significant results were found. Two SNPs, rs2959272 and rs1152003, were significantly associated with insulin sensitivity using a dominant and recessive model respectively. Minor allele carriers for SNP 2952972 had significantly lower M values, and thus were more insulin resistant than individuals homozygous for the major allele (p=0.03). Further, homozygotes for the minor allele of SNP rs1152003 had significantly lower M-Values and were more insulin resistant than major allele carriers (p=0.05). Since the clamp study was a pilot study and was not done in the African American sample, replication in a second cohort cannot be examined.

These results indicated that the null hypothesis H.02a. (Individuals who are homozygous minor allele carriers for SNPs in, PPAR γ will not be more insulin resistant than heterozygote or homozygous major allele carriers), was not rejected.

Secondary phenotypes: blood pressure and components of RAAS.

Salt sensitive systolic, diastolic, and mean arterial blood pressure was measured using the protocol previously described (Methods Section Chapter 3). All three variables were normally distributed. Tests for normality were met using the Shapiro-Wilk test ($p=0.2$ systolic), $p=0.6$ diastolic), $p=0.7$ [mean arterial pressure]). Individuals with complete data for blood pressure measurements on both a high salt diet and low salt diet and PPAR γ genotype were included in this analysis. The total number of individuals analyzed was 245 for systolic and mean arterial pressure blood pressure measurements and 244 for diastolic blood pressure measurements.

Of the eleven SNPs, rs13099634, demonstrated a significant association with systolic salt sensitive blood pressure. Both major allele homozygote and heterozygote individuals demonstrated greater increases in systolic blood pressure in response to salt loading (GG=15.9, GA=17.4, AA=12.6 $p=0.03$ additive model, $p=0.02$ recessive model). This association was not replicated in the African American sample ($p=0.7$).

SNP rs1373641 (also a marker for rs2938395) demonstrated a significant association with diastolic salt sensitivity with homozygote major allele carriers demonstrating the greatest response to salt loading (AA=10 AG=7.7 GG=7.3 $p=0.05$ additive model; dominant $p=0.03$). Further, this association was replicated in the same direction in the African American sample using a dominant model (AA=11.3 GG/AG=4.3 $p=0.02$) (**Figure 8**).

Two SNPs, rs1373641 and rs13099634, were significantly associated with MAP salt sensitive blood pressure. Homozygote major allele carriers for SNP rs1373641 had significantly greater response to salt loading than minor allele carriers (AA=12.1 AG=9.2 GG=8.8, $p=0.04$ additive, $p=0.02$ dominant model). Homozygote major allele carriers for SNP rs13099634 had significantly lower response to salt loading than minor allele carriers (GG=9.1 GA=11.9

AA=11.8, $p=0.05$ additive, $p=0.03$ dominant model). Neither of the results were replicated in the African American sample (rs10510418 $p=0.3$; rs13099634 $p=0.7$).

Components of the RAAS: plasma renin activity (PRA).

Alterations in the RAAS are known to affect renal salt handling and alter vascular functioning (Williams, 1982; Hollenberg, 1984). Therefore, this study assessed the association of the PPAR γ gene with components of the RAAS, specifically PRA and aldosterone, on high and low salt diet. Studying the parameters on a low salt diet provides a context to study the association of PPAR γ and components of the RAAS under RAAS activation.

High salt diet: baseline PRA and aldosterone.

Both baseline PRA and baseline aldosterone were examined on a high salt diet. Three of the 11 SNPs were significantly associated with increased baseline PRA levels on a high salt diet (rs7649970 $p=0.03$ additive; $p=0.02$ dominant; rs1373641 $p=0.03$ additive, $p=0.02$ dominant; rs4135275 $p=0.03$ additive; $p=0.01$ dominant). For all SNPs, individuals that were either homozygous or heterozygote minor allele carriers were associated with increased PRA. However, none of these SNPs were significantly associated with baseline PRA in the African American sample. One of the eleven SNPs (rs3856806) was significantly associated with increased baseline aldosterone levels. Again, minor allele carriers were associated with increased aldosterone levels (GG=4.5, GA=5.5 AA=5.5. $p=0.008$ additive, $p=0.005$ recessive). This result was not replicated in the African American sample ($p=0.6$). Since p values from these associations did not meet significance once adjustments for multiple comparisons were made and further, none of these results were replicated in the second cohort, further investigation of other high salt phenotypes was not done.

Low salt diet: baseline PRA and aldosterone

An examination of the association of the PPAR γ SNPs with PRA and aldosterone on a low salt diet provided different results than those seen in the high salt diet, suggesting that an activated RAAS may influence the association of the PPAR γ gene on PRA and aldosterone levels. Multivariate linear regression demonstrated that rs2959272 genotype was significantly associated with baseline PRA levels (AA=1.55 \pm 1.1 ng/ml/hr, AC=1.66 \pm 1.08 ng/ml/hr, CC=2.19 \pm 1.12 ng/ml/hr; trend p= 0.025) and was consistent with a recessive genetic model. Henceforth, analyses were done with CC as the reference group (AA/AC vs. CC). CC individuals had significantly higher baseline supine PRA levels when compared to the AA/AC group (p=0.016) (**Table 15**). PRA levels were also significantly higher in CC individuals during the upright posture study (p=0.042) (**Table 15**).

As seen with Caucasian cohort, baseline supine PRA levels on a low salt diet were significantly higher in the CC group compared with the AA/AC group (p=0.027) in the African American sample. The upright posture PRA levels were also replicated in the African American cohort (p=0.042).

The minor allele of rs10510419 was associated with lower baseline aldosterone levels on a low salt diet (CC=16.4, CA=13.5 AA=11.0 p=0.02 additive, p=0.03 dominant). However, this result was not significant in the African American sample (p=0.4).

These results indicate that the null hypothesis was rejected and the hypothesis H.2b. (Individuals who are homozygous minor allele carriers for SNPs in the PPAR γ gene will be more salt sensitive than heterozygote or homozygous major allele carriers), was accepted.

Pilot analyses for mechanism

The results for an association between baseline supine PRA and rs2959272 genotype on a low salt diet were consistent across two populations demonstrating that individuals homozygous for the minor allele have higher baseline PRA levels (**Table 15**). Replication of the initial findings decrease the likelihood that the original findings are the result of a Type I statistical error (Munro, 2005) Since PRA levels can be affected by variables other than genotype, mechanistic studies were conducted to determine if influencing factors differed by genotype.

Delta PRA

PRA levels fall in proportion to baseline levels after AngII infusion (Seely et al., 1989). Thus, delta PRA levels were analyzed by rs2959272 to determine the PRA response to AngII infusion in relation to baseline PRA levels. PRA data after AngII infusion was available for 311 Caucasian individuals with hypertension and 47 African Americans with hypertension. While PRA levels after AngII infusion was significantly associated with rs2959272 genotype in both the Caucasian ($p=0.025$) and African American cohorts($p=0.012$), delta PRA levels did not differ between genotype in either group (**Table 15**).

BP and heart rate

Since PRA is known to be affected by both BP (Williams, 1982) and sympathetic nervous system (SNS) activity, this study examined the relationship of rs2959272 with BP and heart rate (HR). CC individuals had significantly higher SBP in the African American cohort ($p=0.004$), however, in Caucasian hypertensives, SBP tended to be lower, though this result was not significant ($p=0.097$) (**Table 15**). There was no significant difference in HR by genotype in both cohorts.

Aldosterone

Since aldosterone and PRA are known to be correlated (Williams, 1982), aldosterone concentrations were analyzed by genotype. Unadjusted analysis of aldosterone concentrations were significantly higher in CC carriers ($p=0.04$) in Caucasian hypertensives. However, after including the covariates age, gender and BMI, statistical significance was reduced ($p=0.08$) (**Table 15**). No association was observed between rs2959272 genotype and aldosterone levels in the African American population ($p=0.35$).

Regression Diagnostics.

Since positive results were found across two cohorts for salt sensitive diastolic blood pressure and low salt PRA levels, regression diagnostics were analyzed to insure the results were not influenced by outliers or multi-collinear independent variables. For both models tested, the independent variables (SNP, age, gender, and BMI) had VIF less than 10 indicating that collinearity does not exist. For both models tested, linear relationships existed between the independent variables and the dependent variable as demonstrated by the partial residual plots and correlation plots.

Meta-analysis.

A meta-analysis of the two cohorts (HyperPATH Caucasian HTN and HyperPATH African American HTN) was carried out for the primary significant phenotype in the HyperPATH study, elevated PRA levels using the Fishers' combined p value approach (Fisher, 1948). As expected, rs2959272 demonstrated highly significant associations with elevated PRA using a recessive model (Supine PRA: rs2959272 $p=0.002$; Posture PRA $p=0.02$).

Summary of Results

In summary, a complete examination of the PPAR γ gene with measurements of insulin resistance and component of the RAAS was conducted. Analysis of the PPAR γ gene demonstrated significant associations with 2 SNPs and decreased M values (insulin resistance) of the clamp in the Caucasian hypertensive population. However, the p values from this analysis do not withstand correction for multiple testing and the results were not replicated in a second population. Further, no significant associations were found between SNPs of the PPAR γ gene and HOMA-IR suggesting that in the hypertensive population of the HyperPATH cohort the PPAR γ gene is not a marker for insulin resistance.

Conversely, the examination of the PPAR γ gene with vascular dysfunction and components of the RAAS indicates that SNPs within the PPAR γ gene may be a marker for salt sensitivity and altered renal functioning on a low salt diet in individuals with hypertension. SNP rs1373641 was significantly associated with diastolic salt sensitive blood pressure in both the Caucasian and African American population. A second SNP, rs2959272, was associated with low salt and posture PRA levels in both Caucasian and African American hypertensive populations. These findings support a relationship between the PPAR γ and vascular dysfunction in a hypertensive population.

Tables and figures.

Table 11. Cohort Characteristics. Data represents mean +/- standard deviations (Underwood, Sun, Williams, Pojoga, Chamarthi, Lasky-Su et al., 2010).

Characteristics HS Diet	Caucasian Normotensive (n=151)	Caucasian Hypertensive (n=395)	African American Hypertensive (n=55)
Age, years	39.4±11.5	48.20± 9.06	46.24 ± 8.12
Female gender (%)	75(50)	157 (39.75)	40 (72.73)
Body mass index (kg/m ²)	25.2±4.0	28.25 ± 4.0	29.2 ± 4.1
Systolic blood pressure (mm Hg)	110.9±13.6	146.3± 19.6	152.2± 20.5
Diastolic blood pressure (mm Hg)	66.2±8.4	87.7± 11.7	88.6± 10.9
Mean arterial pressure (mm Hg)	81.1±9.4	107.2± 13.2	109.8± 13.2
Baseline Triglycerides (mg/dl)	114.2±72.6	167.4± 119.8	92.5± 27.9
Baseline Total Cholesterol (mg/dl)	165.6±32.5	201.6± 40.1	191.3± 33.6
Baseline HDL (mg/dl)	47.0±17.3	40.8± 12.8	49.3± 5.5
Baseline LDL (mg/dl)	97.2±28.3	123.6± 36.3	126.5± 36.3
Baseline PRA (ng/ml/hr)	.46±.44	0.64± .9	.3± .3
Baseline Aldosterone (ng/dl)	3.9±2.8	5.7± 4.2	3.4± 3.6

Figure 6: Linkage Disequilibrium plot of 29 PPAR γ tagging SNPs. Numbers represent R² values.

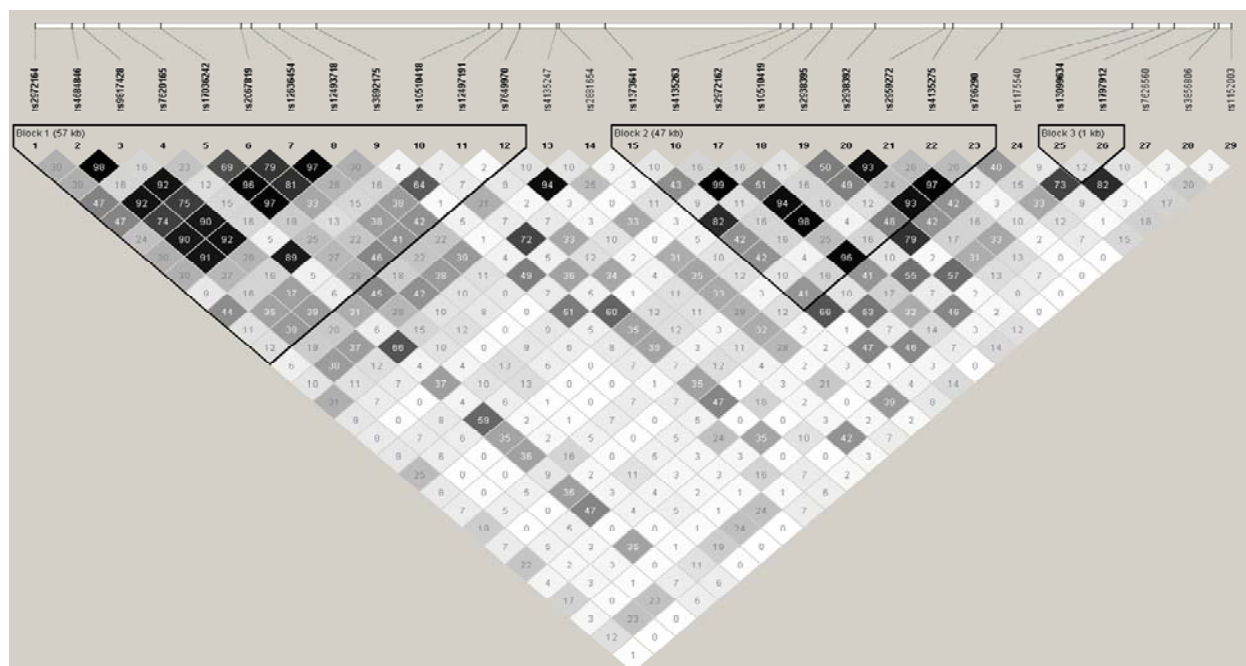


Figure 7: Linkage Disequilibrium plot of 11 PPAR γ tagging SNPs. Numbers represent R^2 values. SNPs with R^2 values greater than 0.8 have been removed.

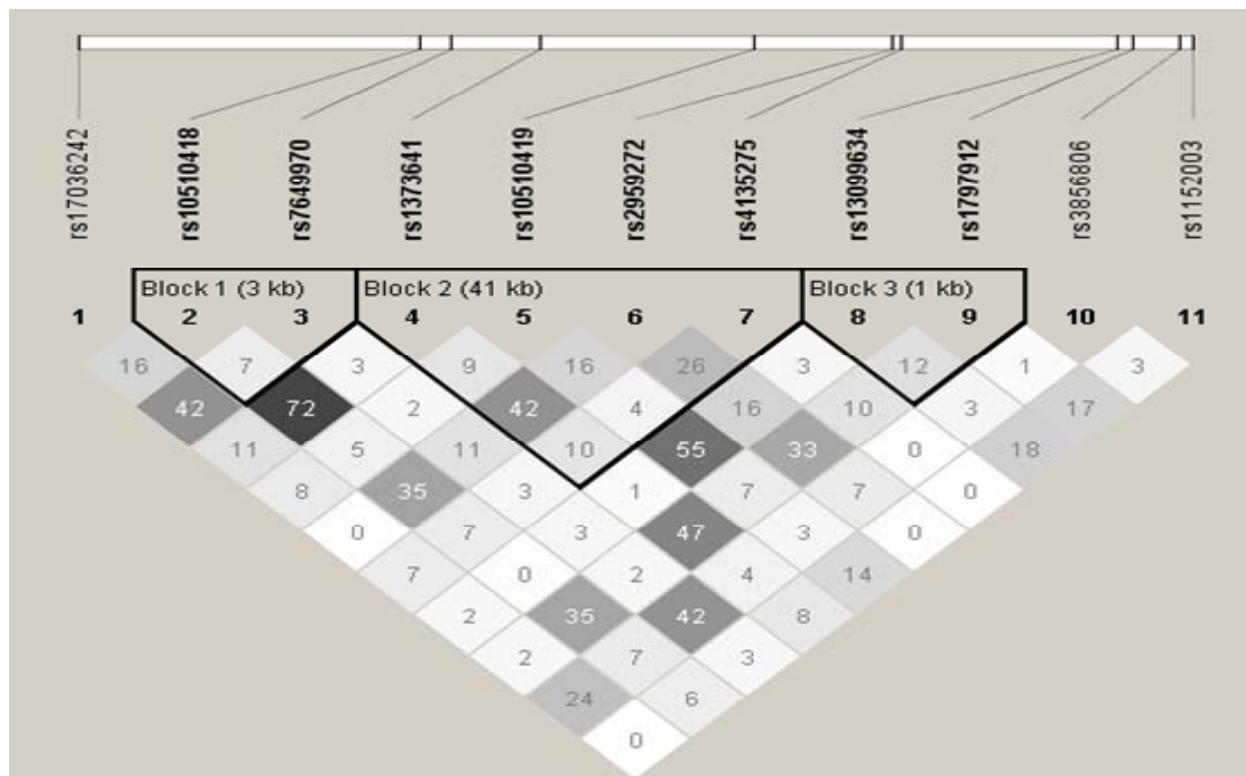


Table 12: SNP location within PPAR γ gene. Maj=major allele, Min=minor allele, HWE=Hardy-Weinberg equilibrium values, p values obtained from chi-square analysis.

Polymorphism	Location	Maj/Min	MAF	HWE p value
rs17036242	intron 12324490	G/A	0.26	0.983
rs10510418	intron 12363563	A/C	0.32	0.933
rs7649970	intron 12367272	G/A	0.13	0.648
rs1373641	intron 12377474	A/G	0.34	0.928
rs10510419	intron 12401936	C/A	0.16	0.877
rs2959272	intron 12417833	A/C	0.48	0.679
rs4135275	intron 12418844	A/G	0.18	1
rs13099634	intron 12443463	G/A	0.19	0.357
rs1797912	intron 12445239	A/C	0.35	1
rs3856806	coding- synonymous 12450557	G/A	0.14	0.243
rs1152003	intron 12452055	C/G	0.35	1

Table 13. 11 PPAR γ SNPs and their association with HOMA-IR (natural log transformed). P-values were obtained from a mixed model regression and accounting for age, gender, and BMI.

Polymorphism	HS HOMA-IR HS p value Additive Model p value
rs17036242	0.9
rs10510418	0.4
rs7649970	0.5
rs1373641	0.7
rs10510419	0.5
rs2959272	0.6
rs4135275	0.9
rs13099634	0.7
rs1797912	0.8
rs3856806	0.9
rs1152003	0.2

Table 14. 11 PPAR γ SNPs and their association with the glucose infusion rate (**M value**) of the clamp (natural log transformed). P-values were obtained from an ANOVA analysis for the additive model and a student's t-test for the dominant and recessive genetic models.

Polymorphism	p value Additive Model	p value Dominant Model	p value Recessive Model
rs17036242	0.9		
rs10510418	0.5		
rs7649970	0.9		
rs1373641	0.5		
rs10510419	0.6		
rs2959272	0.3	0.03*	
rs4135275	0.7		
rs13099634	0.1		
rs1797912	0.3		
rs3856806	0.6		
rs1152003	0.2		0.05*

Figure 8. The association of PPAR γ rs1373641 with delta Diastolic Blood Pressure: the Caucasian-HTN and African American cohort are represented. p-values were obtained from a mixed model linear regression accounting for age, gender, and BMI.

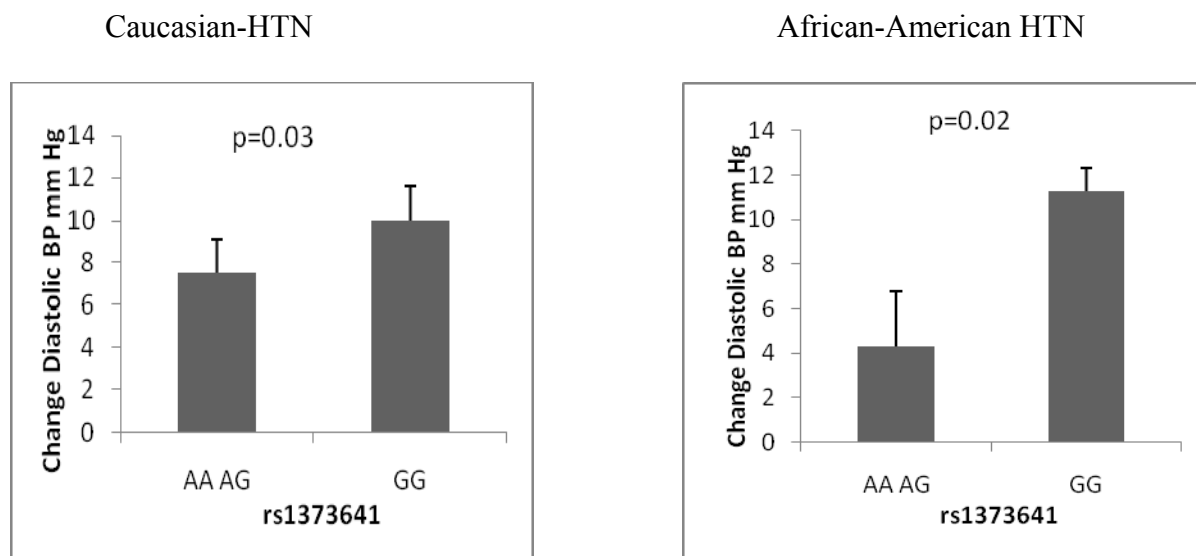


Table 15. Baseline supine PRA obtained during low salt diet, differed by SNP rs2959272 in both the Caucasian and African American populations. A second environment, upright posture PRA, demonstrated similar results. Fisher's combined p value was significant for both tests (p=0.002 baseline supine PRA, p=0.02 upright posture PRA). Delta PRA, baseline supine aldosterone, and baseline heart rate did not differ by genotype. SBP did significantly differ by genotype in the African American population. Point estimates (least square means), 95%CI, and p-values were obtained from a mixed model regression (Underwood et al., 2010).

	Caucasian Hypertensives					Af. American Hypertensives				
	N	Mean Estimate	LCI	UCI	p value	N	Mean Estimate	LCI	UCI	p value
Baseline Supine PRA										
AA/AC	280	1.617	1.44	1.82		34	0.886	0.6	1.31	
CC	75	2.189	1.75	2.73	0.016*	18	1.653	0.95	2.86	0.027*
Upright Posture PRA										
AA/AC	280	4.78	4.1	5.58		34	2.31	1.36	3.94	
CC	75	6.18	4.83	7.9	0.042*	18	4.09	2.1	7.94	0.042*
Delta PRA										
AA/AC	241	-0.849	-1.085	-0.609		31	-0.522	-0.738	-0.306	
CC	70	-1.126	-1.45	-0.799	0.06	16	-0.531	-0.838	-0.223	0.468
Systolic Blood Pressure										
AA/AC	253	131	123.5	136.16		29	130	124.1	135.9	
CC	65	127	118.8	136.16	0.097	17	143	136	151.3	0.004*
Baseline Supine Aldosterone										
AA/AC	280	14.82	13.03	17.22	0.086	34	13.33	9.14	19.49	0.353
CC	75	16.86	13.97	20.36		18	11.66	8.28	16.42	
Baseline Heart Rate										
AA/AC	280	65	64	66	0.29	34	65	63	77	0.20
CC	75	66	64	68		18	70	59	71	

Discussion.

This study demonstrates a relationship between the PPAR γ gene and two phenotypes in two hypertensive populations: 1) salt sensitive diastolic blood pressure and 2) low salt baseline PRA levels. The PRA association was consistent in two environments: a) supine baseline and b) upright posture. Pilot mechanistic studies established that delta PRA, baseline systolic blood pressure, heart rate (a potential surrogate for adrenergic activity), and aldosterone measurements

did not differ by rs2959272 genotype and were not affecting the PRA results. It is possible that the increase in renin is directly related to the PPAR γ genotype. Evidence from both animal and human studies supports a relationship between renin and PPAR γ in the kidney (Todorov et al., 2007; Duan et al., 2003; Zanchi et al., 2004; Hansen et al., 2006).

What effect might the potential relationship between the PPAR γ gene, renin, and edema have on the process of volume retention seen with PPAR γ agonist use? First, renin via AngII, can modify sodium handling first by a direct effect (mainly on the proximal renal tubule) and second by indirect effects via modification of renal blood flow and aldosterone secretion (Seely et al., 1989; Hollenberg, 1984). Also, PPAR γ agonists can directly increase ^{22}Na flux in cultured collecting duct cells, activating the epithelial sodium channel (ENAC), and inducing fluid retention in mice (Guan et al., 2005). Thus, salt retention could be regulated by direct actions of PPAR γ on ENAC and indirectly through PPAR γ 's direct action on renin that leads to increases in aldosterone secretion.

The relationship between PPAR γ and volume retention may also be involved with the association found between the PPAR γ gene and increased diastolic blood pressure in response to changes in dietary salt (salt sensitivity). This is not the first association of the PPAR γ gene with blood pressure. PPAR γ knockout mice demonstrate severe lipodystrophy, insulin resistance, and a decrease in blood pressure (Duan, 2007). Further, PPAR γ partial-agonists (Telmisartan) and PPAR γ agonists (rosiglitazone) have been found to decrease blood pressure and insulin resistance in individuals with hypertension (Sanchez et al., 2008; Raji et al., 2003). These studies suggest an influence of PPAR γ on both glucose regulation and vascular dysfunction in human hypertension. PPAR γ has also been implicated in the development of salt sensitivity in a

hypertensive population (Zanchi, 2010). In this study a SNP of the PPAR γ gene was associated with increased diastolic blood pressure in response to dietary salt. As proposed above, alterations in the PPAR γ gene may cause altered renal functioning and increased volume retention. Altered renal responses may also result in inappropriate responses to salt loading manifested as salt sensitivity. The PPAR γ SNPs found to be significantly associated with baseline PRA and salt sensitivity are in slight LD with one another ($R^2=0.42$), supporting a relationship between the two phenotypes.

This analysis found no significant association between the PPAR γ gene and measurements of insulin resistance. This is in stark contrast to many epidemiologic studies that have found an association of this gene with T2DM (Altshuler, 2000). It is possible that the effect of the PPAR γ gene on glucose metabolism is small and significant results may be found with a larger sample size. Of note, the PPAR γ SNP most well known for its association with T2DM (Pro12Ala) has a small minor allele frequency (0.12 in the Caucasian population of HAPMAP) and this sample had only two homozygote minor allele carriers for this SNP. Thus, lack of power may explain the non-significant association seen in this population. Alternatively, few studies have analyzed this well known association in a hypertensive population and it is possible that this gene is not associated with glucose metabolism in this population.

This study has several limitations. First, edema incidence with PPAR γ agonist use was unavailable to assess whether rs2959272 genotype can predict increased volume retention. Second, p values do not withstand a correction for 11 SNPs. However, since replication was demonstrated, it is unlikely the findings are the result of a type I error. Third, without access to

renal or endothelial tissue it is impossible to determine the effects of the intronic SNP rs2959272 or rs1373641 on kidney or endothelial PPAR γ levels.

In summary, this study demonstrates that CC carriers of SNP rs2959272 in the PPAR γ gene have significantly higher PRA levels than AA /AC carriers. This association provides insight into a possible mechanism for the inter-individual variability of volume retention with PPAR γ agonist use. Second, this study demonstrates that GG carriers of rs1373641 have increased diastolic blood pressure response to salt loading. This association highlights the PPAR γ gene as a marker for vascular dysfunction and may help explain inter-individual variability between the association of PPAR γ agonist use and increased cardiovascular risk seen in some, but not all, individuals (Graham et al., 2010).

Chapter 6

Results: The Association of Single Nucleotide Polymorphisms of the Angiotensinogen Gene with Insulin Resistance and Vascular Dysfunction in Humans: A SNP and Haplotype

Analysis

Population characteristics.

Population characteristics are summarized in **Table 16**. Individuals with hypertension have higher blood pressure and a worse metabolic profile than individuals without hypertension.

Gene characterization.

Sixteen tagging SNPs were identified from HapMap (Phase II, November 2008) using the chromosomal co-ordinates chr1:228,904,892-228,916,564 and including 5 kb flanking regions. Sixteen SNPs captured 100% of the common HapMap Caucasian variation in this region defined as minor allele frequencies >0.1 at $R^2 > 0.9$. SNP rs2493134 was used as a surrogate for the well known AGT SNP M235T (rs699) ($R^2=1$) (The International HAPMAP Consortium, 2005). All genotyped SNPs had a completion rate of greater than 95%. All SNPs conformed to Hardy-Weinberg expectations (HWE) in the study population. Further, SNP allele frequencies did not differ by site ($p > 0.05$ for all SNPs tested via chi-square analysis). Repeat genotyping for 10% of the SNPs demonstrated concordance with the original genotype call.

Primary phenotype: insulin resistance.

Sixteen SNPs were genotyped (**Table 17**). Three SNPs were removed prior to the start of analyses due to monomorphism in the population (rs11568045, rs11568026) and a MAF less than 0.1 (rs11122576), resulting in 13 SNPs. Ten of the SNPs were in linkage disequilibrium

(LD) ($R^2 > 0.80$) with other SNPs (**Figure 15**). Nine SNPs were significantly ($p=0.0004-0.02$) associated with lower HOMA-IR and therefore, insulin sensitivity (**Table 17**).

Association of rs2493134 with HOMA-IR, fasting insulin, fasting glucose levels

This study used rs2493134 for further analyses, because it is the most significant and is in complete LD ($R^2=1$) with the non-synonymous mutation AGT M235T. **Table 18** demonstrates an association of rs2493134 with HOMA-IR (untransformed estimates TT=2.21 [1.9-2.6] CT=1.80[1.6-2.2] CC =1.65 [1.4-2], $p=0.0004$ and fasting insulin levels (TT=9.97 [7.7-12.89] mU/ml CT=8.51 [6.6-10.95] mU/ml CC= 7.67 [5.9-10.01] mU/ml $p=0.0005$ accounting for age, gender, BMI, and study site in the Caucasian hypertensive population). No significant association was seen between rs2943134 and fasting glucose levels ($p=0.3$). Fasting insulin, HOMA-IR, and fasting glucose were all natural log transformed to meet normality assumptions.

Further, there was no significant association between rs2493134 and HOMA-IR or fasting insulin in the African American hypertension population. In the Caucasian normotensive population, no significant associations were found between rs2493134 and fasting insulin, HOMA-IR, or fasting glucose however, a similar trend in mean estimates (lower HOMA-IR and fasting insulin) were seen for the minor allele. It is likely that with a larger sample size, the SNP association would become significant.

Association of rs2493134 with glucose infusion rate of the euglycemic

hyperinsulinemic clamp

Of the ten subjects genotyped for the AGT gene in the Caucasian-HTN population who underwent the euglycemic hyperinsulinemic clamp, no significant differences existed for the M value of the clamp by rs2493134 genotype (mg/kg/min, mean \pm SD; TT= 6.8 \pm 1.5, CT/CC=6.9 \pm 2.5, $p=0.9$). The M value for the clamp was normally distributed and thus, was not transformed.

Since the clamp study was a pilot study and was not done in the African American sample, replication in a second cohort cannot be examined.

The results thus far indicate that the null hypothesis H.03a. (Individuals who are homozygous minor allele carriers for SNPs in AGT gene will not be more insulin resistant than heterozygote or homozygous major allele carriers) was rejected, and an alternate hypothesis (individuals who are homozygous minor allele carriers for SNPs in AGT gene will be more insulin sensitive than heterozygote or homozygous major allele carriers) was accepted.

Exploratory analysis: covariates known to influence AGT genotype: gender and BMI.

Since both gender (Tsai et al., 2009) and obesity (Hopkins et al., 1996) are known to interact with SNPs of the AGT gene, the influence of these covariates on the association between rs2493134 and HOMA-IR was investigated. Although the multivariate analysis demonstrated that a significant portion of the variance of HOMA-IR was accounted for by gender with men having higher HOMA-IR values ($p=0.00001$), the interaction between SNP rs2493134 and gender was not significant ($p=0.9$) indicating that the SNP's association with HOMA-IR was not influenced by gender.

In contrast, the analysis of the effects of BMI on the association of rs2493134 and HOMA-IR suggested that BMI may be moderating the results. An interaction between BMI as a continuous variable and SNP was not significant ($p=0.6$). However, when the population was stratified by obesity status (normal: $BMI < 25 \text{ kg/m}^2$, overweight: $BMI 25-29 \text{ kg/m}^2$, obese: $BMI \geq 30 \text{ kg/m}^2$) (National Institutes of Health, 1998), an interaction was close to significant between the obese group and rs2493134 ($p=0.15$ additive SNP model; $p=0.06$ dominant SNP model). Further, SNP rs2493134 exhibited a greater beta estimate ($\beta = -0.20$ $p=0.01$) for the

regression model tested in the obese group compared to the beta estimates for the same SNP tested in normal and overweight individuals (beta=- 0.19 p=0.06, beta=-.10 p=0.06) (**Table19**).

Haplotype analysis.

The SNP LD plot from this hypertensive population indicated that three haplotype blocks existed. **Table 20** displays all three haplotype blocks and each block's association with HOMA-IR. Haplotype rs7079C|rs3789670C|rs3789671G|rs2478545C in block 1 and haplotypes rs6687360T|rs11122576A|rs2004776A and rs6687360C|rs11122576A|rs2004776G in block 2 are significantly associated with HOMA-IR (p=0.05, beta=0.099; p=0.014, beta=-0.201; p=0.008, beta=0.1564). The association of haplotype block 3 with HOMA-IR is significant only when individuals carry the major allele (**T**) for SNP rs2493134 (p=0.0009 unadjusted; p=0.002 adjusted for age, gender, and BMI). Further, it important to note that both rs6687360 and rs11122476 of block 2 are in strong LD with rs2493134 of block 3, suggesting rs2493134 is likely influencing the results of haplotype 2.

Secondary phenotypes: salt sensitive blood pressure.

All three salt sensitivity variables were normally distributed. Tests for normality were met using the Shapiro-Wilk test (p=0.4 [systolic blood pressure], p=0.7 [diastolic blood pressure], p=0.2 [mean arterial pressure]. Individuals with complete data for blood pressure measurements on both a high salt diet and low salt diet and AGT genotype were included in this analysis. The total number of individuals analyzed was 237 for systolic and 236 for diastolic and mean arterial pressure blood pressure measurements.

Of the thirteen SNPs, no significant associations between SNP and systolic, diastolic, or mean arterial salt sensitive blood pressure were seen (**Table 21, 22, 23**). Therefore, the null hypothesis, H.03a (Individuals who are homozygous minor allele carriers for SNPs in AGT gene

will not be more salt sensitive than heterozygote or homozygous major allele carriers), was not rejected.

Regression Diagnostics.

Since positive results were found for HOMA-IR values, regression diagnostics were analyzed to insure the results were not influenced by outliers or multi-collinear independent variables. The independent variables (SNP, age, gender, and BMI) had VIF less than 10 indicating that collinearity does not exist. Further, linear relationships existed between the independent variables and the dependent variable as demonstrated by the partial residual plots and correlation plots.

Summary of results.

This study demonstrates a significant association between SNPs of the AGT gene and insulin sensitivity in a Caucasian population. This relationship is robust as evident by the numerous significant associations even after multiple comparison adjustment. The current study also demonstrates an association of AGT haplotypes, specifically rs2493134T|rs3789678C|rs5050T|rs2493137T|, with HOMA-IR. This haplotype is driven by the major allele of rs2493134 (a proxy for AGT M235T) furthering the role of this SNP in mechanisms of insulin sensitivity.

Table and figures.

Table 16. AGT Cohort Characteristics. Data represents mean +/- standard deviations.

NTN=normotensive; HTN=Hypertensive; Af. American=African American; HDL=high density lipoprotein; LDL=low density lipoprotein.

POPULATION CHARACTERISTICS				
	Caucasian NTN	Caucasian HTN	Af. American	HTN
	N	132	317	44
age (years)	39.1±11.1	48.6± 8.06	46.5±7.2	
Femal Gender (%)	65(49.3%)	130(41%)	32(72.7%)	
BMI (kg/m ²)	25.1±3.8	28.05±3.8	29.5±3.9	
Fasting Glucose(mg/dl)	85.3±10.7	90.8±11.2	88.2±12	
Fasting Insulin(mg/dl)	10.4±4.9	9.8±5.7	11.2±6.7	
Baseline Systolic Blood Pressure (mm Hg)	109.5±11	145.6±20.2	154.1±21.4	
Baseline Diastolic Blood Pressure (mm Hg)	65.7±8.1	86.5±11.2	89.7±11.5	
Baseline Map Blood Pressure (mm Hg)	80.3±8.4	106.2±13.3	111.2±13.8	
HDL cholesterol (mg/dl)	47.1±18.3	40.5±12.7	50.1±16.2	
LDL cholesterol (mg/dl)	96.2±32.8	123.5±36.4	122.9±36.1	
Total cholesterol(mg/dl)	165.5±32.8	198.5±36.2	189.5±37.3	
Triglycerides (mg/dl)	150.5±73.8	164.9±111	93.1±31.2	

Figure 9. Linkage Disequilibrium plot of tagging SNPs in Caucasian HTN population.

Numbers represent R^2 values.

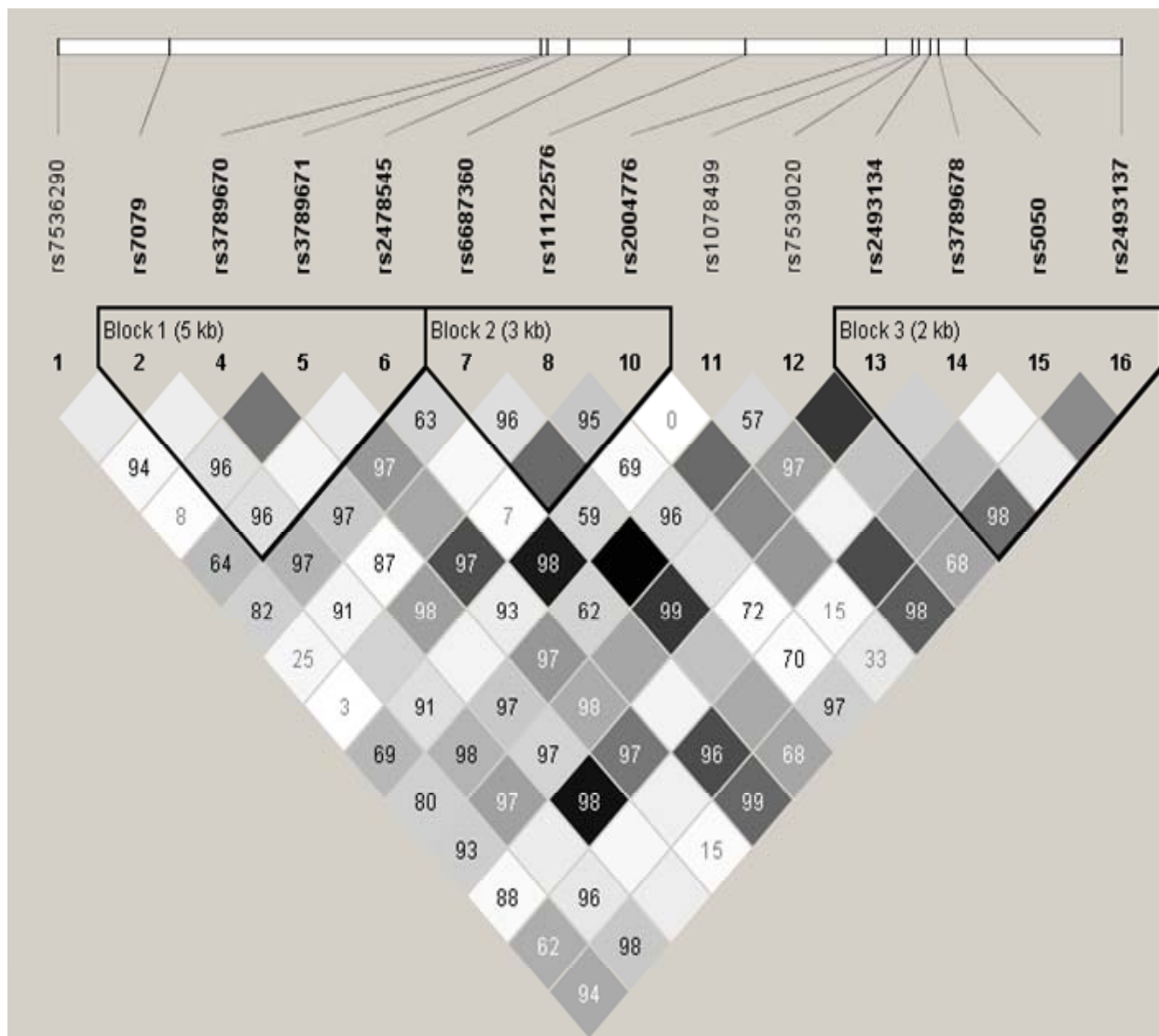


Table 17. AGT SNPs, their Hardy-Weinberg p values, and their association with HOMA-IR (natural log transformed). MAF=major allele frequency. NTN=normotensive population. P-values were obtained from a mixed model regression and accounting for age, gender, study site, sibling relatedness, and BMI.

Name	Alleles	Hardy Weignberg Equilibrium p value	HS LOG HOMA-IR P VALUES (ADDITIVE MODEL)
rs7536290	A:G	0.22	0.1
rs7079	C:A	0.02 (0.3 NTN)	0.7
rs11568045	A:A	monomorphic	monomorphic
rs3789670	G:A	0.03 (.81 NTN)	0.09
rs3789671	C:A	0.83	0.02*
rs2478545	G:A	0.25	0.008*
rs6687360	G:A	0.52	0.0009*
rs11122576	A:G	0.3	maf<0.10
rs11568026	A:A	monomorphic	monomorphic
rs2004776	G:A	0.8	0.02*
rs1078499	A:G	0.5	0.01*
rs7539020	G:A	0.5	0.001*
rs2493134	T:C	0.9	0.0004*
rs3789678	G:A	0.02 (NTN)	0.6
rs5050	A:C	0.2	0.028*
rs2493137	A:G	0.5	0.007*

Table 18. rs2493134 and Fasting Insulin; Fasting Glucose, and HOMA-IR in Caucasian-HTN population. Mean estimates (log transformed) and P-values were obtained from a mixed model regression and accounting for age, gender, study site, and BMI. SE=standard error, LCI=lower 95% confidence interval; UCI=Upper 95% confidence interval; N=sample size.

HS ln insulin						
rs2493134	N	estimate	SE	LCI	UCI	P trend
TT	99	2.2999	0.1304	2.0433	2.5566	0.0005*
TC	153	2.1411	0.1281	1.8889	2.3932	
CC	65	2.0374	0.1352	1.7712	2.3035	
HS ln homa						
rs2493134	N	estimate	SE	LCI	UCI	P trend
TT	99	0.7908	0.09033	0.613	0.9686	0.0004*
TC	153	0.61	0.08558	0.4416	0.7784	
CC	65	0.5011	0.0977	0.3088	0.6933	
HS ln glucose						
rs2493134	N	estimate	SE	LCI	UCI	P trend
TT	99	4.5	0.05	4.4	4.6	0.1
TC	153	4.5	0.05	4.4	4.6	
CC	65	4.5	0.05	4.4	4.6	

Table 19. SNP and HOMA-IR associations in Hypertensive Population Stratified by Obesity Status. LCI: lower confidence interval; UCI: upper confidence interval. Point estimates, 95% CI, beta and p values were obtained from a mixed model regression.

SNP and HOMA-IR Association Stratified by Obesity Status						
Normal BMI<25						
rs2493134	N	HOMA estimates	LCI	UCI	Beta	p value
TT	23	1.67	1.14	2.46	-0.19	0.06
TC	35	1.38	0.96	1.97		
CC	12	1.15	0.73	1.67		
Overweight BMI 25-29						
rs2493134	N	HOMA estimates	LCI	UCI	Beta	p value
TT	41	2.01	1.70	2.36	-0.10	0.06
TC	74	1.73	1.51	2.01		
CC	29	1.68	1.39	2.05		
Obese BMI>=30						
rs2493134	N	HOMA estimates	LCI	UCI	Beta	p value
TT	35	2.89	2.41	3.46	-0.20	0.01
TC	44	2.25	1.92	2.64		
CC	24	1.95	1.55	2.44		

Table 20. Haplotype analyses for association with HOMA-IR in individuals with hypertension.

BLOCK1	HAPLOTYPE	BETA	P VALUE	FREQUENCY
	CCGT	-0.8844	0.15	0.21
	CTTC	-0.1488	0.06	0.11
	CCTC	-0.0244	0.77	0.08
	ACGT	0.02343	0.64	0.32
	CCGC	0.09857	0.05*	0.28
	rs7079 rs3789670 rs3789671 rs2478545			
BLOCK2	HAPLOTYPE	BETA	P VALUE	FREQUENCY
	TGA	-0.098	0.35	0.09
	TAA	-0.201	0.014*	0.20
	TAG	-0.038	0.7	0.11
	CAG	0.1564	0.008*	0.60
	rs6687360 rs11122576 rs2004776			
BLOCK3	HAPLOTYPE	BETA	P VALUE	FREQUENCY
	CCGC	-0.15	0.09	0.17
	CCTC	-0.13	0.14	0.15
	CTTT	-0.15	0.11	0.14
	TCTT	0.2	0.0009**	0.54
	rs2493134 rs3789678 rs5050 rs2493137			

Table 21. The association of AGT SNPs with change in systolic blood pressure (HS-LS). P

values were obtained from a mixed model linear regression accounting for age, gender, and BMI.

N=237.

Name	Delta Systolic P VALUES (ADDITIVE MODEL)
rs7536290	0.8
rs7079	0.5
rs11568045	monomorphic
rs3789670	0.2
rs3789671	0.3
rs2478545	0.4
rs6687360	0.4
rs11122576	maf<0.10
rs11568026	monomorphic
rs2004776	0.4
rs1078499	0.6
rs7539020	0.4
rs2493134	0.5
rs3789678	0.3
rs5050	0.9
rs2493137	0.9

Table 22. The association of AGT SNPs with change in Diastolic Blood Pressure (HS-LS). P values were obtained from a mixed model linear regression accounting for age, gender, and BMI.

N=236.

Name	Delta Diastolic BP P VALUES (ADDITIVE MODEL)
rs7536290	0.1
rs7079	0.2
rs11568045	monomorphic
rs3789670	0.2
rs3789671	0.3
rs2478545	0.08
rs6687360	0.8
rs11122576	maf<0.10
rs11568026	monomorphic
rs2004776	0.3
rs1078499	0.07
rs7539020	0.8
rs2493134	0.6
rs3789678	0.1
rs5050	0.4
rs2493137	0.1

Table 23. The association of AGT SNPs with change in Mean Arterial Blood Pressure (HS-LS).

P values were obtained from a mixed model linear regression accounting for age, gender, and BMI. N=236.

Name	Delta Salt MAP BP P VALUES (ADDITIVE MODEL)
rs7536290	0.3
rs7079	0.8
rs11568045	monomorphic
rs3789670	0.2
rs3789671	0.3
rs2478545	0.1
rs6687360	0.7
rs11122576	maf<0.10
rs11568026	monomorphic
rs2004776	0.4
rs1078499	0.1
rs7539020	0.7
rs2493134	0.9
rs3789678	0.2
rs5050	0.5
rs2493137	0.3

Discussion.

The marker, M235T, was previously associated with essential hypertension, adrenal and renal response to Ang II, and angiotensinogen levels (Watkins et al., 2010; Hopkins et al., 1996; Hopkins et al., 2002); however an association with this gene and glucose metabolism has been unclear. Sheu et al (1998) found no association with M235T of the AGT gene and insulin sensitivity although a positive association of AGT SNP T174M with insulin resistance was found. Guo et al (2005) demonstrated that AGT M235T was associated with increased insulin resistance in Mexican-Americans with and without hypertension; however, this association was lost when BMI was included as a covariate. Pollex et al (2006) found that the AGT SNP T174M was associated with the Metabolic Syndrome in the Oji-Cree population furthering the gene's

involvement with glucose metabolism. These findings clarify the role of the AGT gene with insulin sensitivity by capturing the entire AGT gene, as characterized by HAPMAP, in a well-phenotyped population.

In vivo and *in vitro* studies provide insight into a possible mechanism underlying the association of the AGT gene with insulin sensitivity. AGT gene variants M235T and rs2943134 have been associated with increases in AGT levels (Watkins et al., 2010). Interestingly, infusion of AngII in humans has been shown to improve insulin sensitivity. Morris et al (1994) studied normotensive individuals with type 2 diabetes demonstrating that sub-pressor (1ng/kg/min) and pressor (5ng/kg/min) doses of AngII improve insulin sensitivity measured by euglycemic insulin clamp. The sub-pressor dose demonstrated an effect without an increase in blood pressure, demonstrating that hemodynamic alterations are not the sole mechanism for improved insulin sensitivity. An additional study, in normal men, confirmed an increase in insulin sensitivity with AngII infusion (Fliser, Arnold, Kohl, Hartung, & Ritz, 1991). Studies in animal and cell culture further these findings. Juan et al (2005) found that acute injection of AngII (2ug/100g body weight) increased insulin stimulated glucose uptake in rat adipocytes. Further, when adipocytes were removed and incubated in AngII, stimulated tyrosine phosphorylation of the insulin receptor increased, AKT phosphorylation increased and glut-4 transport increased demonstrating a role for AngII in whole body glucose homeostasis (Juan et al., 2005). It is possible that increased plasma AGT levels, a known effect of the AGT SNPs M235T and rs2493134, are increasing AngII levels and affecting glucose homeostasis via the mechanisms outlined above.

Of interest, the results suggest an influence of obesity, albeit not significant on the study results. An interaction between the AGT gene and BMI has been shown in previous studies (Hopkins et al., 1996) and it is possible that with a larger sample size the interaction suggested in

these analyses would become significant. AGT gene expression has been shown to be increased on both a high fat diet in human visceral adipocytes (Rahmouni, Mark, Haynes, & Sigmund, 2004) and in a hyperinsulinemic state in human 3T3-L1 adipocytes (Jones, Standridge, Taylor, & Moustaid, 1997). These data suggest that with obesity, the effect of the AGT M235T on insulin sensitivity is enhanced.

The results of the haplotype analysis are consistent with the single SNP analyses. The association is primarily driven by haplotype block 3 and more specifically, SNP rs2493134 within this block. The results demonstrate that major allele carriers (T) are most likely to have elevated HOMA-IR results and are insulin resistant or as described in these SNP results, the minor allele is associated with insulin sensitivity. Interestingly, a haplotype that includes AGT M235T has been found to be more strongly associated with angiotensinogen levels than the SNP alone (Watkins et al., 2010). This may explain why some individuals known to have an increased frequency of the minor allele of SNP AGT M 235T, African Americans, have an increased risk of insulin resistance while this data suggest that the SNP should be protective from altered glucose metabolism. Further studies are necessary to assess the association of the AGT gene with insulin sensitivity in an African American population; specifically, whether extensive haplotype analyses provide the most relevant information.

Strengths of this study include the control of experimental conditions (including control of medications and diet known to affect components of the RAAS and glucose metabolism) and clarification of the relationship between the AGT gene and HOMA-IR values in humans. Functional data are not present in this study; however, previous studies demonstrate that AGT M235T is associated with increased angiotensinogen levels (Guo et al., 2005). Further studies are necessary to determine whether AGT levels differ in individuals by AGT genotype and BMI

status. Additional studies are also necessary to determine whether an individual's genotype in other RAAS genes known to be associated with glucose metabolism, including the angiotensin converting enzyme (ACE), affect the association seen between the AGT gene and insulin sensitivity.

In conclusion, this study confirms that SNPs of the AGT gene are associated with insulin sensitivity in Caucasians. Haplotype analysis extends this finding and implicates SNP rs2493134, a proxy for M235T, as the most influential SNP. The results indicate that both hypertension status and BMI may be influencing the association with the genotype effect being the strongest in hypertensive, obese individuals. These results demonstrate a potential role for the AGT gene to explain why some individuals, even with an abnormal cardio-metabolic profile, are insulin sensitive. As clinicians attempt to use AGT genotype as a genomic marker for individualized hypertension treatment, the effects of this gene on glucose metabolism should be considered.

Chapter 7

Conclusion and Future Directions

The aim of this dissertation was to identify genomic markers for the co-aggregation of insulin resistance and hypertension in humans. First, a candidate gene and intermediate phenotype approach was used to examine whether single nucleotide polymorphisms of the CAV1, PPAR γ , and AGT genes were associated with insulin resistance in a Caucasian population with hypertension. Replication was examined where available using two cohorts for two of the genes: 1) IR-HTN (Hispanic) for CAV1 and 2) African American hypertensives from HyperPATH for PPAR γ . Secondary analyses, including associations with salt sensitivity and altered RAAS response, were conducted to examine the mechanistic underpinnings of a SNP's primary association with insulin resistance. The associations were tested using a mixed effects multivariate regression analysis accounting for age, gender, BMI, and sibling relatedness.

Future directions: CAV1.

Examination of the CAV1 gene found two SNPs significantly associated with insulin resistance in both Hispanic (HTN-IR) and Caucasian (HyperPATH) hypertensive populations. The identification of CAV1 as a genomic marker for the co-aggregation of insulin resistance and hypertension is a novel finding and verifies the involvement of CAV1 with processes of glucose utilization in humans with hypertension. Interestingly, the findings existed only in individuals with hypertension. Future work is necessary to evaluate the interaction between hypertension and CAV1 gene variants.

It is possible that a hypertension/CAV1 gene interaction exists. An activated RAAS, seen in hypertension, may interact with CAV1's involvement in the development of insulin resistance. Alternatively, the effect of hypertension and CAV1 may be additive. For example, the

combination of altered insulin mediated glucose uptake in individuals harboring CAV1 variants in addition to altered glucose utilization seen with hypertension may result in greater insulin resistance. More analyses in a larger population are necessary to determine whether an interaction or additive effect exists.

A second mechanism underlying the environment-gene interaction of hypertension and CAV1 on insulin resistance may be their association with increased inflammation. Inflammation is a known contributor to insulin resistance in humans. In addition, increasing CAV1 levels in endothelial cells of mice resulted in decreased inflammation (Bucci, 2000), suggesting a relationship between CAV1 and inflammation. It is plausible that decreased CAV1 levels would result in increased inflammation potentially contributing to insulin resistance in humans. Hypertension also has been associated with increased inflammation (Guo et al., 2008). If individuals have two mechanisms contributing to increased inflammation (decreased CAV1 levels and hypertension), a greater severity of inflammation may ensue resulting in insulin resistance. This hypothesis can be tested by analyzing markers for inflammation in CAV1 genotyped individuals with and without hypertension to determine whether differences exist by hypertension and genotype status.

Finally, since hypertension status has been shown to affect the association of CAV1 and insulin resistance, future analyses must be conducted to determine whether CAV1 genotype predicts an individual's risk for the metabolic syndrome; a complex syndrome with both hypertension and insulin resistance components. As discussed, no genomic markers for the metabolic syndrome have been discovered using a GWA study approach. However, the candidate gene approach identified a marker that is a potential marker for the metabolic

syndrome. It is possible that *CAV1* genotype also predicts the metabolic syndrome and this association must be examined in future studies with well-phenotyped populations.

Future directions: PPAR gamma

The examination of tagging SNPs of the *PPAR γ* gene with insulin resistance indicates that this gene was not associated with insulin resistance in the Caucasian HyperPATH cohort. This is contrary to many studies demonstrating a significant association of the Pro12Ala SNP with lowered fasting glucose and protection against T2DM (Altshuler et al., 2000). Insufficient sample size may have contributed to this negative finding. However, it is also possible that the association does not exist within a hypertensive population. Few studies have tested the association between Pro12Ala and fasting glucose in a hypertensive sample. The mechanism underlying insulin resistance and hypertension is likely different than the mechanism contributing to insulin resistance and type 2 diabetes in a normotensive population (Yanai et al., 2008).

This study did identify an association between the *PPAR γ* gene and renin levels in two cohorts (HyperPATH-HTN Caucasian and African American). This is a novel finding with important clinical significance, for it identifies a mechanism for the inter-individual differences in volume retention with *PPAR γ* use. Future studies, including randomized control trials comparing the incidence of volume retention with thiazolidinedione use in individuals with and without *PPAR γ* SNPs, are necessary to determine if *PPAR γ* genotype predicts risk for developing volume retention and whether renin inhibition is beneficial in this population.

Future directions: AGT.

This study found that the *AGT* gene is associated with insulin sensitivity in the Caucasian HyperPATH population. Haplotype analysis confirmed the findings further supporting a role for

the AGT gene as a marker for insulin sensitivity. Although it is accepted that AngII infusion increases glucose uptake in humans without hypertension (Buchanan et al., 1993; Jonk et al., 2010), the effects of the infusion in a hypertensive population is unknown. This study suggests that AngII infusion would also increase glucose uptake in a hypertensive population however, this hypothesis must be directly tested.

Alternatively, some studies suggest that other components of the RAAS, mainly elevated renin, may inhibit glucose metabolism in individuals with hypertension (Price et al., 1999). Future studies must examine this area closely to identify the specific components of the RAAS that are inhibiting or improving whole body glucose uptake in humans. Specifically, studies that test renin inhibition directly and the effects of this inhibition on insulin resistance would be beneficial and should be conducted.

Implications for the use of the conceptual framework:

This study supports the use of the intermediate-phenotype/candidate-gene approach for the identification of genomic markers for complex disease. This approach has now been shown to be effective for both hypertension and the metabolic syndrome and will likely be effective with other complex diseases; possibly even T2DM. Further, this study supports the thrifty genotype theory. The results support the premise that common variants (MAF>10%) are contributing to metabolic disease. The genotype approach used for this study, identifying common variants in the gene from HAPMAP, may be useful to identify additional genomic markers for a variety of complex disease.

Future directions for clinical practice.***Validation.***

Replication is necessary to validate the study finding and support a role for similar physiologic processes contributing to the outcome of interest. Thus, testing the positive results in larger populations of various ethnicities is essential. Replication of the CAV1 finding in a larger African American sample would support similar underlying physiology for insulin resistance and hypertension in different ethnicities. Alternatively, if CAV1 is determined not to be a marker of insulin resistance and hypertension in African Americans, the finding would highlight an important difference in biomarkers and potentially underlying pathophysiology between the two populations.

Epigenetics.

The relationship between genes and human disease is extremely complex (Manolio, 2010). Epigenetics, the study of the regulation of gene transcription, has become an area of interest to many clinical geneticists. Initially, epigenetics was thought to involve only gene-environment interactions; however, it soon became known that DNA methylation and histone modification were important regulators of epigenetic forces and the term is currently used in relation to these processes (Liu, Li, & Tollefsbol, 2008). It is likely that epigenetic factors are regulating genomic effects on the development of complex diseases such as the metabolic syndrome. Future studies examining epigenetic factors of complex disease development are essential. New techniques analyzing the epigenetics of complex disease are arising including chromatin immunoprecipitation followed by microarray analysis (ChIP-chip) that may provide novel mechanisms to uncover the complex relationship between genes and their regulatory elements (Morse, 2010). As scientists embark on the examination of epigenetics in complex

human disease it is essential that the same mistakes made with the GWA studies (unclean phenotypes, lack of hypothesis driven experiments) and are not repeated. This will take great effort from scientists and money from funding sources, but will likely be rewarded through valid findings and lower chance for type I errors.

Implications for clinical practice: nursing.

The identification of genomic markers for the metabolic syndrome may lead to more effective individualized prevention and treatment strategies, decreasing the morbidity and mortality related to this condition. Since health promotion and disease prevention are primary concerns for professional nurses, it is essential for nurse scientists to conduct research in clinical genomics that may lead to individualized health care (Conley & Tinkle, 2007; Underwood & Read, 2008). Many studies are beginning to highlight the effectiveness of using genomic information to provide individualized prevention and treatment strategies. Specifically, genetic information has led to improved health behaviors including earlier cancer screening for individuals with an identified genetic risk (Beery & Williams, 2007), individualized and more effective cancer treatment strategies (Wadelius & Pirmohamed, 2007; Suarez-Farinas, Shah, Haider, Krueger, & Lowes, 2010), and earlier and targeted cancer prevention strategies (Anderson, Jacobson, Heitjan, Zivin, Hershman, Neuget et al., 2006; Olopade, Grushko, Nanda, & Huo, 2008). Nursing's involvement in identifying and using genomic information in health promotion, disease prevention, and individualized disease treatment is essential to ensure effective, personalized, and targeted care that leads to improved health outcomes.

Nursing has a long history of promoting individual health through behavioral change (Hill, Han, Dennison, Kim, Roary, Blumenthal et al., 2003; Ramirez-Garcia & Cote, 2009). Nola Pender conceptualized this tenet through the development of the Health Promotion Model

(Pender, 1996). This multi-faceted model describes the involvement of individual and environmental variables that interact to affect an individual's overall health and health behaviors. It is now reasonable to include an individual's genomic profile into this model. Research indicates that knowledge of genetic risk for disease can change health behaviors (Attia et al., 2009). In one study, all individual carriers of a genetic mutation known to cause hereditary nonpolyposis colon cancer (HNPCC) adhered to recommended colorectal cancer screening guidelines (Claes, Denayer, Evers-Kiebooms, Boogaerts, Philippe, Tejpar et al., 2005). Further, a systematic review of risk reduction and health promotion behaviors with genetic testing of adult-onset disease found that knowledge of genetic risk for hereditary breast and ovarian cancer (HBOC) as well as hereditary colon cancer increased an individual's use of cancer screening services and treatments (Beery & Williams, 2007). It is yet to be determined whether genetic knowledge related to risk of developing insulin resistance would lead to improved health behaviors, but it is possible. More work must be done to examine the effects of receiving genomic information on specific patient populations.

In addition to providing disease risk, genomic markers can provide insight into the underlying physiology contributing to disease onset. This information may lead to improved individualized prevention and treatment strategies. For example, homozygote C allele carriers of the non-synonymous SNP -176G>C of the interleukin-6 gene (Il6) have been shown to predict progression to T2DM (Kubaszek, Pihlajamaki, Komarovski, Lindt, Lindstrom, Eriksson et al., 2003). Further, a gene-environment interaction exists with this association where the greatest SNP effect is seen in obese individuals (Herbert, Liu, Karamohamed, Liu, Manning, Fox et al. 2006). Herbert et al. (2006) suggests that targeting individuals harboring this specific SNP with individualized exercise and weight loss prevention programs may be more beneficial than current

standard of care. Further, understanding the physiology underlying the gene associations will support targeted pharmacologic treatment regimes, improving the effectiveness, and hopefully compliance with medication treatment (Olopade, Grushko, Nanda, & Huo, 2008).

As the role of the nurse practitioner increases in both the primary care and ambulatory setting, they will be on the front lines of using and explaining genomic information related to chronic disease (Calzone, Cashion, Feetham, Jenkins, Prows, Williams et al., 2010). It will be interesting to assess whether the inclusion of genomic information, relayed in the appropriate manner, increases the effectiveness of nurse practitioner led prevention and treatment programs. This is an important question that must be evaluated within a clinical research study. If it is found that genomic markers improve chronic disease prevention strategies, nurses at all practice levels must be proficient in the interpretation of clinical genetic results and incorporate them into the education and treatment components of their practice (Calzone et al., 2010).

In terms of this dissertation, the identification of CAV1 as a genomic marker for the co-aggregation of insulin resistance and hypertension has enormous potential for improving clinical practice in the manner outlined above. Once replicated and confirmed in multiple populations, CAV1 may be used by clinicians to identify individuals most at risk for the co-aggregation of insulin resistance and hypertension. The metabolic effects of pharmacologic manipulation of CAV1 in humans, proposed for cancer treatment (Trimmer, Whittaker-Menezes, Bonuccelli, Milliman, Daumer, Aplin et al., 2010), must be examined as a possible treatment for insulin resistance in the hypertensive population. Alternatively, other pharmacologic agents affecting upstream or downstream effects of CAV1 on glucose metabolism may be found to be beneficial and may not have the potential side effects of CAV1 agonists. Second, the use of this biomarker

by nurse practitioners in clinical practice may lead to improved prevention strategies and better health outcomes for individuals carrying the CAV1 variant.

In summary, acknowledgement and understanding of genomic information in the design and implementation of improved health interventions is essential to modern nursing. Thus, it is essential that nurse scientists be involved directly with research in genomic health (Underwood & Read, 2008). Studies such as the ones described in this dissertation support the development and implementation of nurse led interventions that will directly affect patient care. Further, these studies provide insight into the genomic effect on disease, supporting multi-disciplinary interventions addressing physiologic, pharmacologic, and psychological components of individualized treatment. Finally, the results support the use of the intermediate phenotype/candidate gene approach to identify genomic markers for chronic disease. Genomic science has the promise to make individualized health care a reality (Collins, 2010). Nursing's emphasis on promoting health through individualized and personalized care makes the incorporation of genomics into nursing practice essential.

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Appendix A.**IRB Institutional Agreement**

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Institutional Review Board (IRB) Authorization Agreement

Name of Institution Providing IRB Review:

THE BRIGHAM AND WOMEN'S HOSPITAL, INC. (BWH) - Federalwide Assurance (FWA) #00000484

THE GENERAL HOSPITAL CORPORATION d/b/a MASSACHUSETTS GENERAL HOSPITAL (MGH) - Federalwide Assurance (FWA) #00003136

Name of Entity or Organization Relying on the Designated IRB(s):

Boston College (BC) - Federalwide Assurance (FWA) #00001461

Terms of Agreement

1. Scope. The Officials signing below agree that BC may rely on the IRB(s) of the institutions designated above registered with OHRP (hereafter referred to as the "Partners HealthCare System IRB(s)" or "Partners IRB(s)"), in accordance with the terms and conditions set forth in this Agreement, for review and continuing oversight of its human subject research described below:

IF AGREEMENT IS FOR ONE STUDY, FILL IN THE FOLLOWING AND DELETE THE REMAINING TEXT:

Name of Research Project: Vascular Disease And Hbp: Pai-1, Insulin And Genes (crc)

Protocol #: 1999p-002189

Name(s) of Principal Investigator(s) at each Site: Gordon Williams, MD; Patricia Underwood, RN

Sponsor or Funding Source and Award # (if any): NIH

2. FWA; Review in Accordance with FWA. BC will maintain a current, approved FWA with OHRP for the duration of this Agreement BC will notify the Partners IRB(s) immediately if its FWA is threatened, terminated, or expires for any reason.

The review and continuing oversight performed by the Partners IRB(s) of the research included under this Agreement will meet the human subjects protection requirements of BC's OHRP-approved FWA, a copy of which shall be given to the Partners IRB(s) upon the effective date of this Agreement and thereafter upon any material change or renewal.

3. Concurrent Review. BC reserves the right at any time to assert its jurisdiction over the review of a study included under this Agreement and to require concurrent review of the study by its own IRB, but must do so in writing to the PI and to the Partners IRB(s). In this event, the more stringent requirements of the two IRBs' reviews will govern. BC acknowledges and agrees that the Partners IRB(s)' disapproval of a protocol may not be administratively overruled by BC.
4. IRB Independence; Local Research Context. This Agreement does not require the Partners IRB(s) to allow representatives of BC to attend its meetings or otherwise accept comments or influence from BC representatives in the review of an included study, except to the extent necessary or requested to ensure sufficient knowledge of the local research context.
5. IRB Decisions; Minutes. The Partners IRB(s) will notify appropriate officials at BC and the responsible BC PI in writing of its decision to approve or disapprove any study accepted for review under this Agreement or of modifications required to secure approval of the study, as well as of

subsequent IRB-reviewed and approved changes in the research activity, namely, by sending copies to such officials and the PI of the review notification letters it sends to investigators. Relevant minutes of the Partners IRB(s)' meetings will be made available to BC upon written request.

6. **IRB Authority.** BC will accept the decisions and requirements of the Partners IRB(s) with respect to the study/ies conducted under this Agreement. BC will also ensure that its investigators cooperate in the Partners IRB(s)' continuing review process, cooperate with all other requirements of the Partners IRB(s), and cooperate with the requirements of the Partners Human Research Quality Improvement Program.

Compliance Responsibilities of Relying Entity and Its Investigators. BC remains responsible in connection with the study/ies included under this Agreement for its own compliance, and for ensuring compliance by its investigators (including, but not limited to, physicians, research nurses, coordinators, data managers, or other members of the research team), with the determinations of the Partners IRB(s); with the terms of BC's OHRP-approved FWA; with 21 CFR Parts 50, 54, 56, 312, and 812 and 45 CFR Part 46 (collectively, the "Federal Research Regulations"), HIPAA and its regulations, research billing requirements, and all other applicable international, federal, state, and local legal requirements; with the report of the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research entitled, Ethical Principles and Guidelines for the Protection of Human Subjects of Research (the "Belmont Report") and other applicable ethical principles; and with Partners HealthCare System's (Partners') confidentiality policies, the Partners Responsible Conduct of Human Studies, Partners' Policy Regarding Noncompliance in Human Subjects Research, the Partners Conflict of Interest Policy, and the Partners IRB(s)', Partners', and BWH's and MGH's other institutional policies, procedures, and documents for conducting research as applicable, including clinical or administrative policies and procedures related to the carrying out of the research activities at a Partners institution by investigators from BC, such as requirements for professional staff appointments, credentialing, insurance coverage, and background checks. In addition, and without limiting the foregoing, any agreement that BC may enter with a sponsor in connection with a study included in this Agreement shall comply with the Partners IRB(s)', Partners', and BWH's and MGH's policies governing such agreements, including but not limited to, policies on subject injury coverage, indemnification from the sponsor, intellectual property, freedom to publish, and ownership of records and data. Copies of the Partners IRB(s)', Partners', and BWH's and MGH's policies, procedures, and documents referenced herein will be available upon request to BC and its investigators. Nothing herein shall be interpreted to prevent BC or its investigators from complying with any policy or procedure of BC applicable to the covered research; however, to the extent there is a conflict between such policy or procedure and the applicable policies and procedures of the Partners IRB(s)', Partners, or BWH or MGH, the parties will work together to ensure that the more stringent requirements of the conflicting policy or procedure will govern.

7. **Reporting of Information on Noncompliance and Subject Safety.** BC will promptly report to the Partners IRB(s) and to Partners, BWH, and MGH any noncompliance by BC or its investigators with the determinations, documents, laws, policies, and procedures listed in Section 7 in connection with the research activity/ies conducted under this Agreement of which BC is aware. BC will also ensure that its investigators immediately report to the Partners IRB(s) any injuries to subjects or unanticipated problems involving risks to subjects or others in the research activity conducted under this Agreement of which the investigators are aware, and promptly report any proposed changes in such research activity/ies; such changes shall not be initiated without the Partners IRB(s)' review and approval except where necessary to eliminate apparent immediate hazards to the subjects.
8. **Notification of Information on Noncompliance, Suspension/Termination, and Subject Safety Determined/Discovered by IRB.** In turn, the Partners IRB(s) will notify BC in writing of serious or continuing non-compliance by BC or BC's investigators discovered by Partners, any suspension or termination of IRB approval, and injuries to subjects or unanticipated problems involving risks to subjects or others discovered by Partners.

9. Cooperation in Investigations and Corrective Actions. BC will cooperate with and ensure its investigators' cooperation with any inquiry by the Partners IRB(s), the Partners Human Research Quality Improvement Program, or any government authority into research compliance in a study conducted under this Agreement. Such cooperation will include, but is not limited to, providing research records and related information, meeting with Partners research representatives upon request, permitting reasonable on-site audits of BC's facilities, and helping to carry out remedial action if reasonably indicated. Such remedial action may include termination of participation by BC or its investigators in designated research activities. BC may also take further action where appropriate to deter and remedy such deficiencies; however, to the extent there is a conflict between remedial actions sought to be taken by the parties, the more stringent of the remedial actions shall apply.
10. Recordkeeping. BC will instruct its investigators to maintain records of all human subjects research and related activities conducted under this Agreement for at least six years, and longer if required by law, after completion of the research. Upon request, BC shall provide a copy of such records to the Partners IRB(s), and to others if legally required.
11. Term. This Agreement shall become effective on the last date signed below and shall continue until completion of the research (as determined by the Partners IRB(s) provided that the parties' FWAs remain current and in good standing and provided that the Agreement is not earlier terminated as provided below.
12. Termination. Either Partners, BWH, or MGH, on the one hand, or BC, on the other, may terminate this Agreement (i) without cause upon thirty (30) days prior written notice to the other or (ii) upon fourteen (14) days prior written notice to the other in the event of a breach by the other that is not cured to the reasonable satisfaction of the non-breaching party within said fourteen (14)-day notice period. In the event that BC's FWA is threatened, terminated, or expires, Partners or BWH or MGH may terminate the Agreement immediately.
13. Effect of Expiration or Termination; Survival. In the event of any termination of this Agreement, the parties will ensure that OHRP is notified and will work together to determine the effect of such termination on any research being conducted under the Agreement at the time of termination. In the event of any expiration or termination of this Agreement BC will remove the Partners IRB(s) from the list of designated IRBs on its FWA and will notify the Partners IRB(s) that this has been done.
17. Notices. All communications, reports and notices required under this Agreement shall be delivered by hand, by facsimile, or by first-class mail, postage prepaid and addressed as follows:
- If to Partners/BWH/MGH: P. Pearl O'Rourke, M.D.
 Director of Human Research Affairs
 Partners HealthCare System, Inc.
 Research Management
 50 Staniford Street – Suite 1001
 Boston, MA 02114
 Fax: (617) 726-3246
- If to Partners IRB(s): Elizabeth L. Hohmann, M.D.
 Director and Chair, Partners Human Research Committee
 116 Huntington Avenue – Suite 1002
 Boston, MA 02116
 Fax: (617) 424-4199
- Phone/fax of Partners IRB(s): (617) 424-4171 / (617) 424-4199


With a copy to: Deborah Barnard, MS, CIP
 Director, Quality Assurance and Quality Improvement
 Partners Human Research Committee
 116 Huntington Avenue – Suite 1002
 Boston, MA 02116
 Fax: (617) 424-4199

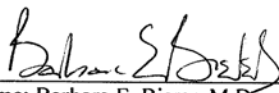
If to **Boston College**: Dr. Stephen Erickson, Interim Director
 Office for Research Protections
 Boston College
 Waul House, 3rd Floor
 Chestnut Hill, MA 02467

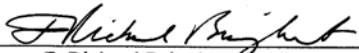
18. Miscellaneous. This Agreement has been executed and delivered in and shall be governed by and construed and interpreted in accordance with the laws of the Commonwealth of Massachusetts. This Agreement may be amended only by a written agreement signed by authorized representatives of all parties. If any provision of this Agreement shall be held to be invalid, illegal, or unenforceable, the validity, legality and enforceability of the remaining provisions of this Agreement shall not be affected thereby. The failure of a party to insist upon the strict performance of any of the terms of this Agreement shall not be construed to be a waiver or relinquishment of any of the terms of the Agreement or of the whole Agreement. All the titles and headings contained in the Agreement are inserted only as a matter of convenience and reference and do not define, limit, extend, or describe the scope of this Agreement or the intent of any of its provisions. This Agreement is not assignable in whole or in part, and any attempt to do so shall be void.

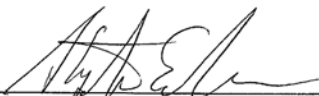
This Agreement will be kept on file at each institution/entity and provided to OHRP upon request.

EXECUTED BY AUTHORIZED SIGNATORY OFFICIALS

 Date: 12-29-08
 Name: P. Pearl O'Rourke, M.D.
 Institutional Title: Director of Human Research Affairs
Partners HealthCare System, Inc.
 Research Management
 50 Staniford Street – Suite 1001
 Boston, MA 02114

 Date: 1/12/09
 Name: Barbara E. Bierer, M.D.
 Institutional Title: Senior Vice President, Research
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 Date: 1/1/09
Name: F. Richard Bringhurst, M.D.
Institutional Title: Senior Vice President of Medicine and Research Management
The General Hospital Corporation d/b/a Massachusetts General Hospital 55 Fruit Street – BUL 240
Boston, MA 02114

 Date: 12/5/08
Name: Stephen Erickson, Ph.D.
Institutional Title: Director, Office for Research Integrity and Compliance
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