ALPHA 1A- AND BETA 2-ADRENOCEPTOR GENE EXPRESSION DIFFERENCES IN HYPERTENSIVE AND NORMOTENSIVE PERSONS BY RACE

By

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This document is dedicated to my husband, Craig for his endless love and support, to my Great-grandmother, Margaret Gray for inspiring me to become a nurse, to my Grandmother, Julia Chodzinski, who has shown me the power of perseverance and the importance in believing in my dreams, and finally, to my mother, Cheryl Crossland, who instilled in me a strong sense of hard-work and determination, and who has given me confidence when I needed it most.

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Abstract of Dissertation Presented to the Graduate School
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By

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Major Department: Nursing

It has been hypothesized that genes of the adrenergic receptor (ADR) system contribute to hypertension (HTN). This notion is supported by genetic (gene-knockout and association), physiological, and pharmacological studies of ADRs in animal and human models. The α_{1A}- and β_2-subtypes are two of nine ADRs. Briefly, vascular contraction is mediated by the α_{1A}-ADR, whereas vascular dilation is mediated by the β_2-ADR. Gene expression studies of these subtypes in animal strains (particularly between normotensive and hypertensive strains) suggest an important role in the development of HTN; however, studies of this nature have not been conducted with human tissues in a between-groups design. This study explores the feasibility of conducting such a study in humans and the relative gene expression differences of the two aforementioned ADR genes in people with and without HTN and explores the impact of self-identified race. Gene expression refers to transcription of ribonucleic acid (RNA) from deoxyribonucleic

acid (DNA). This process is a necessary step in the making of proteins. Gene expression is influenced both by genetic and environmental factors during transcription.

Relative levels of RNA of the α_{1A}- and β_2-ADR genes were measured in arterial tissue samples obtained from 41 subjects who had coronary artery bypass surgery at either Shands at Alachua General Hospital or the Malcom V. Randall Veterans Hospital. Subjects were grouped according to the diagnosis of HTN (n = 24) or NT (n =17), as defined by national guidelines. During surgery, a small amount (10-30 mg) of normally-discarded internal mammary artery tissue was provided to the researcher, processed, and analyzed with Real-Time, reverse-transcription polymerase chain reaction to obtain relative quantitation of gene expression.

Hypertensive subjects showed 3.92- and 2.05-fold differences in relative α_{1A}- and β_2-ADR gene expression (respectively) compared to normotensives (statistically significant with alpha of 0.05), with hypertensives demonstrating reduced expression of both genes. Fold differences for both ADR subtypes remained significant when comparing White/Caucasian hypertensive versus normotensive subjects. Further exploratory aims produced some significant findings. This study experienced methodological issues with the reference gene, thereby affecting accuracy of relative gene expression quantitation; therefore, interpretation of results is cautioned.

CHAPTER 1
INTRODUCTION

This chapter will introduce the main research problem and background and delineate the hypotheses to be tested. The definitions of variables, major terms, assumptions, and significance of the study will also be presented.

AA= African American
ADR = adrenergic receptor, or adrenoceptor
$\alpha 1_A$-ADR = adrenergic receptor, Alpha 1A-subtype
β_2-ADR = adrenergic receptor, Beta2-subtype
BP = blood pressure
CABG = coronary artery bypass graft
cDNA = complementary DNA
CVR = cardiovascular reactivity
DBP = diastolic blood pressure
HTN = hypertension
IMA = left internal mammary artery
mRNA = messenger RNA
NT = normotension
PCR = polymerase chain reaction
RNA = ribonucleic acid
RT = reverse transcription
SBP = systolic blood pressure
SIR = self-identified race
T2DM = type 2 diabetes
TCV = Thoracic and Cardiovascular Surgery
TPR = total peripheral resistance
VAMC = Veterans Administration Medical Center

Figure 1-1. Abbreviations used.

Background and Problem Statement

Just over 26% of adults worldwide (approximately 972 million adults) have hypertension (HTN). Essential HTN is synonymous with "primary" HTN, in which the cause of the high blood pressure (BP) is unknown. This type represents 90-95% of all cases of HTN (American Heart Association [AHA], 2005). Alternately, secondary HTN accounts for the other 5-10% of all cases. For these cases, the cause is known and often

correctable. Renal disease (of various types) is the most common cause of secondary HTN. Secondary HTN can also arise from single-gene disorders such as glucocorticoid remediable aldosteronism, the most common autosomal dominant form of inherited HTN. Secondary causes can also be conditions (such as pregnancy or stress) that, when corrected, bring BP back to normal levels.

Heritability (h^2) is the ratio of additive genetic variance to total phenotypic variance. It can be thought of as the amount of variation in high BP attributable to the variation in our genetic makeup. Pedigree, twin and sibling studies have discerned that the heritable portion of essential HTN is approximately 30% (Ambler & Brown, 1999). More recent data suggest that BP traits such as SBP and DBP have high estimated heritability at about 72% and 63%, respectively (Zeegers, Rijsdijk, Sham, Fagard, Gielen, de Leeuw, et al., 2004). This could mean that the estimated heritability of HTN is actually higher than we previously thought; or that this particular study had inflated values due to study design and/or analyses. Furthermore, the recurrence risk of HTN increases as the number of parents with HTN increases, so that an offspring has approximately 4% chance of developing HTN with no hypertensive parents, a 10-20% risk with one hypertensive parent and the risk increases to 25-45% when both parents are hypertensive (Lucassen, 1999). These findings point to an obvious link to genetics in explaining some of the variance in HBP.

HTN is known as the “silent killer,” as few, if any, symptoms are noted by its sufferers. It is widely accepted that essential HTN is multifactorial, developing as a result of multiple genes and multiple environmental factors, their interactions producing altered homeostasis of BP regulation in the body. Furthermore, HTN has a complex

pathophysiology involving the cardiovascular, renal, endocrine, neurohumoral, and immune systems. Within these systems are subsystems that contribute to the grand schema of developing HTN, each mechanism having a number of genetic components. These include the renin-angiotensin-aldosterone (RAA) system, the angiotensin converting enzyme (ACE), the sodium balance either by the kidneys or by hormonal influences, or the vascular system, to name a few. Nearly every mechanism has its own candidate gene(s) for HTN. Some examples are those coding for the following proteins: renin, angiotensinogen, angiotensin I and II, angiotensin-converting enzyme, atrial natriuretic peptide (B and C types), nitric oxide (inducible and endothelial), endothelins, dopamine, kallikrein, adducing α-subunit, and adrenergic receptors (ADRs).

The ADRs are particularly important in regulating BP. They are the main binding sites for the catecholamines epinephrine and norepinephrine, which work in delicate balance to regulate vasodilation and vasoconstriction. These vasomotor reactions can influence the rising and falling of BP levels in the body. The ADRs can also mediate BP regulation through renal sodium excretion and release of renin from the juxtaglomerular cells in the kidneys (DiBona, 1989). Decades of research have shown that ADRs are important in the regulation of BP in humans and animals, and that alterations in ADRs at the cellular and genetic levels may lead to HTN. Various functional differences in ADRs have been reported between normotensive and hypertensive humans and animals. Of novel interest are recent animal studies of gene expression differences in two specific ADRs in HTN: the α_{1A}- and β_2-subtypes. Both subtypes are involved in vasomotor tone via expression in the arteries and veins and both are implicated in the grand schema of HTN. Each has a gene that codes for its receptor protein. How these genes are expressed

in the tissues can lead us to important information about their role in HTN. Gene expression of these ADR subtypes can be measured by their messenger ribonucleic acid (mRNA) levels found in the tissues where they are present. The mRNA levels provide us with direct information about the level of transcription of the genes that code for these subtypes. Current technology allows us to preserve tissue samples in such a way that we can accurately measure this mRNA (or level of transcription) and compare these levels between groups of people (for example, between people with normotension (NT) versus HTN). (More detailed background information on these subtypes and gene expression is provided in Chapter 2: Review of the Literature.)

The previous paragraph explained that differences in ADR exist between hypertensive and normotensive humans and animal strains; that two specific subtypes have been implicated in the pathophysiology of HTN; and that examining gene expression of these genes may provide a novel insight into one aspect of the disease process. ADR differences in HTN have also been reported among self-identified races and ethnicities. While it is accepted that there is great interindividual variability among people with regard to ADR function, expression, physiologic response and pharmacologic response (Small, McGraw, & Liggett, 2003), potential racial and ethnic differences attract attention because of the disproportionate statistics regarding hypertensive disease in racial and ethnic subpopulations. Disease prevalence, management, morbidity and mortality among the black or African American (AA) population are particularly problematic because AAs exhibit the highest rate of HTN and the worst health outcomes in regards to morbidity and mortality in the U.S. The possible explanations of race-specific differences in health and disease outcomes are at the center

of great debate. Possible variables include socioeconomic factors as well as differences in pathophysiologic mechanisms, pharmacologic responses, and recently genetic variability. In the ADR literature, there are reports of adrenergic-specific differences in cardiovascular reactivity (CVR) within black/AA populations (McAdoo, Weinberger, Miller, Fineberg, & Grim, 1990; Stein, Lang, Singh, He, & Wood, 2000; Knox, Hausdorff, & Markovitz, 2002) and adrenergic-specific diversity in medication response in AAs (Humphreys & Delvin, 1968; Jennings & Parsons, 1976; Seedat, 1980; Cushman, Reda, Perry, Williams, Abdellatif, & Materson, 2000).

The estimated heritability for HTN in people of sub-Saharan African descent is 45-68% (Rotimi, Cooper, Cao, Ogunbiyi, Ladipo, et al., 1999; Gu, Borecki, Gagnon, Bouchard, Leon, Skinner, et al., 1998). While this heritability estimate is specific to people having origins of the sub-Saharan region of Africa, which is not generalizable to any- or everyone having origins in Africa, it warrants further investigation into genetic sub-population differences. Genetic studies of association have used self-identified race (SIR) as a variable. Reports of racial differences in allele frequency of ADR polymorphisms exist (Hindorff, Heckbert, Psaty, Lumley, Siscovick, Herrington, et al., 2005; Xie, Kim, Stein, Gainer, Brown, & Wood, 1999). If all of these aforementioned ADR differences (cellular, functional, pathophysiologic, and pharmacologic) truly exist between HTN and NT people and among SIRs and ethnicities of hypertensives, can genetics explain these differences? Relatedly, do differences exist regarding how these ADR genes are expressed in the vascular tissue? These are some exploratory issues that will be addressed by this study.

Purpose of the Study

The purpose of this study is to examine the relationships among HTN and gene expression of the α_{1A}- and β_2-adrenergic receptors (ADRs) in the human population, and to explore if SIR helps to explain some of the differences. The study will address three specific aims:

Specific aim 1: To quantify differences in gene expression of α_{1A}-ADR and β_2-ADR in the internal mammary artery (IMA) between subjects with normotension (NT) and HTN.

- To quantify relative differences in α_{1A}-ADR gene expression between study groups with NT and HTN.
- To quantify relative differences in β_2-ADR gene expression between study groups with NT and HTN.

Specific aim 2: To explore relative differences in gene expression of α_{1A}-ADR and β_2-ADR in the IMA between subjects with NT and HTN by SIR.

- To explore relative differences in α_{1A}- and β_2-ADR gene expression between White/Caucasian subjects with NT and HTN.
- To explore relative differences in the α_{1A}- and β_2-ADR gene expression between White/Caucasians with HTN versus Black/AAs with HTN.

Specific aim 3: To explore the relationship between level of α_{1A}- and β_2-ADR gene expression and need for post-operative positive inotropic medication administration.

Exploratory aim 1 (E1): To explore the association between diagnosis of HTN and α_{1A}- and β_2-ADR genotypes. Three genotypes will be explored: α_{1A}-ADR (Codon 347, refSNP ID:1048101), β_2-ADR (Codon 16, refSNP ID:1042713 and Codon 27, refSNP ID: 1042714) for their association with HTN.

- E1-1: To explore the impact of SIR on genotype by diagnosis association.

Exploratory aim 2 (E2): To explore the association between genotype and gene expression.

- E2-1. To explore the association between α_{1A}-ADR (Codon 347) single nucleotide polymorphism (SNP) and α_{1A}-ADR gene expression.
- E2-2. To explore the association between β2-ADR (Codon 16) SNP and β2-ADR gene expression.
- E2-3. To explore the association between β2-ADR (Codon 27) SNP and β2-ADR gene expression.

Hypotheses

For all specific aims, the null hypothesis that no statistically significant differences exist between groups will be tested. In keeping with the neurohumoral model of HTN, if α_{1A}-ADRs contribute to vasonconstriction and β_2-ADRs contribute to vasodilation, it could be hypothesized that subjects with HTN would display greater levels of α_{1A}-ADRs and lower levels of β_2-ADR gene expression than NT subjects; however, as the reverse phenomenon can also lead to HTN via regulatory feedback loops (Anderson, McNeilly, & Myers, 1992), a unidirectional hypothesis is not appropriate. Similarly, racial differences in cardiovascular reactivity patterns could possibly support directional hypotheses of AAs showing greater α_{1A}-ADR gene expression than Caucasians, and Caucasians showing greater β_2-ADR gene expression than AAs; however, there is not enough evidence in the literature to support a unidirectional hypothesis at this time. For E1, HTN is expected to be positively associated with each of the three ADR SNPs, based both on previous findings and the compelling evidence supporting the role of these SNPs in the disease process of HTN (Small, McGraw, Liggett, 2003). For E2 (E2-1 through E2-3), it is hypothesized that gene expression may be affected by the variants in SNPs in

the gene, so that variations in genotypes may affect gene expression; however, the direction of this relationship is not established enough for unidirectional hypotheses.

Definitions of Terms

Terms discussed in this study are defined as below.

Hypertension – Defined by the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC VII) as having three consecutive BP readings of 140/90 mmHg or greater, the diagnosis of high BP by a health care practitioner, or taking antihypertensive medications specifically for BP control (JNC VII, 2003).

Normotension – Having BP below 130/90 mmHg, having never been diagnosed with high BP, and not taking antihypertensive medications specifically for BP control, also defined by the JNC VII (2003).

Adrenergic receptors- A group of nine G-protein receptors from the super family of cell-surface receptors that signal the sympathetic nervous system in response to the need for BP homeostasis. α_{1A}-ADRs contribute to vasoconstriction. β_2-ADRs contribute to vasodilatation.

Cardiovascular reactivity- A complex cardiovascular trait in which individual cardiac and vascular responses to physiological and psychological stressors may lead to changes in systolic blood pressure (SBP), diastolic blood pressure (DBP), total peripheral resistance (TPR), and other hemodynamic measurements that represent vascular response to maintain cardiovascular homeostasis.

Gene expression- Process by which genes are "expressed" in the body. DNA is a double-stranded sequence of nucleotides that codes for proteins. DNA strands are transcribed (or copied), making single-strand messenger RNA (mRNA). The

transcription process begins at the promoter region of the gene. The mRNA template produced by transcription is then translated into proteins. The levels of mRNA found in a biological specimen are indicators of the level of transcription. How the genes are expressed (or how much transcription is taking place) can be informative of how much the gene is functioning or how much is being copied to produce specific proteins.

Race – Used in this study as a self-identification of one of 5 categories that reflects their geographic *origin* based on the corresponding regions and populations listed in the groups. Generally, cultural aspects of affiliations with these groups of origin is implied. These categories are set by the U.S. Office of Management and Budget (OMB) and are meant to reflect population-specific self-identification, not skin color. (See Appendix A.) This variable is referred to as "self-identified race," or SIR for short. See sections titled "Recommendations for the collection of racial and ethnic data" and "Implications for studying disease by race" in Chapter 2.

Assumptions

This study focuses only on essential HTN. The first major assumption is that essential HTN is a multifactorial disease process with multiple genetic and environmental factors that are likely to interact. While many models of HTN exist, this study focuses on the neurohumoral model of HTN. The second major assumption is that the neurohumoral model plays a major role in the pathophysiology of HTN. This model concentrates on the importance of activation of the sympathetic nervous system and neurohumoral substances (namely epinephrine and norepinephrine). When released, these endogenous catecholamines interact with α- and β-ADRs to elicit a cascade of cellular membrane and intracellular events (Berecek & Carey, 2003) that affect the cardiovascular system. A third assumption is that mRNA levels are indicative of receptor regulation and a fourth

assumption is that receptor regulation impacts disease mechanisms at the receptor and/or cellular level. It is already known that the "transcription rate and steady-state level of β-ADR messenger RNA" is modified when the β receptors are stimulated (2003, p. 3). This is yet to be confirmed in regards to α-ADRs, but induction of transcription is likely to play a similar role in regulating these receptors. Finally, a fifth assumption is that RT-PCR only quantitates steady state mRNA levels, and therefore only a "snapshot in time" (Bustin, 2002). Furthermore, these levels may not reflect levels of protein produced by the cell (Gygi, Rochon, Franza, & Aebersold, 1999).

Significance of the Study

HTN is one of the most prevalent chronic diseases in the United States (U. S.). The AHA reports there are an estimated 65,000,000 Americans over age 6 and 1 in 3 adults that have HTN (2005). Although HTN is easily detected and usually controllable, the cause of 90-95% of cases is unknown (AHA, 2005). Economic costs of hypertension in the U.S. are estimated to total $59.7 billion in 2005 (AHA, 2005). Vascular-related comorbidites of HTN include diabetes, peripheral vascular disease, and stroke. In short, HTN and its vascular consequences have major impacts on our society's health and economy. HTN's complex pathophysiology leads to a complex phenotype with many clinical variations. Its silent nature and disease complexity often result in poor rates of diagnosis and control. This is evident by a control rate of merely 34% in all known hypertensives (JNC VII, 2003). Overall, the complexity of the disease process makes HTN difficult to manage and study. Much promise has been placed in the study of genetics, particularly in regards to popular gene association studies, where associating the frequency of a particular allele and/or haplotype (combination of alleles) with a disease is the focus. As summarized by Small and colleagues (2003), some allele-based association

studies report positive associations between ADR alleles and HTN, while others report no significance of these alleles. Specifically, the α_{1A}- ADR has been hypothesized to play a role in HTN due to its role in vasomotion; however, only one study has associated a polymorphism of the α_{1A}- ADR gene to HTN (Jiang, Mao, Zhang, Hong, Tang, and Li, et al., 2005). Polymorphisms of the β_2-ADR have been positively associated with HTN (Timmermann, Mo, Luft, Gerdts, and Busjahn, et al.,1998.) Gene association studies are numerous, but many are inconclusive, inconsistent, and poorly powered. Animal and human studies focusing on physiologic, pharmacologic, genomic, and genetic factors have shown promise in providing evidence for α- and β-ADR mechanisms in mediating CVR in HTN, as will be delineated in Chapter 2: Review of Literature. Still, little attention has been paid to the role of gene expression of α- and β-ADRs in vascular tissue in HTN and CVR in *human* studies. Gene expression studies have been conducted to identify the role of α- and β-ADRs, but predominantly in non-human models (Gaballa, Peppel, Lefkowitz, Aguirre, Dober, and Pennock, et al., 1998) or specifically to focus on the effect of medication (Wang & Brown, 2001; Nishio & Watanabe, 1999) or aging (Miller, Hu, Okazaki, Fujinaga, & Hoffman, 1996) on gene expression of these ADRs. Few studies have been found in the literature regarding gene expression analysis of ADRs in humans. Only one study demonstrated the differences in gene expression of these ADRs between persons with and without HTN. This group of researchers examined the presence of three α1-ADRs in *peripheral blood lymphocytes* of human NT and hypertensive subjects (Veglio, Tayebati, Schiavone, Ricci, Mulatero, and Bronzetti, et al., 2001). They studied gene expression of the ADR genes located in the blood. They also compared the human blood sample gene expression data to that of NT and hypertensive

strains of rats, finding similar α_1-ADR densities in human blood and animal tissues. Also, significant differences in expression of certain α_1-ADR subtypes were found both between humans with HTN and their normotensive controls, as well as between the normotensive and hypertensive strains of rats (Veglio et al., 2001). This study provided important information about the use of peripheral blood lymphocytes in the analysis of gene expression of α1-ADR subtypes, as well as relation of human to animal models. Their findings supported the link between α_1-ADR subtypes in HTN at the gene expression level (Veglio et al., 2001). Some limitations of this study include isolation of the α_{1A}- subtype in rat vas deferens tissue and not arterial, venous, aortic, or myocardial tissue. Finally, measuring mRNA levels via peripheral lymphocytes is an indirect measure of transcription because the measurement is not occurring in the tissue of interest (or the tissue thought to be directly involved in the disease pathway). This is less reliable than direct methods, where mRNAs are examined in the tissue. Veglio and colleagues (2001) reported chosing this method because human tissue was not possible to obtain. Theoretically, there are concerns with using blood to examine gene expression. One major issue is the source of mRNAs in circulation; the origin of the mRNAs that are found in the bloodstream is lymphocytes. It is not clear if mRNAs expressed in the blood have differential expression than those expressed directly in the tissues. While Veglio and colleagues (2001) were able to show similarities in expression between vas deferens tissue and blood lymphocytes, further investigation is needed to compare blood lymphocytes with other tissues. Many consider it less reliable to use circulating blood mRNAs to examine the direct relationship between transcriptional processes and disease

mechanisms because other factors circulating in the blood could potentially vary the expression at any given time and thus make the research less replicable.

Other human gene expression studies involving ADR subtypes in human tissues have focused on other disease processes such as congestive heart failure, and have obtained samples from human myocardial tissue, most commonly obtained from endomyocardial-biopsy specimens (Lowes, Gilbert, Abraham, Minobe, Larrabee, and Ferguson, et al., 2001; Moniotte, Vaerman, Kockx, Larrouy, Langin, and Noihomme, et al., 2001). Gene expression studies examining the differences in mRNA level present in the actual tissue could help to explain if the actual expression of the proteins that make up the ADRs play a role in HTN, rather than merely the presence of a particular allele. Researching the mechanisms that account for these differences has the potential to increase our understanding of the impact of gene expression on phenotypic variance in HTN and adrenergically-driven vascular tone.

The first aim in this study investigates whether or not gene expression of α_{1A}- and β_2-ADRs are related to the diagnosis of HTN by examining their expression in human arterial tissue between subjects with and without a diagnosis of HTN. This method provides direct measures of steady-state levels of mRNA and insight into the genetic picture of real-time transcription activity in each subject's individual environment. To my knowledge, an investigation of this nature has not been previously reported in human arterial tissue, comparing hypertensive and normotensive subjects. This study provides preliminary results about the role of α_{1A}- and β_2-ADR gene transcription levels and their relationship to HTN, and could lead to larger-scale studies in the future. On a more

innovative note, this study could further support gene therapy involving the ADR system in the management of HTN.

The second aim of the study explored if differences in α_{1A}- and β_2-ADR gene expression levels exist when SIR was taken into account. This aim provides information about whether or not similar ADR gene expression trends exist in self-identified racial groups. This study may contribute to the overall goal of reducing health disparities related to SIR and HTN that may be due to genetic variation, as identified by *Healthy People 2010* (National Heart, Lung, and Blood Institute [NHLBI], 2003). The health disparities among hypertensive black and AA groups are real. What is uncertain is whether or not a genetic basis exists to help explain those disparities. This study provides preliminary data to begin to answer that question.

The third specific aim of the study is to explore the relationship between α_{1A}- and β_2-ADR gene expression levels and the need for post-operative positive inotrope medication administration. The need for positive inotrope administration in the post-operative stage of recovery from coronary artery bypass surgery (CABG) is most often the result of a negative cardiac event (such as acute congestive heart failure, cardiac arrest, hypovolemia, or arrhythmias) that necessitates increased vascular resistance to correct the problem. Since α_{1A}- and β_2-ADRs are involved in vascular resistance, and inotropes increase vascular resistance, this aim provides indirect information about subjects' cardiovascular reactivity. In addition, it provides information about whether or not gene expression of the α_{1A}- and β_2-ADRs is related to poorer cardiovascular outcomes, as measured by their need for positive inotropes, in subjects undergoing CABG.

The global health and economic consequences of this disease are incredible. HTN is a disease that affects every continent and population, some more disproportionately than others. Any novel information regarding the impact of α_{1A}- and β_2-ADR gene expression on people with HTN could prove to be a valuable building block for future studies. This study bridges the gap between bench and the bedside using advanced genetic technology to examine if and how gene expression of α_{1A}- and β_2-ADRs relate to HTN. In addition, the study seeks to explore the impact of SIR on gene expression of the two chosen ADR genes, and the impact of gene expression on the need for emergency cardiac medication in the post-operative stage of recovery.

CHAPTER 2
REVIEW OF LITERATURE

Introduction

This chapter will present a review of the literature regarding all relevant topics of the study, including: genetics, hypertension, cardiovascular reactivity, adrenergic receptors, the α_{1A}- and β_2-ADR subtypes, pharmacotherapeutic aspects of the α_{1A}- and β_2-ADRs, population differences in HTN development and management, recommendations for the collection of racial and ethnic data, implications for studying disease by SIR, positive inotrope administration, and genetic analysis techniques.

Genetic Influences on Essential Hypertension

Early heritability studies carried out with essential hypertensive twins estimated at least 63% of the variability in BP was due to genetic factors and reported "little evidence for environmental influence on the familial aggregation of BP" (Grim et al., 1984, p. 453). The most cited estimate of essential HTN heritability is approximately 30% (Ambler & Brown, 1999). Some recent researchers examined the heritability of BP *traits*. In a classical twin study of 173 dizygotic (DZ) and 251 monozygotic (MZ) twin pairs aged 18-34 years, randomly selected from the East Flanders Prospective Twin Survey, heritability estimates were: SBP 74% (95% CI: 0.68-0.79) and DBP 63% (95% CI: 0.55-0.59). These heritability estimates were not confounded by the following potential risk factors: body mass index (BMI), cholesterol ratio, birthweight, physical activity, sex, and cigarette smoking (Zeegers, Rijsdijk, Sham, Fagard, Gielen, and de Leeuw, et al., 2004).

Genetic Models of Hypertension

HTN has two distinct genetic classes: monogenic (meaning caused by one gene) and polygenic (caused by multiple genes). A number of monogenic forms of HTN have been identified, such as glucocorticoid remediable aldosteronism, Liddle's syndrome, Gordon's syndrome, and Bilginturan syndrome (all autosomal dominant gene abnormalities), and apparent mineralocorticoid excess, caused by an autosomal recessive gene abnormality (Beevers, Lip & O'Brien, 2001). These are rare in the general population. Essential HTN is considered polygenic. This "polygenic model" of disease stems from R. A. Fischer's "quantitative genetics" theory proposed in 1918. He postulated that a phenotype (observable expression of a genotype as a trait or disease) "was influenced by a large number of genes, each behaving according to basic Mendelian rules, but each having only a small individual effect on the phenotype" (McClearn, Vogler, & Plomin, 1996, p. 96). Not long after, scientists realized that the environment could influence a phenotype, and the debate began concerning 'nature versus nurture'. This led to introduction of the gene-environment interaction model.

The earliest research designs that supported these models of familial aggregation were pedigree studies involving twins, siblings, and families. MZ twins share as much as 99% of genetic information; DZ twins and non-twin siblings share as much as 50% of the same genetic information. Large-scale pedigree studies in which families are observed longitudinally created the basis for the common linkage analysis that now uses pedigrees and genetic testing to link specific genes to phenotypes of disease. From these studies, a great number of candidate genes were discovered for high BP. Association studies take this concept one step further. These studies correlate candidate genes to hypertensive

phenotypes in the general population, looking for associations between alleles (or variants of the gene) and HTN.

Another current model of HTN is the animal model. Animal models ultimately serve as models for human disease. Animal researchers use sophisticated breeding methods and more recently, gene knock-out and knock-in techniques to manipulate and control genetic, environmental and even phenotype variables. Using this basic research model, researchers can investigate numerous theories in HTN that could not otherwise be studied in humans.

Cardiovascular Reactivity, HTN and Adrenergic Receptors

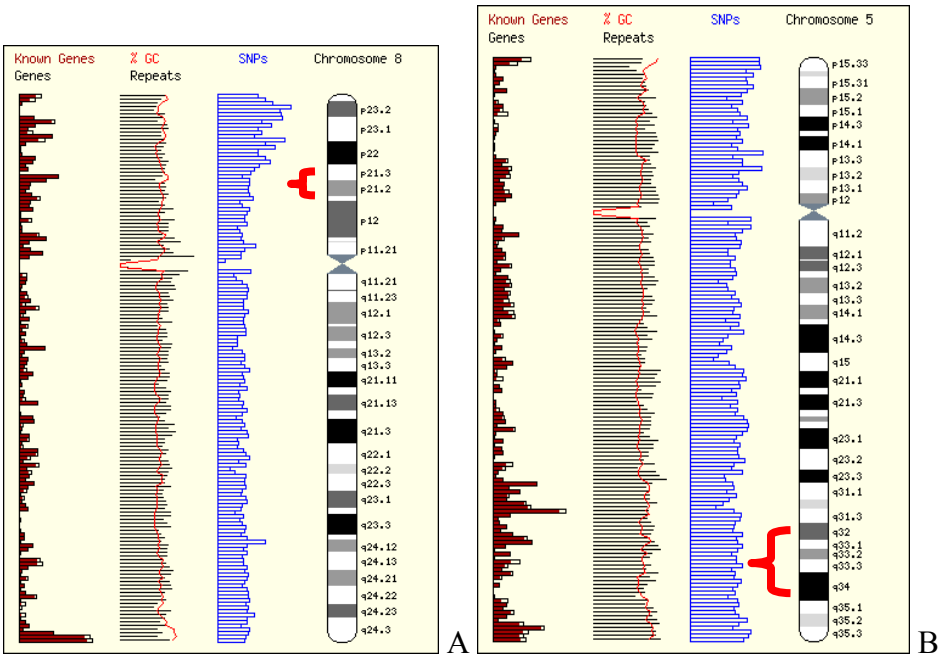
As previously defined, CVR is a complex cardiovascular trait in which individual cardiac and vascular responses to physiological and psychological stressors may lead to changes in SBP, DBP, TPR, and other hemodynamic measurements that represent vascular response to maintain homeostasis**.** These changes differ between NTs and hypertensives, in both human (de Visser, van Hooft, van Doornen, Hofman, Orlebeke, & Grobbee, 1995) and non-human animal models (McDougall, Paull, Widdop, & Lawrence, 2000). Furthermore, these responses have been categorized as being predominantly α- and β-adrenergic in nature (Linden, Gerin, & Davidson, 2003). α- and β -ADRs are members of the super family of cell surface receptors that carry out signaling via coupling to guanine nucleotide binding proteins (G-proteins) (Small, McGraw, & Liggett 2003). They are critical components in the sympathetic nervous system's response to disease and maintenance of homeostasis, as they are the target receptors for epinephrine and norepinephrine (Small et al., 2003). Theoretically, alterations in peripheral vascular mechanisms are the proposed basis for the α- and β-ADR sensitivity-modulated CVR, in which exaggerated responses to a stressor produce differing CVR

(Lovallo & Gerin, 2003). These exaggerated responses are proposed to be a result of preclinical alterations in vascular resistance that can cause a disproportionate rise in BP relative to an otherwise normal demand for blood flow (Lovallo & Gerin, 2003). Fredrikson, Tuomisto, and Sundin (1990) report vascular dysregulation to both conditioned and unconditioned vasoconstriction in their comparison study of vascular response to classical conditioning in mild hypertensives versus NT. Miller and Ditto (1991) report patterns of increasing vascular resistance in response to an active-coping psychological stressor, which were purported to be due to α -adrenergic activity, and not neurohumorally independent autoregulation.

Alpha 1A- and Beta 2-Adrenergic Receptors

There are nine subtypes in the family of human ADRs; the α_{1A}- and β_2-subtypes are specifically located in the vasculature (Small, McGraw, & Liggett, 2003). The α_{1A}-ADR gene is located on chromosome 8 at location 8p21 (OMIM # 104221, 2002). The β_2-ADR gene is located on chromosome 5 at location 5q32-34 (OMIM # 109690, 2003). Figure 2-1 shows the approximate locations of each ADR gene. Vascular contraction is controlled primarily by α1-ADRs, and their importance in BP regulation is emphasized by the efficacy of α1-AR antagonists in human HTN (Rokosh & Simspon, 2002; Guthrie & Siegel, 1999; ALLHAT Collaborative Research Group; 2000). The α_{1A}-ADR receptor gene product is required to maintain arterial BP, as evidenced by a recent mouse gene knockout study (Rokosh & Simspon, 2002). Leech & Faber (1996) reported that constriction of rat skeletal muscle arterioles is mediated predominantly by an $\alpha 1_D$-ADR. However, Reja, Goodchild, and Pilowsky (2002) reported α_{1A}-receptor messenger ribonucleic acid (mRNA) expression was significantly greater in spontaneously hypertensive (SHT) rat tissue samples compared with NT rats, and was positively

correlated with SBP in all central tissue investigated. The α_{1A}-ADR mRNA expression level appears to be an important determinant of SPB, and is one of the genetic markers examined in this study.



A B

Figure 2-1. Chromosome (Ensembl human map view) showing the locations of both ADR genes. A) Chromosome 8, with bracket indicating approximate location of the α_{1A} -ADR gene. B) Chromosome 5, with bracket indicating approximate location of the β_2-ADR gene.

During activity or stress, β-AR signaling is responsible for regulating changes in heart rate, BP, and contractility (Reja, Goodchild, & Pilowsky, 2002). After selective β-ADR receptor activation, both β_1- and β_2-ADR elicited dilation of large coronary arteries (Young, Vatner, & Vatner, 1990). Monopoli and colleagues (Monopoli, Conti, Forlani, & Ongini, 1993) reported that human coronary artery contains equimolar amounts of β_1- and β_2-receptor subtypes and that β_2-ADR specifically mediates vasodilation in vascular smooth muscle. Likewise, another study reported predominantly β_2-mediated relaxation in human IMA exposed to both epinephrine and norepinephrine in vitro (Ferro, Kaumann, & Brown, 1993). Polymorphisms of the β_2-adrenoroceptor gene have been

associated with: 1) Interindividual variability in resting SBP and DBP in response to mental challenge (McCaffery, Pogue-Geile, Ferrell, Petro, & Manuck, 2002); 2) The level of resting and stress-related BP (Li, Faulhaber, Rosenthal, Schuster, Jordan, Timmermann, and Hoehe, et al., 2001); and 3) Vascular reactivity as indicated by lower basal blood flow and attenuated increases in forearm blood flow in hypertensive adults (Cockroft, Gazis, Cross, Wheatley, Dewar, and Hall, et al., 2000). A polymorphism of the β_2- ADR gene, the Gln27Glu (glutamine, codon 27, glutamate) which causes a point mutation of cytosine (C) -to-guanine (G), was examined by Bray and colleagues (2000). They reported an occurrence of HTN with the Glu27 allele was 1.8 times higher than with one or two copies of the Gln27 allele (95% confidence interval, 1.08 to 3.00, p = 0.023). Chruscinski and colleagues (2001) demonstrated a positive role for β2-ADR in mediating vascular dilation when BP response to was blunted in a mouse gene knockout model. Knowing that the vascular system is rich with β_2- ADRs and that they mediate vasodilatation, Iaccarino and colleagues (2002) chose to overexpress β_2-ADRs via adenoviral-mediated gene transfer in normotensive Wystar-Kyoto and spontaneously hypertensive rats. They reported successful gene transfer of the β_2- ADR gene and enhanced vasorelaxation in the carotid arteries of hypertensive strain of rats versus the NT strain (n = 8 to 10 per group) after β_2- ADR overexpression (F = 3.088, $P < 0.05$). β_2-ADR appears to be an important determinant of BP, and was the second genetic marker examined in the study.

Pharmacotherapeutic Aspects of Alpha-1A and Beta-2 Adrenergic Receptors

The primary indication for both α- and β-blocking drugs is HTN. In the case of β-blockers, cardioselective types are preferred to reduce side effects caused by blockade of multiple ADR subtypes. Selective peripheral α1 blockers such as prazosin and terazosin

induce vasodilation by blocking the α1 receptors in vascular smooth muscle (arterioles and veins). Their selectivity to α1 causes less reflex tachycardia than drugs that inhibit α2 (Kalkanis, Sloane, Strichartz & Lilly, 1998).

More commonly used in the treatment of HTN are β blockers, like metoprolol, propanolol, and atenolol. The JNC VII (2003) supports their use in various populations and they have been shown to reduce morbidity and mortality in randomized controlled trials. Cardioselective β-blockers principally block β1 receptors and partially block β2 receptors. This reduces side effects of blocking all β2 receptors in the lungs and blood vessels. Although β ADRs, when stimulated cause vasodilation, β-blockers also reduce renin release from the juxtaglomerular cells of the kidney, thus reducing the renin angiotensin system's effect on increasing BP. In addition, β-blockers interfere with sympathetic vasoconstrictor nerve activity and block the effects of catecholamine surges (Khan, 1999). β blockers also reduce heart rate, plasma norepinephrine, muscle sympathetic nerve traffic and systemic norepinephrine spillover, all indices of adrenergic activity in essential HTN (Grassi, 2004).

Population Differences in HTN

The prevalence of HTN in AAs in the U. S. is among the highest of all groups. AAs tend to have worse clinical sequelae than their White, non-Hispanic counterparts (Cooper & Rotimi, 1997). Americans who self-identify with African descent have a 1.5-2 fold increase in prevalence of HTN compared to Americans who self-report descent from Europe; comparing women in these two groups leads to the highest prevalence differences (Eberhardt et al., 2001). Disease management is often particularly problematic, with AAs often requiring a more than one (and often multiple) anti-hypertensive medications to effectively manage their high BPs, many of whom cannot

afford them. HTN is a particular problem in health care in the Southeastern U. S., as the prevalence of HTN among blacks *and* whites is greater, and death rates from stroke are higher in this region than others (AHA, 2003).

In regards to developing HTN, examples of racial differences in physiologic mechanisms and pharmacologic responses involving ADRs exist in the literature. Differences in CVR are noted in relation to BP and heart rate, and include α- and β-adrenergic patterns that are associated with racial cohorts--particularly AAs versus Caucasians. Anderson, McNeilly, & Meyers (1992) explain two dichotomous CVR patterns that are reported to be associated with SIR: the myocardial and the vascular. These patterns are summarized in Table 2-1.

Table 2-1. Adrenergic cardiovascular stress patterns.

Myocardial Reactivity Pattern (β-adrenergically driven)	**Vascular Reactivity Pattern (α-adrenergically driven)**
↑ *in BP associated with:* ↑ Cardiac Output (C.O) ↑ Stroke Volume ↑ Heart Rate ↑ Epinephrine and Norepinephrine ↓ in Total Peripheral Resistance	↑ *in BP associated with:* ↑ Norepinephrine ↑ Total Peripheral Resistance
Characteristic of Caucasian reactivity pattern	Characteristic of Black/AA reactivity pattern

There are reported differences in drug disposition and responsiveness in relation to adrenergic-agonists and antagonists (Wood & Zhou, 1991), implying ADR differences across SIRs. Some support the above model, and some do not. Sentinel research shows that β-blockade and combined α- and β-blockade via pharmacological agents appear to be less efficacious in AAs, South Africans, Jamaicans, and West Indians as compared to

Caucasians (Humphreys & Delvin, 1968; Jennings & Parsons, 1976; Seedat, 1980). Another seminal study in 1977 by the Hypertension Detection and Follow-up Program Cooperative Group reported β-blockers to be less efficacious in African Americans. Gibbs, Beevers, and Lip (1999) purported that this may be due to decreased cardiac output and renin release, causing increased total peripheral resistance. In support, Wood (2002) reported a marked impairment of β_2AR-mediated vasodilation in blacks, accompanied by increased α-adrenergically mediated vasoconstriction, as well as racial differences in response to endogenous and exogenous agonists. Also, vasoconstrictor response to endogenously stimulated norepinephrine is higher in blacks than whites (Stein, Lang, Singh, He & Wood, 2000). Male, black Veterans residing inside the 'Stroke Belt' (southeast U.S.) are reported to have lower treatment success rates with captopril ($p = 0.07$); and, regardless of region, blacks in the study were less likely than whites to achieve successful lowering of their BP with atenolol ($p = 0.02$), prazosin ($p = 0.03$), and more likely with diltiazem ($p = 0.05$) (Cushman et al., 2000). Jamerson and DeQuattro (1996) disagree, explaining that while observed responses of blacks to both ACE inhibitors and β-blockers in the treatment of HTN are less favorable than is seen in whites, the responses are still clinically significant. Literature on nonpharmacologic differences by SIR is also present. Ferro and Walton (2001) report significant differences in short-term BP responses to a 10-week regimen of nonpharmacological treatments (dietary, activity, stress reduction, and education sessions) in HTN for African/Caribbean blacks compared to whites. Blacks and the control group experienced no change in either systolic or diastolic BP at 10 weeks, and statistically significant decline in systolic ($p < 0.005$) and diastolic BP ($p < 0.05$) were seen in the Caucasian group. In a 2005 meta-

analysis of 137 monotherapy clinical trials and 28 combination therapy trials (totaling 11,739 participants), Wu and colleagues reported that AAs had better BP reduction with calcium channel blockers than their non-AA counterparts ($p = 0.001$); and, that non-AAs responded better than AAs to α1-blockers, β1-blockers, and angiotensin converting enzyme inhibitors ($p = 0.0001$) (Wu, Kraja, Oberman, Lewis, Curtis, Ellison, and Arnett; 2005).

The estimated heritability for HTN in people of sub-Saharan African descent is 45-68% (Rotimi, Cooper, Cao, Ogunbiyi, and Ladipo, et al., 1999; Gu, Borecki, Gagnon, Bouchard, Leon, and Skinner, et al., 1998). Genetic studies of association have reported racial differences in allele frequency of ADR polymorphisms (Hindorff, Heckbert, Psaty, Lumley, Siscovick, and Herrington, et al., 2005; Small, McGraw, & Liggett, 2003; Xie, Kim, Stein, Gainer, Brown, & Wood, 1999).

This review of the literature highlights the many inconsistencies across studies in regards to the influence of race on health and disease. A number of things can explain these inconsistencies: study design issues; differing measurement/report of race; inadequate power; and different medications studied. Another plausible explanation is that the differences are really attributable to non race-based, interindividual variability.

Recommendations for the Collection of Racial and Ethnic Data

Much like its original use for classifications of groups, some mainstream definitions of race today infer major biological underpinnings. The online Merriam-Webster dictionary (2003) defines race as “a division of mankind possessing traits that are transmissible by descent and sufficient to characterize it as a distinct human type”. This definition inherently includes genetics as a factor in race by use of the phrase ‘transmissible by descent’. Numerous similarities in other definitions exist, involving

such phrases as: ‘physically distinguishable,’ ‘having common ancestries,’ and ‘having certain biological characteristics that set them apart from other groups.’ It is easy to see why and how the paradigm of linking genetics to race exists even now, as these are definitions from no longer than a century ago. Very recently, there has been an upsurge of efforts to discard notions of race as biologically-associated. Some researchers and ‘civilians’ wish the term to be recognized solely as an antiquated system of skin-color-based classification that inherently carries with it socio-politically charged notions of racism. Others argue for *less* biologically-based definitions of race that incorporate social beliefs about language, history, and culture (such as Witzig, 1996). Definitions like these seem very similar to those of ethnicity, where language, history, culture, and socio-political factors are main constructs of this term. To further complicate the matter, some utilize the term ethnicity with the presumption that they are avoiding any biological undertones inherent in the term ‘race’; however, definitions of race and ethnicity are markedly similar and most people use the two terms interchangeably (Sankar & Cho, 2002). In a meta-analyses of articles published in *Nursing Research*, authors Drevdahl, Taylor, and Phillips (2001) present this case well, comparing three operational definitions of race and three of ethnicity used (some cited from other sources) by nursing researchers within the 1990s and 2000. Race and/or racial group was defined as: “Imply[ing] biological characteristics…that are genetically transmitted from one generation to another” (Schubert & Lionberger, 1999, p. 116); “Concept that signifies and symbolizes sociopolitical conflicts and interests in reference to different types of human bodies” (Winant, 2000, p. 172); and, “Group that is socially defined as having certain biological characteristics that set them apart from other groups, often in invidious ways (Pincus &

Ehrlich, 1999, p12). The three ethnicity and/or ethnic group definitions were: “Contains information about the history of the population, and hence the genetics of the group, as well as sociocultural information” (Crews & Bindon, 1991, p. 45); “Group that has certain cultural characteristics that set them off from other groups and whose members see themselves as having a common past” (Pincus & Ehrlich, 1999); and, “ Segment of a larger society whose members are thought…to have a common origin and to share important segments of a common culture and who…participate in shared activities in which the common origin and culture are significant ingredients” (Yinger, 1994, p. 3). While debate continues over the construct of race and ethnicity, it is generally agreed upon that: Whatever definition is used, it should be clearly delineated (Sankar & Cho, 2002); Racial classifications should be critically evaluated for their usefulness and contribution to the testable theories (Duster, 2001); and, The methods of capturing race and/or ethnicity should be carefully outlined (Williams, & Jackson, 2000).

Standardization of definitions is a major issue and is recommended for consistency in reporting results in research (U.S. Department of Health and Human Services [USDHHS], 2003). The OMB Race and Ethnicity Classification system can be utilized for standardization. It includes both race and ethnicity categories, and defines race by context of geographical origin. Cultural associations are accounted for in the Ethnicity self-report section. Users have the option of selecting more than one self-affiliated category of race; or, the option to not answer the question at all. Based on this knowledge, this study will utilize the OMB Classfication system. This self-report system allows the user to choose more than one race, if applicable. It utilizes standardized

constructs of race and ethnicity that are rooted in ancestral and geographic origins of their predecessors and is widely used in research.

Implications for Studying Disease by Race

An abundance of health disparities literature exists on the social, ethical, and legal ramifications of studying disease by SIR. Historically speaking, some experiments that target racial groups have resulted in serious social and ethical problems for that population (for example, the Tuskegee syphilis experiments). Current debate in regards to identifying genetic differences by race has identified many concerns, including the potential to send the message that researchers are trying to find clear biological differences that would imply certain races are 'unequal' to others. People fear that finding biological differences will justify certain social inequalities. The key opposition to studying diseases by race are the following points: 1) There may be many inherent problems in measuring and grouping races in a multi-racial society for the purpose of genetic clustering (Wilson, Weale, Smith, Gratrix, Fletcher, and Thomas, et al., 2001; Williams & Jackson, 2000); 2) The *sociopolitical* context of race is an important variable that is often disregarded in research (Burchard, Ziv , Coyle, Gomez, Tang, and Karter, et al., 2003) ; 3) Fear of justifying inequality (Bonham, 2003); and 4) Racial boundaries are *not* likely to be equally useful in all kinds of genetic research (Sankar & Cho, 2002). Others argue for research that examines race *carefully*, making sure to address the above concerns. Duster (2001) purports that race should not be discarded as a variable in research just because the categories do not biologically map exactly, and that race remains alive in the context of practical application. Fullilove (1994) warns against *a priori* consideration of race as important in medicine without question, but also states that

little is truly done to explain the meanings in associations between health outcomes and race.

In certain disorders and diseases, race is very much a risk-associated variable. Sickle-cell anemia (SSA) maintains a significantly higher prevalence in people of African and Mediterranean descent. Likewise, Cystic Fibrosis (CF) is more likely to affect those from Western European descent. That is not to say that whites have not been diagnosed with SSA, or that cases of CF have not been seen in blacks or other non-whites. It is only to say that there is a higher risk associated with particular diseases among certain populations. Population genetics has ascertained that greater genetic differentiation occurs between *continentally separated groups* (Burchard et al., 2003), and that more variation is present within racially-stratified populations than between them. Nonetheless, others have reported great genetic variation among the five racial groups (five groups as categorized by the OMB classification system) (Risch, Burchard, Ziv, & Tang, 2002). Genetically speaking, some racial groups possess low frequencies of certain alleles associated with disease, (2003). Whether or not the low frequency of alleles is truly associated with race or simply a result of sampling methods or statistical errors in the research is currently under debate. While it is more easily seen in "simple" diseases like SSA and CF, the genetic influence of race is much more difficult to ascertain in complex diseases like type 2 diabetes (T2DM), asthma, HTN, and Alzheimer's disease. Nonetheless, specific susceptibility gene variants for chronic diseases have been found in specific populations. Phimister (2003) explains that a variant of the calpain-10 gene (associated with T2DM) is specific to a population of Mexican Americans in Texas. Moreover, a variation of the *ETS* family of genes that predispose carriers to asthma has

been discovered in a population inhabiting the island of Tristan da Cunha of the South Atlantic (2003). In recent editorial, Fine, Ibrahim & Thomas (2005) cite three studies of complex genetic disorders (Crohn's disease and Factor V Leiden) where genetic variation by race was reported (Ridker, Miletich, Hennekens, & Buring 1997; Shen, Lin & Tsay, 1997; Hugot, Chamaillard, Zouali, Lesage, Cezard, and Belaiche, et al., 2001).

For some, results of these studies are not convincing enough to conclude that race is an appropriate variable for use in research. Cooper, Kaufman, and Ward (2003) argue against studying disease by race, first asserting that race-specific findings in research are better explained by environmentally-determined socioeconomic factors. They (2003) also report examples of inconsistencies in research in which Type I error affected the outcomes and interpretations, such as with the reported race-specific effect of ACE inhibitors in the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial. Cooper and colleagues also argue that true race-specific genetic results have not been found, and are "mathematically and biologically implausible" (p. 1167, 2003). In 2001, Wilson and colleagues performed a unique study designed specifically to test the validity of race and ethnicity as genetic research variables. They studied eight populations of varying origin, some of which were extremely specific (for example, Amharic- and Oromo-speaking Ethiopians from Shewa and Wollo provinces collected in Addis Ababa). Using a model-based clustering method known as STRUCTURE, they were able to estimate the proportion of each individual's genome having ancestry in each cluster. One underpinning in this model is the idea that admixture plays an important role in the variability of race. Genetic admixture reflects multiplicity or variation in race and ethnicity (Burchard et al., 2003). Wilson and his co-authors ascertained the

apportionment of individuals (average per-individual proportion of ancestry) by using the STRUCTURE model to characterize 'clusters' based on a set of allele frequencies at each locus. From there, the researchers matched the clusters with specific geographical areas (in this case, four broad regions), and interpret the similarities seen in each cluster. Because 62% of the Ethiopians fell into the cluster with the Jews, Norwegians, and Armenians, they concluded that identifying these people as "black" in race would be an "inaccurate reflection of the genetic structure" (2003, p. 266). They concluded similar results with subjects from China and New Guinea in which "Asian" race grouping would have been inappropriate (2003). While almost truly convincing, this study requires replication, and use of "presumably neutral microsatellite markers" (p. 265) needs to be validated as truly neutral. Based on these and other data, many researchers have moved toward the use of ancestry, rather than race or ethnicity in their research models. In fact, self-reported ethnicity and ancestry constructs have been related in biological models. Helgadottir and colleagues (2006) found that self-reported ethnicity was highly correlated with genetic determination of estimated individual ancestry (via ancestry informative markers) and even group ancestry (determined by weighted least squares). They also reported ethnicity-based differences in risk for myocardial infarction in African Americans who had European admixture. This implies that self-reported ethnicity can be informative and useful in genetic research as a means to group individuals for comparison.

The PI agrees that numerous other factors may impact the poor health outcomes seen in the AA population, including various socioeconomic indicators and factors related to health care access and delivery. It is important to reiterate that the literature

presented clearly identifies specific pathophysiologic and biologic differences among racial groups involving HTN that direct this line of research. In further support of this venture, at least one other (Krieger, 2005) suggests that in the case of gene expression, observed phenotype differences seen among populations could possibly reflect variation in gene expression (more so than simple gene frequency) because of the nature of gene expression patterns occurring in the context of certain (perhaps shared) environmental conditions. Moreover, one charge of the Task Force of the American Sociological Association was to comment on the further investigation of race and ethnicity in the contribution of disparate outcomes (2003). They presented the example of AA health disparities in regards to HTN and affirm that research needs to continue in this line of research to distinguish between social and biological forces at play (2003). Properly designed research in this area could provide answers regarding molecular differences and poor health outcomes, a better understanding of the disease process in certain populations, and tailoring of medications or gene therapy. Furthermore, proper statistical analyses and careful interpretation of findings can strengthen results and help to reduce potential 'racial profiling.'

The ethical principle of social justice, in its simplest form, states that all people should be afforded equal benefits, (such as goods and services) regardless of their personal characteristics, choices, or beliefs. Current research that examines race as a variable has also been criticized for limiting social justice. For example, research that excludes subjects based on their race (a practice that is declining) prevents certain groups from reaping the benefits of a study. Similarly, certain socioeconomically limited groups tend not to benefit from research because of the inherent cost associated with new

technology that comes from it. As certain races have been associated with lower socioeconomic status (SES), social justice is compromised for these groups. It is from research such as this that we learn of the social and ethical ramifications that can be incurred if research is not properly conducted involving race and disease. In the research community we must be most careful in our interpretation of data involving race, ethnicity, ancestry, and/or socioeconomic status so that misleading conclusions are not made that adversely affect social justice, policy, and practice. We should be, at minimum, cognizant of how the design, implementation, analyses, and interpretation of results involving health disparities can affect the overall well-being of socioeconomically disparate groups.

Positive Inotrope Administration

Short-term positive inotropes are often administered post-operatively to CABG patients to increase vascular resistance in cases of acute congestive heart failure, cardiac arrest, hypovolemia, and arrhythmias. The three subclasses of positive inotropes are: cardiac glycosides (digitalis, digoxin); β-adrenergic agonists (dopamine, dobutamine, epinephrine); and phosphodiesterase inhibitors (milrinone, amrinone, enoximone). The need for positive inotropes in the post-operative phase of recovery from bypass surgery can be informative as an indirect assessment of the subjects' cardiovascular reactivity. As ADRs are involved in vascular tone, knowledge about whether or not a subject needed this medication could help to explain some of the differences in gene expression of the α_{1A}- and β_2-ADR genes.

Genetic Analysis Techniques

There are many types of genetic methodologies used in research today. Linkage studies identify regions of the genome that contain putative candidate genes that are

proposed to be related to a phenotype or disease process based on their location on chromosomes. Association studies investigate the prevalence of certain gene alleles and their association with a phenotype, or disease process. Both of these methods examine deoxyribonucleic acid (DNA). Unlike these, gene expression studies examine the relationship between the level of ribonucleic acid (RNA) and disease. While DNA is a double-stranded nucleic acid made up of nucleotides, RNA is single-stranded, and is the result of transcription of genetic information; the information encoded in DNA is transcribed into mRNA, which is an intermediate and one of the regulatory steps in the synthesis of new proteins. Many cellular characteristics concerning survival, growth and differentiation are reflected in altered patterns of gene expression and the ability to quantify transcription levels of specific genes is central to research into gene function (Zamorano, Mahesh, & Brann, 1996).

Common gene expression methods (quantification of steady-state transcription) are northern blotting, *in-situ* hybridization, RNAse protection assays, cDNA arrays, and RT-PCR (Bustin, 2000). RT-PCR is a type of PCR that allows one to compare the levels of a specific mRNA in different sample populations, to characterize patterns of mRNA expression, to discriminate between closely related mRNAs, and to analyze RNA structure (Bustin, 2000) . This method involves isolating mRNA from the biological sample (in this case, IMA) and performing reverse-transcription (RT) to make complimentary DNA (cDNA) The cDNA represents only the mRNA component of the total RNA, which can then be analyzed by gene expression equipment. TaqMan is a type of RT-quantitative PCR method that continuously measures (in real time) accumulated PCR product. The PCR product reflects the original level of mRNA template (See Figure

2-2). This is measured using a TaqMan probe. The probe is a dual-labeled fluorogenic oligonucleotide. The dual-labels are a reporter dye and a quenching dye. The ABI Prism (Applied Biosystems HT 7900) equipment and software examines the fluorescence intensity of the reporter and quencher dyes and calculates the increase in normalized reporter emission intensity over the course of the PCR amplification (Genomics and Proteomics Core Laboratories, 2003) within all samples located on the 96-well plate. The normalized reporter is known as a housekeeping gene. This is a type of gene in which there is a known and predicted level of expression. Its purpose in gene expression analysis is to act as an internal reference or control, whereby all samples are normalized by this same gene. (Practically speaking, since the level is known, the housekeeping gene values are subtracted from the sample values to obtain the normalized levels.) This allows relative expression to be established, instead of absolute quantification of the data, which is tedious and impractical (Bustin, 2000). A spectrum of standard housekeeping genes are available. The protocol for this study will use glyceraldehydes-3-phosphate dehydrogenase (GAPDH), a housekeeping gene that has been previously used with successful results in normalizing data for ADR gene expression in arterial tissues (Wang & Brown, 2001; Reja, Goodchild & Pilowski, 2002; Peuster, Fink, Reckers, Beerbaum & von Schnakenburg, 2004).

Northern blotting, *in-situ* hybridization, RNAse protection assays, and cDNA arrays are all alternate methods of measuring gene expression. Northern blotting isolates RNA by elecrophoretic separation on an agarose gel, ‘blotting’ or transferring RNA fragments from the gel onto a membrane (usually nitrocellulose), adding a labeded probe to the membrane and detecting the band with the probe bound to the target sequence

(CRISP Thesaurus, accessed 8/2/05). In situ-hybridization determines the presence of an RNA sequence of interest by hybridizing a probe to the target sequence, and visualizing on a microscope the location of the bound RNA target in the chromosome or cell (cytoplasm, for example) (Human Genome Project, Talking Glossary, accessed 8/2/05). RNAse protection assays examine gene expression by hybridizing antisense RNA corresponding to known genes with an unknown sample. Next, the sample is digested with a single-strand specific RNAse and any surviving RNA left is presumed to be complimentary to the antisense and therefore, transcribed from the gene of interest (CRISP Thesaurus, accessed 8/2/05). Finally, cDNA arrays (more commonly known as microarrays) utilize a microarray 'chip' or platform with many small spots that correspond to a different gene on each spot. The spots are pre-treated with cDNA that is the only coding part of the sequence of interest that corresponds to an mRNA transcript. These cDNA spots are hybridized with a probe. The chips are incubated in solution containing the genetic material being investigated. Messenger RNA transcripts floating in the solution hybridize to their cDNA already on the chip. When the chip is exposed to ultraviolet light, the fluorescent probes emit light at varying intensities, allowing qualitative comparison of expression between the different genes on each chip and between subjects (Rice University Connections webpage, accessed 8/2/05). These techniques can be limited in their sensitivity (Melton, Kreig, Rebagliati, Maniatis, Zinn & Green, 1984) and in their cost-effectiveness (Bustin, 2000). RT-PCR is the optimal method when evaluating a limited number of genes and starting mRNA template is low. There are additional advantages in using TaqMan Real Time RT-PCR gene expression analysis. Unlike other forms of quantitative RT-PCR, this method quantitates the *initial*

amount of the mRNA template (the geometric phase), rather than the *final* amplified product (the plateau phase), allowing detection of a 2-fold versus a 10-fold change. This improves the sensitivity, specificity, and reproducibility of the method (Dorak, 2003; Dawson, 2003). Real Time RT-PCR also involves only three major steps, whereas other conventional RT-PCR methods involve nine steps. Reducing the number of steps in the gene expression process assists in minimizing error in sample analysis. Also, once the ABI Prism equipment has completed its fluorescence phases, the data are fed into a computer linked to the equipment, eliminating the need for post-PCR processing (Bustin, 2000). The only clear disadvantage of this method is the cost of the predeveloped reagents and the ABI Prism, but it is still more cost-efficient than cDNA array (microarray) methods when evaluating a small number of genes. The TaqMan RT-PCR principle steps are represented in Figure 2-3.

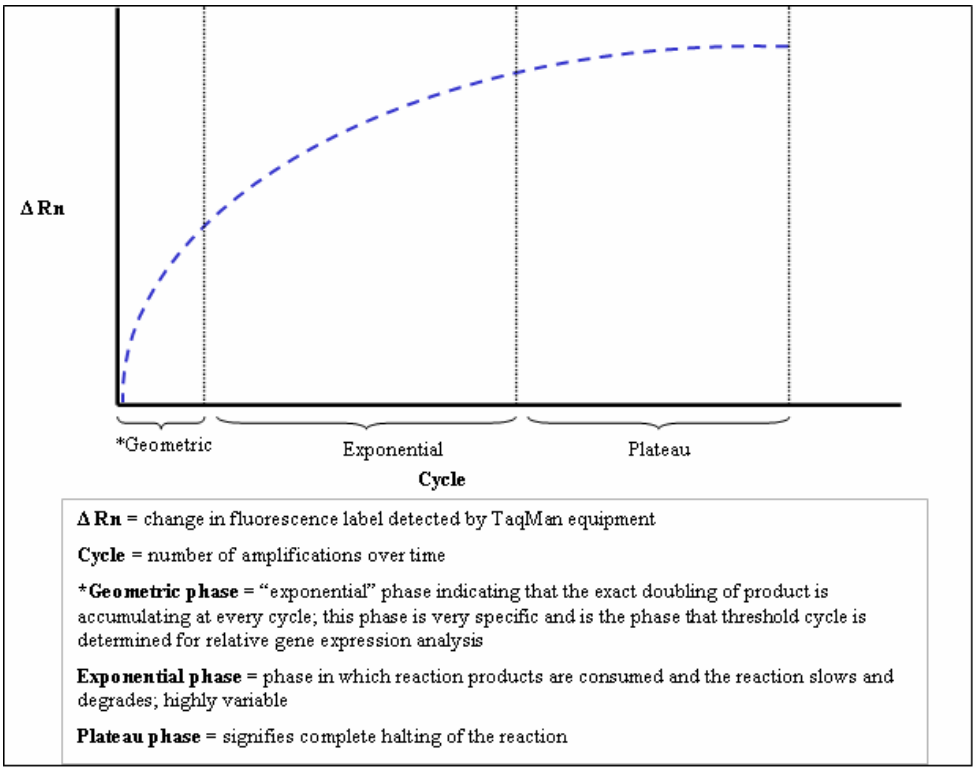


Figure 2-2. Amplification of gene expression using TaqMan Real Time RT PCR.

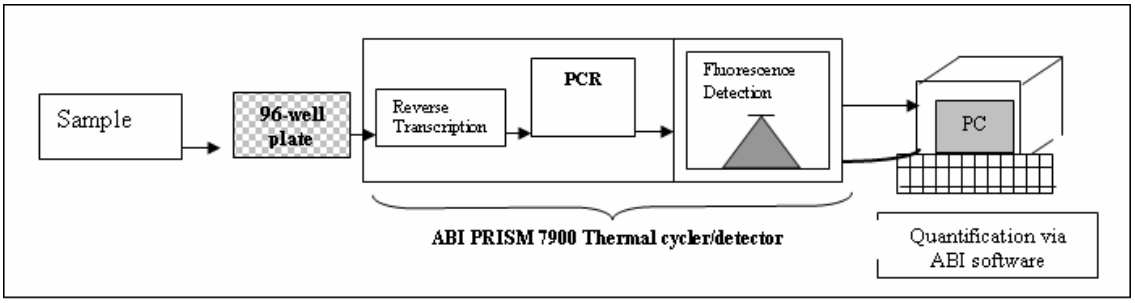


Figure 2-3. TaqMan RT-PCR steps (adapted from Bustin, 2000).

Collection of Human Tissues for Gene Expression Analyses

Tissue samples and/or biopsies are the source of choice for analysis of gene expression/transcription, simply because it is the direct site at which to examine the mRNA levels mediating protein production in the body. It is widely accepted that mRNAs found in circulating blood lymphocytes provide indirect evidence of this process. During CABG, it is common for portions of surgical remnants of IMA to be discarded. The IMA branches off of the left subclavian artery and supplies the thoracic cavity with oxygenated blood. It is most typically used for bypass of the left anterior descending coronary artery. As previously described, α_{1A}- and β_2-ADRs are specifically located in the vasculature (Small, McGraw, & Liggett, 2003). $Alpha_{1A}$ mRNA has been detected in the IMA (Gow, Mitchell, & Wait, 2003). β_2-AR has been detected in the human coronary arteries (Monopoli, Conti, Forlani , & Ongini, 1993) and in the human IMA (Ferro, Kaumann, & Brown, 1993). Very minute amounts of arterial tissue (about 30 mg) are necessary for this type of analysis, which involves isolating messenger RNA from the tissue sample and performing reverse-transcription to make cDNA, which can then be used for TaqMan (Real Time) gene expression analysis.

Summary

HTN has a genetic component, as supported by empirical and experimental data. The α_{1A}-ADR and β_2-ADR subtypes of the ADR receptor genes are hypothesized to play a role in mediating the disease process of HTN via gene expression differences. Race and/or ethnicity may also contribute to the variance seen in HTN and adrenergically-driven vascular tone, as supported by previous studies described. While it is controversial to use race/ethnicity as variables in genetic research, the use of a standardized measure and careful interpretation can be informative and help reduce the potential for social injustice.

The use of Real-Time PCR for analysis of gene expression is an ideal method for examining steady-state transcription levels and comparing relative fold-differences between groups. This analysis can provide direct information about the function of these genes in the given environment at that particular moment. Tissue samples are the desired source for examining mRNA levels; however, human studies of the α_{1A}-ADR and β_2-ADR genes expression in human tissue are not currently reported. This study attempts to fill the gap in this knowledge by using human arterial tissue for analyses of the α_{1A}-ADR and β2-ADR genes expression levels between hypertensive and normotensive adults.

CHAPTER 3
PROCEDURES AND METHODS

Introduction

This chapter presents study design and protocols. Details regarding recruitment techniques, research settings, variables, and methods are discussed. The following methods are thoroughly explicated: collection and storage of samples, processing of tissue and blood for isolation of genomic DNA and RNA, and post-isolation processing of RNA for gene expression analyses.

Design

This study used an exploratory, quantitative design to meet the goals of the aforementioned specific aims. This was a two-arm gene expression study to compare NT versus hypertensive persons primarily, then to explore differences between two self-reported race categories: Black/AAs and White/Caucasians. Recruitment goals were set at a total of 60 subjects with 15 subjects in each arm. This sample size was based on a formulation of 82% power, an effect size of 0.25 (medium), and a significance of 0.05 for a two-tailed test. Gpower computer software was used to calculate the required sample size (Erdfelder, Faul, & Buchner, 1996). A medium effect size was consistent with other gene expression studies of adrenergic mechanisms in HTN (Wang & Brown, 2001).

Following consent, pencil and paper data collection was used to obtain some basic demographic and medical history information. During surgery, a small amount of IMA, (normally discarded during CABG surgery) was collected and later analyzed for α_{1A}- and β_2-ADR gene expression via TaqMan (Real-Time) RT- PCR. Additionally, about 10 cc

of blood was collected intra-operatively for genotyping. A post-operative chart review was completed to determine the subjects' need for positive inotrope pharmacotherapy while in intensive care.

Subject Recruitment

Prior to initiation of the study protocol, Institutional Review Board (IRB) approval was obtained from the UF IRB-01 and the VA Subcommittee for Clinical Investigations. Adult subjects between the ages of 21 and 70 who were scheduled for CABG surgery were recruited from those admitted to the University of Florida Thoracic and Cardiovascular Surgery (TCV) team. These subjects included patients from the following facilities: Shands at Alachua General Hospital and the Malcolm Randall Veterans Administration Medical Center (VAMC), both located in Gainesville, Florida. These facilities mainly serve Alachua County, but often include patients from the entire North Central Florida and surrounding areas.

HIPAA Waivers of Authorization were obtained from the UF and VAMC IRBs. This IRB-approved waiver allowed the applicant to review charts of the scheduled CABG patients to determine which patients met the inclusion/exclusion criteria of the study. Inclusion and exclusion criteria with rationale are presented in Table 3-1. Prior to beginning the study, all surgeons agreed to have their patients screened for this study. These individuals were contacted by telephone or via face-to-face meeting during pre-operative appointments for recruitment. This recruitment process was continued until the planned group allotments were filled. Subjects were considered hypertensive if their medical chart indicated: a) diagnosis of HTN by a practitioner, b) three consecutive office BPs $\geq$ 140/90 mmHg, or c) prescription of antihypertensive medications specifically for high BP. Subjects were also asked to verify that they were diagnosed with high BP.

Subjects were not excluded on the basis of race, religion, ethnicity, socioeconomic status, or level of education. Women and minorities were recruited for this study. The AHA (2003) reports that more men than women have high BP until age 55. From age 55 and older, the percentage of women with high BP continues to increase (2003). Moreover, HTN is primarily an underlying cause of death for more women than men (2003). As indicated in the TCV surgery database, the average percent of women undergoing CABG surgeries by the TCV surgery department is 21%. This means that for every one female undergoing the procedure by this department, there are approximately 5 males. U.S. Census data for Alachua County indicates that approximately 73.5% of the population is Caucasian, 19.3% is AA, and 7.2% is 'other'. The JNC VII (2003) reports that health disparities exist in minority populations with HTN. Inclusion of women and minorities will create a study population that is representative of the entire population of those undergoing CABG procedure. Children were not included in this study. While 50 million Americans age 6 and older have high BP (AHA, 2003), it is rare for children to have CABG surgeries. While it is important to study childhood HTN and its long-term consequences, it is not feasible to recruit such a minimally represented population (children with HTN who undergo CABG).

Table 3-1. Inclusion and exclusion criteria with rationale.

Inclusion	Rationale
age 21-70	-children excluded: rare CABG -There is evidence that genetics still plays a role in HTN even into late 70's, without confounding of isolated systolic hypertension (ISH) past the age of 70; however, ISH results in a clinically and pathophysiologically different phenotype from essential HTN (Sleight, 2004). This difference in phenotype could lead to a difference in gene expression that would confound the data.

Table 3-1 Continued.

Inclusion	Rationale
undergoing scheduled CABG surgery	-prime surgery to obtain arterial tissue that is often discarded (Wang & Brown, 2001) -improper data collection could occur with unscheduled cases
read/write English	-unable to provide interpreter for multiple languages
Exclusion	**Rationale**
undergoing heart surgery that does not include the internal mammary artery (IMA)	-the IMA is most frequent artery used in bypass surgery, usually grafted to the left anterior descending artery
low cardiac output syndrome (LCOS)*	-subjects with this post-operative hemodynamic diagnosis may exhibit increased total peripheral resistance secondary to the diagnosis and confound the inotrope-related data

Consenting Process and HIPAA Regulations

The PI obtained approval from the UF and VAMC IRBs for human subjects' research. Subjects signed an informed consent to participate in the study, including informed consent for chart review and research use of normally discarded surgical remnants of IMA (see Appendices B & C). Subjects were not compensated for their participation in the study. Subjects were informed they could withdraw from the study at any time. There was no anticipated direct benefit to the subjects: they did not receive any information concerning their hemodynamic responses to positive inotrope administration, nor did they receive results of their gene expression of α_{1A}-and β_2-ADRs. This eliminated the need for another informed consent that was designed specifically for disclosure of genetic information, and also eliminated the need for genetic counseling related to testing and/or results. It is uncertain at this time what the expression of these genes in human tissue actually means in terms of health benefits; therefore, lay interpretation would be difficult at this time.

Data collection containers were labeled with a subject ID barcode sticker. A hand-written table containing the coding system for the subjects was kept in a locked filing cabinet with only the PI having access. This was the only source of data that matched the subject to the ID code.

Setting

This study was completed in Gainesville, Florida at the Gainesville VAMC and Shands at Alachua General Hospital facilities. Screening of patient charts for eligibility occurred at the VAMC and the TCV surgery office in the UF Health Science Center. Subjects were approached for recruitment and consented at the VAMC, AGH, or at Cardiology Associates of Gainesville, all places where subjects were either undergoing pre-operative assessments or were admitted. Data collection occurred in the VAMC and AGH 'heart' surgical suites. Blood and tissue samples were stored at the UF Center for Pharmacogenomics. Laboratory analysis occurred at the UF Pharmacogenomics Core facility and the UF Interdisciplinary Center for Biotechnology Research (ICBR). Subject folders with consents and hard-data were stored in locked filing cabinets in the PI's student office space at the UF College of Nursing.

Research Variables

The independent variables were diagnosis of HTN/NT and self-identified OMB racial category. Both were categorical, nominal variables. SIR (self-identified race) was determined by self-report of one or more of the five categories as defined by the OMB (see Appendix A). Diagnosis was a dichotomous variable with either HTN or NT as variable choices. The dependent variables were gene expression of α_{1A}- and β_2-ADR (continuous), genotype (categorical/nominal), and post-operative positive inotrope administration (dichotomous, nominal). Gene expression was determined using the ABI

Prism 7900 and ABI Assays on Demand for α_{1A}-ADR (Hs00169124_m1) and β_2-ADR (Hs00240532_s1) (Applied Biosystems, Foster City, CA).

Relative gene expression of α_{1A}- and β_2-ADRs were utilized to determine the values of the gene expression variables, as outlined by Livak and Schmittgen (2001). Three ADR single nucleotide polymorphisms (SNPs) were examined. The α_{1A}-ADR (Codon 347, refSNP ID:1048101) SNP is located on chromosome 8 at location 8p21, in the second exon, or coding region. The β_2-ADR (Codon 16, refSNP ID:1042713 & Codon 27, refSNP ID: 1042714) SNPs are located on chromosome 5q32-34, both in the first (and only) exon of the gene. Figures 3.1 and 3.2 show the loci of investigated polymorphisms with relation to the α_{1A}- and β_2-ADR genes. Genotypes for the α_{1A}-ADR (Codon 347), β_2-ADR (Codon 16 & Codon 27) were determined by PCR followed by pyrosequencing (PSQHS96A System, Biotage, Uppsala, Sweden) on genomic DNA isolated from blood samples (PSQäHS 96 System, Uppsala, Sweden). Chart review was conducted to determine subjects' need for post-operative positive inotrope medication.

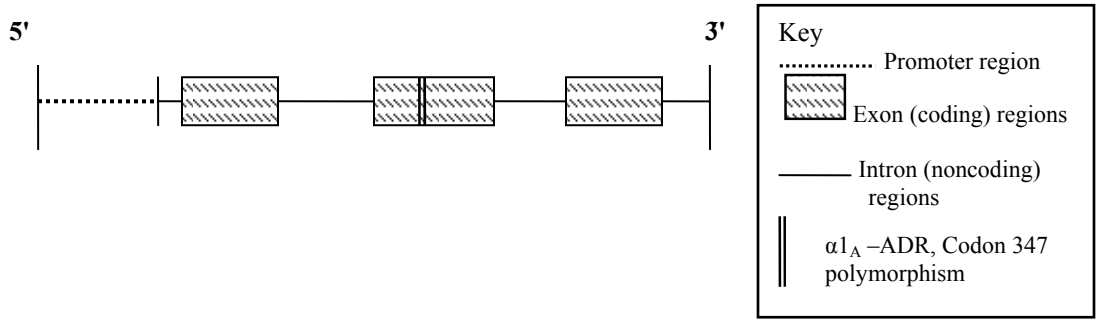


Figure 3-1. α_{1A} -ADR gene with promoter, intron and exon boundaries and investigated polymorphism.

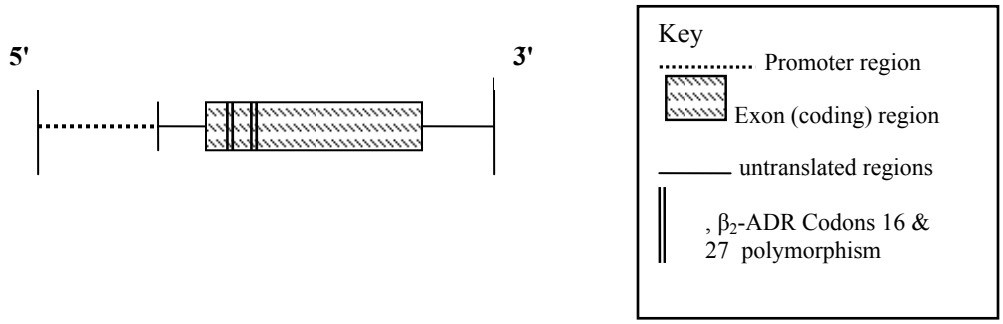


Figure 3-2. β_2-ADR gene with promoter, exon boundary and investigated polymorphisms.

Study Protocol

Data Collection and Laboratory Methods

Subjects completed a pencil and paper demographic form that provided information about their race, age, past medical history, medication use, height, weight, and income. As recommended by the Federal Drug Administration ([FDA], 2003), race was determined by self-report using the OMB revised race and ethnicity categories.

Blood and tissue collection

All blood and tissue samples were collected in the surgical suites. After the patient was anesthetized for surgery, approximately 5-10 cc of arterial blood obtained from the central arterial line was placed in a purple-top tube containing EDTA and placed in a cooler with ice. The surgical remnants of IMA pedicle were cleaned by either the PI or the surgeon after removal from the patient and then placed in a sterile specimen container by the PI. The PI quickly (in a sterile field), cut the tissue to pieces smaller than 0.5 cm, and transferred the pieces immediately to a microtubule containing 100 microliters of RNAlater solution (Qiagen ®, Valencia, CA, USA) (see Figure 3-3). RNAlater is a nontoxic, aqueous tissue and cell storage reagent that protects cellular RNA in intact and unfrozen samples. It stabilizes RNA and preserves its integrity by halting mRNA degradation upon its infusion into the sample. At this point, according to Qiagen's

instructions, the RNA is protected from degradation for 24 hours at 37º C, one month at 4º C, and indefinitely at -20º C. Completing these steps in a very quick manner minimizes RNA degradation and any changes in the mRNA expression level; thus, the PI worked very quickly to complete this process, which often took less than 1 minute to complete. The samples were then placed in a cooler with ice and transported to the UF Center for Pharmacogenomics Core Laboratory, where they were incubated at 2-8°C at least overnight, but no more than 12 days, then placed in a freezer at -80º C.

Figure 3-3. Tissue pieces immersed in RNAlater preservation solution.

Genomic DNA analyses

Genomic DNA was isolated from blood lymphocytes using a commercially available kit (Qiagen DNA Blood Isolation Kit ® (Qiagen, Valencia, CA, USA). Genotype was determined by polymerase chain reaction (PCR), followed by pyrosequencing (Pyrosequencing, Uppsala, Sweden) (Langaee & Ronaghi, 2005) using a PSQ HS96A single nucleotide polymorphism (SNP) reagent kit according to the manufacturer's protocol (Biotage AG, Upspsala, Sweden). In summary, 10 µl of biotinylated PCR product was immobilized to streptavidin-coated Sepharose beads® (Amersham

Biosciences, Piscataway, NJ). After incubation, the beads were isolated and treated with 70% ethanol, denaturation buffer, and wash buffer. The beads then were released into designated wells containing annealing buffer and 10 pmol of sequencing primer, followed by a 2-minute incubation at 80ºC (Langaee & Ronaghi, 2005).

The α_{1A}-ADR (Arg347Cys) polymerase chain reaction (PCR) amplification was determined by using the primers listed in Table 3-2.The PCR mixture consisted of 6.25 ul HotStarTaq Master Mix® (Qiagen GmbH, Hilden, Germany), 0.75 µl of dimethylsulfoxide (Sigma-Aldrich, St. Louis, MO), 10 pmol of each primer (Operon Biotechnologies, Huntsville, AL), 1.5 µl of water, and 50-100 ng of genomic DNA. The PCR amplification was performed under the following conditions: initial denaturation at 95ºC for 15 minutes, 45 cycles of denaturation at 95ºC for 30 seconds, annealing at 56ºC for 30 seconds, and extension at 72ºC for 1 minute, followed by a final extension step at 72ºC for 7 minutes. The β_2-ADR (Arg16Gly and Glu27Gln) polymerase chain reaction (PCR) amplifications were determined by using the primers listed in Table 4. Note that the same forward and reverse biotinylated primers were used; however, two different forward sequencing primers were used. The PCR mixture consisted of 12.5 ul HotStarTaq Master Mix® (Qiagen GmbH, Hilden, Germany), 1.5 µl of dimethylsulfoxide (Sigma-Aldrich, St. Louis, MO), 10 pmol of each primer (Operon Biotechnologies , Huntsville, AL), 7 µl of water, and 50-100 ng of genomic DNA. The PCR amplification was performed under the following conditions: initial denaturation at 95ºC for 15 minutes, 40 cycles of denaturation at 95ºC for 30 seconds, annealing at 63ºC for 30 seconds, and extension at 72ºC for 1 minute, followed by a final extension step at 72ºC for 7 minutes.

Table 3-2. Genotyping primers.

Gene	Primers	Amplicon length
α_{1A}-ADR (Codon 347)	Forward: CCCCATCATATACCCATGCT Biotinilated Reverse: GTAGCCCAGGGCATGTTTG Forward Sequencing Primer: TGTCTTGAGAATCCAGTGT Sequence to analyze: CTCT/CGCAGAAAGCAGTCT	109
β_2-ADR (Codon 16)	Forward: CGAGTCCCCACCACACCC Biotinilated Reverse 5': AGCACATTGCCAAACACGATG Forward Sequencing Primer: CGGACCACGACGTCAC Sequence to analyze: G/AGAAGCCATGCG	297
β_2-ADR (Codon 27)	Forward 3': CGAGTCCCCACCACACCC Biotinilated Reverse 5': AGCACATTGCCAAACACGATG Forward Sequencing Primer: TGGCTGGCACCCAAT Sequence to analyze: GCAGC/GAAAGGGACGA	297

RNA isolation and reverse-transcription

Once all tissue samples were collected, tissue processing for RNA extraction began. To avoid any degradation of RNA by RNAse, all surfaces and tools were thoroughly cleaned with either RNAZap® (Ambion, Inc., Austin, TX, USA) or RNase AWAY® (Molecular BioProducts, Inc., SanDiego, CA) and rinsed with diethyl-pyrocarbonate (DEPC) water. Tissues were removed from RNALater® solution (Qiagen , Valencia, CA, USA), gently blotted on kimwipes to remove excess solution (see Figure 3-4), weighed, quickly sliced into smaller pieces, then transferred to a ceramic mortar. After the addition of a small amount of liquid nitrogen, the frozen samples were ground into a fine powder with a ceramic pestle (see Figures 3-5 & 3-6). The powdered tissue was then combined with 500 ul of proprietary Lysis/Binding solution from the RNAqueous Kit® (Ambion, Inc., Austin, TX). The slush was then homogenized with a PowerGen 125® electric rotor-stator homogenizer (Fisher Scientific, Pittsburgh, PA) and Omni-Tips™ Plastic Disposable Generator Probes (Fisher Scientific, Pittsburgh, PA).

(see Figure 3-7). After a 30-second centrifugation to remove large debris, the supernatant was removed from the lysate and processed per the manufacturer's protocol. All samples were eluted in 50 ul total volume of proprietary Elution Solution, included in the kit. Total RNA was quantified by Nanodrop® (Nanodrop Technologies, Wilmington, DE). The Nanodrop determined seven samples to have concentrations less than 10 ng/ul. These seven samples were placed in a Cenrivap Console® speed-vacuum (Labconco, Kansas City, MO) on the no-heat setting for approximately 20 minutes. These samples were then reconstituted in 20 ul of RNAqueous Kit's Elution Solution®. All 260/280 ratios were above 1.7. UF ICBR Core staff evaluated quality of 18s and 28s peaks generated by a 2100 Bioanalyzer® (Agilent Technologies, Palo Alto, CA). Quality of peaks were consistent across all samples, indicating little RNA degradation. Next, RNA aliquots were made to equal 10 ng/ul and brought up to 50 ul total volume with RNAse-free water. Samples were then reverse-transcribed with the cDNA Archive Kit® (Applied Biosystems, Foster City, CA) at 25°C for 10 minutes, followed by 2 hours at 37°C in a thermal cycler. All samples were stored at –20°C.

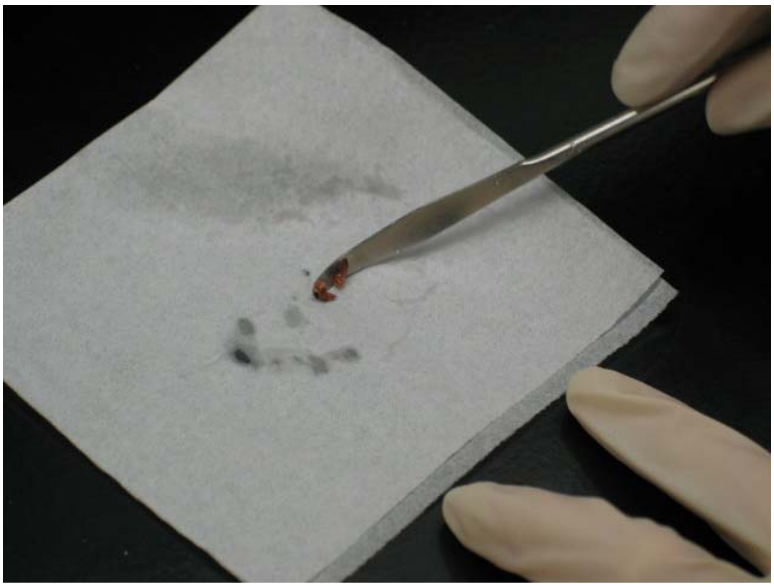


Figure 3-4. Blotting tissue on Kimwipe.

Figure 3-5. Grinding tissue in mortar and pestle on liquid nitrogen.

Figure 3-6. Powdered tissue in mortar.

Figure 3-7. Homogenizing tissue slush with rotar-stator homogenizer.

Real-time polymerase chain reaction

Twenty microliter (ul) reactions were prepared for single-plex Real-Time PCR with the ABI PRISM 7900® system (Applied Biosystems, Foster City, CA), located in the UF Interdisciplinary Center for Biotechnology Research (ICBR). Primers and probes for each of the three assays (α_{1A}-ADR, β_2-ADR, and GAPDH) are listed in Table 3-3. The housekeeping gene GAPDH was used for normalization of gene expression data, as described in the section titled "Genetic Analysis Techniques" in Chapter 2.

For each reaction, 10.0 ul of TaqMan Universal PCR Master Mix® (2X) with AmpErase UNG® was prepared with 1.0 ul of each respective TaqMan Gene Expression Assays on Demand® (20X) (Table 3-3). Eleven microliters of each master mix and 9.0 ul of cDNA template was added to each well of the 96-well plate. Triplicate samples were run, as recommended by Bustin (2000) to increase accuracy of the methodology. Table 3-4 shows the plate set-up for single-plexing of these three assays. PCR conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Fluorescence data were processed and analyzed with the ABI

PRISM Sequence Detection Software® (Applied Biosystems, Foster City, CA). Results were expressed as Ct number (number of cycles needed to generate a fluorescent signal above a predetermined threshold) or ΔCt (target Ct (α_{1A}-ADR or β_2-ADR) minus normalizer Ct (GAPDH)). The Ct value was determined with the ABI 7900 software. The software determines the baseline automatically by assessing the normalized fluorescence signal versus cycle data, per plate. From this baseline, each sample's Ct value is obtained.

Table 3-3. Target gene assay information.

Gene	Assay #	Probes/Quenchers	Amplicons (base pairs)
α_{1A}-ADR	Hs00169124_m1	Probe: FAM	112
β_2-ADR	Hs00240532_s1	Probe: FAM	65
GAPDH	4310884E (ABI product #)	Probe: VIC Quencher: TAMRA	226

Table 3-4. Single-plex plate set-up, one sample.

Sample 1 + GAPDH	Sample 1 + GAPDH	Sample 1 + GAPDH
Sample 1 + α_{1A}-ADR	Sample 1 + α_{1A}-ADR	Sample 1 + α_{1A}-ADR
Sample 1 + β_2-ADR	Sample 1 + β_2-ADR	Sample 1 + β_2-ADR

Positive inotrope data collection

The PI examined the subjects' need for standard-of-care positive inotrope administration in the immediate post-operative period. Chart review was conducted in the post-operative phase for intensive care unit (ICU) documentation of administration of positive inotropic medications. The pharmaceutical agent and dosage were documented. ICU chart was reviewed for presence of the exclusion criteria, diagnosis of low cardiac output syndrome. The need for positive inotrope administration (dichotomized) was

tested for relationship with α_{1A}- and β_2-ADRs gene expression of hypertensive versus NT groups.

Calculations for Relative Gene Expression and Selection of Calibrator

All gene expression data were imported into EXCEL for relative gene expression analyses. As previously stated, samples were run in triplicates for determination of the threshold cycle (Ct) in TaqMan RT-PCR. To control for outlier Ct values, the largest Ct value from each triplicate was removed and the duplicate values were averaged to determine the 'average Ct'. The largest Ct values were chosen for removal because for the majority of the samples, one of the three raw Ct values was greater than 2 Ct's away from the next closest value, indicating, in essence, an outlier. To maintain consistency in this process, each triplicate had the highest value removed. Next, the Ct averages were used to calculate the following:

a) Delta Ct = average target Ct – average endogenous control (GAPDH) Ct; b) Delta Delta Ct = (Delta Ct (sample x) – (Delta Ct (calibrator)); c) 2^-(Delta Delta Ct) = two to the negative power of the DDCt; gene expression relative to the calibrator.

A requirement of the DDCt method of relative quantitation requires selection of a calibrator. One subject (#006142) was chosen as the calibrator. This subject was a normotensive, White/Caucasian male who was not taking any medications at the time of the study, and reported no other cardiac diagnoses. His BMI was comparable to the mean (30.9 versus mean of 29.0). He also reported not having ever been a smoker and did not drink or exercise.

The determination of "fold difference" between groups is expressed as a ratio of the measures of central tendency for the groups compared. Said another way, the fold difference is a ratio of the one measure of central tendency to another, so that if the

median 2^-DDCt of group A was 25.0 and the median 2^-DDCt of group B was 5.0, then the ratio is 25.0: 5.0, indicating a 5-fold difference between groups; or similarly, a 5-fold *decrease* in gene expression in group A versus B. All fold-difference data were calculated in this fashion. The relationship between the Ct value and gene expression is indirect, in that the lower the Ct, the higher the gene expression and vice versa. This same principle applies even after normalization, so that the 2^-DDCt value holds the same interpretation. Briefly stated, during amplification in the Real-Time RT-PCR system, the earlier the mRNA's amplification is detected (thus, the lower the Ct), the more abundant the mRNA. Conversely, if it takes longer for the amplification to be detected (producing a higher Ct value), then the mRNA is less abundant.

Methods for Statistical Analyses

Data were analyzed using Microsoft Excel (Microsoft Corporation, Redmond, WA) and SPSS Version 14 (SPSS Inc., Chicago, IL). Descriptive statistics were used to obtain summary measures for the data. Tests of normality for the gene expression data indicated non-normal distributions, necessitating use of nonparametric tests. To quantify the differences in gene expression of α_{1A}- and β_2-ADRs in the IMA by diagnosis of HTN versus NT (specific aim 1), the Mann-Whitney U test was performed. The Mann-Whitney U test was also used to explore relative differences in gene expression of the α_{1A}- and β_2-ADRs genes and diagnosis, by SIR (specific aim 2), and to explore the relationship between level of α_{1A}- and β_2-ADR gene expression and the need for post-operative positive inotrope administration (specific aim 3). To test the association of diagnosis (HTN vs. NT) and the α_{1A}- Arg347Cys C→T genotype, Pearson Chi-square, and where necessary for nonparametric data, Fisher's Exact tests were used. To test the association

of diagnosis (HTN vs. NT) and the β_2-ADR Arg16Gly G→A, and the β_2-ADR Glu27Gln C→G genotypes, Fisher's exact test was used. The Kruskall-Wallis test was used for testing the association between: a) α_{1A}-ADR relative gene expression and the α_{1A}-ADR Arg347Cys C→T genotype; b) β_2-ADR relative gene expression and each of the β_2-ADR Arg16Gly G→Aand Glu27Gln C→G genotypes. All hypotheses were two-tailed and tested with alpha set at 0.05.

CHAPTER 4
RESULTS

Introduction

The primary aim of this exploratory, pilot study was to determine relative differences in gene expression of the α_{1A}- and β_2-ADR genes between people with and without high blood pressure. The secondary aim was to determine the influence of race on differences in gene expression. A tertiary aim was to examine the impact of gene expression of the α_{1A}-ADR and β_2 subtypes on the need for post-operative positive inotrope pharmacotherapy. This chapter will first present descriptive results, including means, standard deviations, and frequency data for all variables investigated. The three hypotheses posed in Chapter 1 will be addressed using the Mann-Whitney U, Pearson Chi-square, and Fisher's exact tests. Cohen's *d* effect sizes will also be provided. For SIR, the OMB Classification tool allowed for multiple choices of race. Three subjects self-identified as having two races: White/Caucasian and American Indian or Alaska Native. When statistical analyses included the variable SIR, these three subjects were recoded as "White". Explanatory aims E1 and E2 involving genotype by diagnoses associations and genotype by gene expression associations are also included.

Descriptive Results

Subject Demographics

Fifty one subjects were enrolled in the study between August 2004 and July 2005. Four subjects were excluded because blood and tissue samples were unable to be collected due to surgery scheduling changes. As a result, 47 subjects were included in the

data analyses. This sample consisted of 37 males and 10 females with an overall mean age of 56.5 years (range 44-70). Thirty-seven of the subjects were recruited from Shands at Alachua General Hospital and 10 from the VAMC. Twenty subjects were normotensive and 27 subjects were hypertensive. The normotensive group ranged in age from 44-67 with a mean of 55.8 years. The age of the hypertensive group ranged from 44-70 with a mean of 57.3 years. These subjects are included in analyses involving genotyping. Table 4-1 shows the demographic characteristics of this data set, expressed in numbers and percentage. Table 4-2 presents subjects' clinical characteristics, including height, weight, and BMI, expressed as mean and standard deviation, and prescribed β-blocker/dose, concomitant diagnosis of diabetes mellitus-Type2 and surgery facility, expressed as number and percentage.

Table 4-1. Demographics of all enrolled subjects.

	All enrolled *N* = 47		Normotensive *n* = 20		Hypertensive *n* = 27	
	N	%	*n*	%	*n*	%
Gender						
Male	37	78.7	18	90.0	19	70.4
Female	10	21.3	2	10.0	8	29.6
Race						
White/Caucasian	34	72.3	17	85.0	17	63.0
Black/AA	10	21.3	2	10.0	8	29.6
White/Caucasian & Native American	3	6.4	1	5.0	2	7.4
Ethnicity						
Non-Hispanic	45	95.7	20	100	25	92.6
Hispanic	1	2.1	0	0	1	3.7
Did not know	1	2.1	0	0	1	3.7

Table 4-2. Clinical characteristics of all enrolled subjects.

	All *N* = 47	Normotensive *n* = 20	Hypertensive *n* = 27
Height (in)	68.8 + 4.1	69.8 + 3.6	68.1 + 4.3
Weight (lbs)	197.4 + 40.0	192.6 + 36.9	199.7 + 42.8
BMI (kg/m2)	29.2 + 5.9	27.4 + 5.3	30.3 + 6.1

Table 4-2 Continued.

	All *N* = 47	Normotensive *n* = 20	Hypertensive *n* = 27
β-locker (Rx & dose)			
Not prescribed	16 (34.0%)	10 (52.6%)	6 (22.2%)
Metoprolol 12.5mg BID	5 (10.6%)	3(15.8%)	2 (7.4%)
Metoprolol 25mg BID	12 (25.5%)	3 (15.8%)	8 (29.6%)
Metoprolol 50mg BID	10 (21.3%)	2 (10.5%)	8 (29.6%)
Metoprolol 75mg BID	1 (2.1%)	0 (0%)	1 (3.7%)
Metoprolol 100mg BID	1 (2.1%)	0 (0%)	1 (3.7%)
Labetalol 100mg TID	1 (2.1%)	0 (0%)	1 (3.7%)
Missing data	1 (2.1%)	1 (5%)	0 (0)%
T2DM			
No	30 (63.9%)	19 (95%)	11 (40.7%)
Yes	16 (34.0%)	1 (5%)	15 (55.6%)
Pre-DM	1 (2.1%)	0 (0%)	1 (3.7%)
Surgery facility			
AGH	37 (78.7%)	15 (75 %)	22 (81.5%)
VA	10 (21.3%)	5 (25%)	5 (18.5%)

BMI = body mass index; T2DM = Diabetes mellitus-Type 2; AGH = Alachua General Hospital; VA = Veterans Administration Hospital, BID = twice per day

Six additional subjects were excluded from analyses involving gene expression for the following reasons: 1) The PI could not collect tissue from one subject due to change in surgery schedule; and 2) Tissues from five subjects did not yield sufficient RNA material to complete the analyses or had "undetermined" readings in the TaqMan RT-PCR gene expression output. The final sample size for gene expression analyses was 41 subjects. This subset of 32 males and 9 females had a mean age of 57.3 (range 44-70). Of these 41 subjects, 17 were normotensive and 24 were hypertensive. The normotensive group ranged in age from 44-67 with a mean of 56.7 years. The age of the hypertensive group ranged from 45-70 with a mean of 57.7 years. See table 4-3 for the demographic summary by groups, expressed in numbers and percentage. Table 4-4 presents these subjects' clinical characteristics, including height, weight, and BMI, expressed as mean and standard deviation, and prescribed β-blocker/dose, concomitant diagnosis of diabetes

mellitus-Type2 and surgery facility, expressed as number and percentage. Student's t-tests confirmed that hypertensive and normotensive groups did not significantly differ in age (t = -0.803, df = 45, p = 0.426), height (t = 1.374, df = 44, p = 0.177), weight (t = -0.463, df = 45, p = 0.646), or BMI (t = -1.500, df = 44, p = 0.141).

Table 4-3. Demographics for gene expression subset.

	Total subset N = 41		Normotensive n = 17		Hypertensive n = 24	
	N	***%***	***N***	***%***	***N***	***%***
Gender						
Male	32	78.0	15	88.2	17	70.8
Female	9	22.0	2	11.8	7	29.2
Race						
White/Caucasian	29	70.7	14	82.5	15	62.5
Black/AA	9	22.0	2	11.8	7	29.2
White/Caucasian & Native American	3	7.3	1	5.9	2	8.3
Ethnicity						
Non-Hispanic	39	95.1	17	100	22	91.7
Hispanic	1	2.4	0	0	1	4.2
Did not know	1	2.4	0	0	1	4.2

Table 4-4. Clinical characteristics for gene expression subset.

	Subset n = 41	Normotensive n = 17	Hypertensive n = 24
Height (in)	68.7 ± 4.3	69.8 ± 3.7	67.9 ± 4.5
Weight (lbs)	195.8 ± 41.5	193.1 ± 39.1	197.7 ± 43.9
	Subset n = 41	Normotensive n = 17	Hypertensive n = 24
BMI (kg/m^2)	29.0 ± 6.0	27.5 ± 5.5	30.2 ± 6.2
β-blocker (Rx & dose)			
Not prescribed	15 (36.6%)	9 (52.9%)	6 (25.0%)
Metoprolol 12.5mg BID	4 (9.8%)	2 (11.8%)	2 (8.3%)
Metoprolol 25mg BID	10 (24.4%)	3 (17.6%)	7 (29.2%)
Metoprolol 50mg BID	8 (19.5%)	2 (11.8%)	6 (25.0%)
Metoprolol 75mg BID	1 (2.4%)	0 (0%)	1 (4.2%)
Metoprolol 100mg BID	1 (2.4%)	0 (0%)	1 (4.2%)
Labetalol 100mg TID	1 (2.4%)	0 (0%)	1 (4.2%)
Missing data	1 (2.4%)	1 (5.9%)	0 (0%)
T2DM			
No	27 (65.9%)	17 (100%)	10 (41.7%)
Yes	13 (31.7%)	0 (0%)	13 (54.2%)
Pre-DM	1 (2.4%)	0 (0%)	1 (4.2%)
Surgery facility			
AGH	32 (78.0%)	12 (79.6%)	20 (83.3%)
VA	9 (22.0%)	5 (29.4%)	4 (16.7%)

BMI = body mass index; T2DM = Diabetes mellitus-Type 2; AGH = Alachua General Hospital; VA = Veterans Administration Hospital, BID = twice per day

Assessment of GAPDH for Relative Quantitation

The duplicate GAPDH Ct values had a mean and standard deviation of 27.53 ± 2.80 and median of 27.4 with values ranging from 22.1-34.2. Figure 4-1 displays the average duplicate Ct values for each sample, showing this 12-point range. Not only should the raw triplicate Ct values be close (no more than one-half Ct different), the averages should show little variation across samples. Figure 4-2 shows these data again, grouped by 96-well plate number. Evaluation of these graphical data shows that this variance was not plate-specific, meaning each plate showed variation in Ct values for the GAPDH.

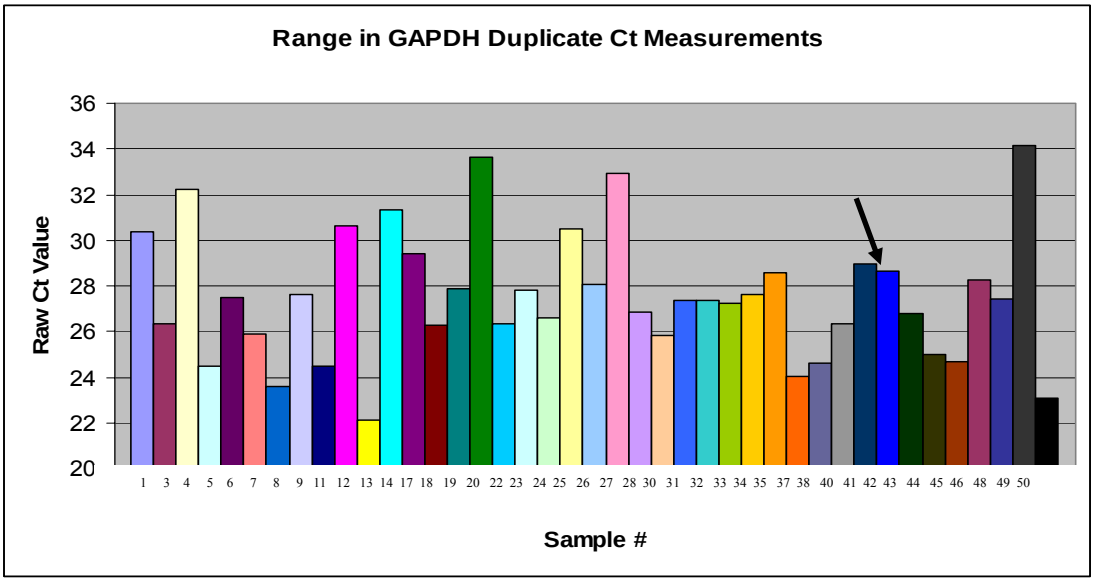


Figure 4-1. Range of average duplicate Ct values of GAPDH per sample number. Note: Arrow indicates calibrator sample.

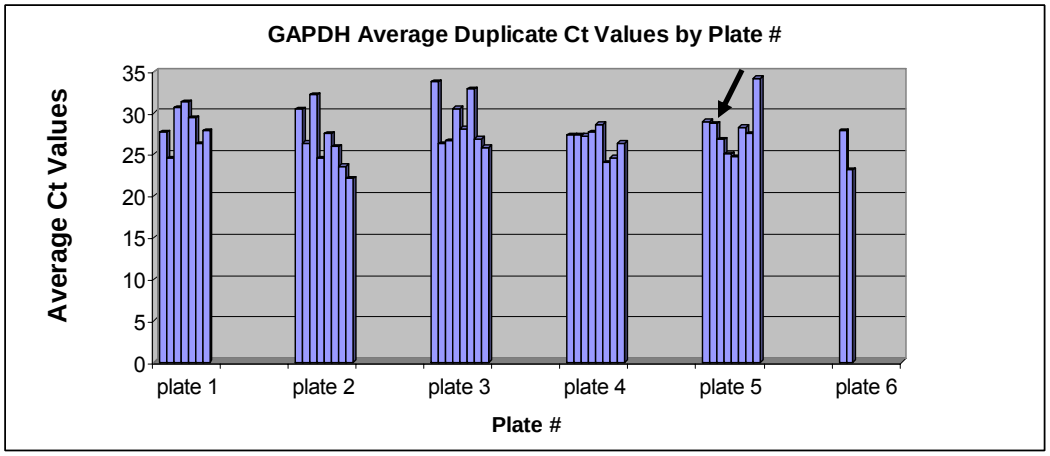


Figure 4-2. Range of average duplicate GAPDH Ct measurements grouped by plate number. Note: Arrow indicates calibrator sample.

Nonparametric Spearman's rho was performed to test correlations between the duplicate GAPDH Ct and each of the α_{1A}- and β_2-ADR duplicate Ct variables. GAPDH was significantly correlated with α_{1A}-ADR duplicate Ct ($R = 0.628$, $p < 0.05$). Conversely, GAPDH was not significantly correlated with β2-ADR duplicate Ct ($R = 0.247$, $p = 0.120$). In addition, Student's t-test confirmed that GAPDH duplicate Ct differed significantly between subjects with HTN versus NT ($t = -2.634$, $df = 39$, $p < 0.05$). Figure 4-3's boxplot represents the groupwise difference in GAPDH between hypertensive and normotensive subjects. Each boxplot contains a box with a bisecting line and two "whiskers" extending from either end. The upper and lower ends of the box represent the upper and lower quartiles, respectively; or, the cutoffs for the 75th and 25th percentiles, respectively. The line that bisects this box represents the median, or middle value. The whiskers extend to the minimum and maximum values for the data. Figure 4-4's boxplot shows the groupwise differences in GAPDH, α_{1A}- and β_2-ADR raw Ct values. These figures illustrate the variability in raw Ct values between groups and are not

informative of relative gene expression differences. Further interpretation of these data and discussion of their importance is thoroughly presented in Chapter 5: Discussion.

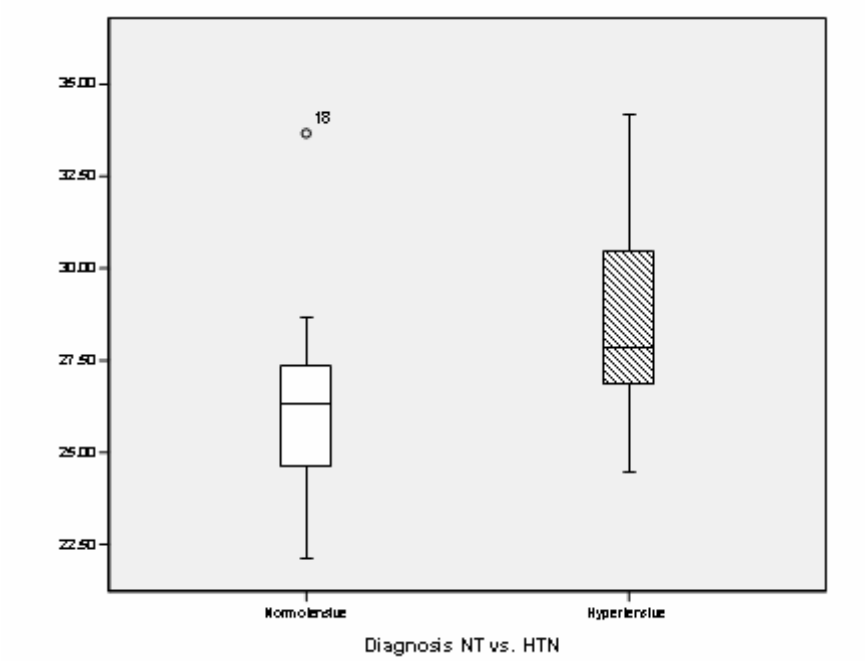


Figure 4-3. Boxplot for average duplicate GAPDH by diagnosis.

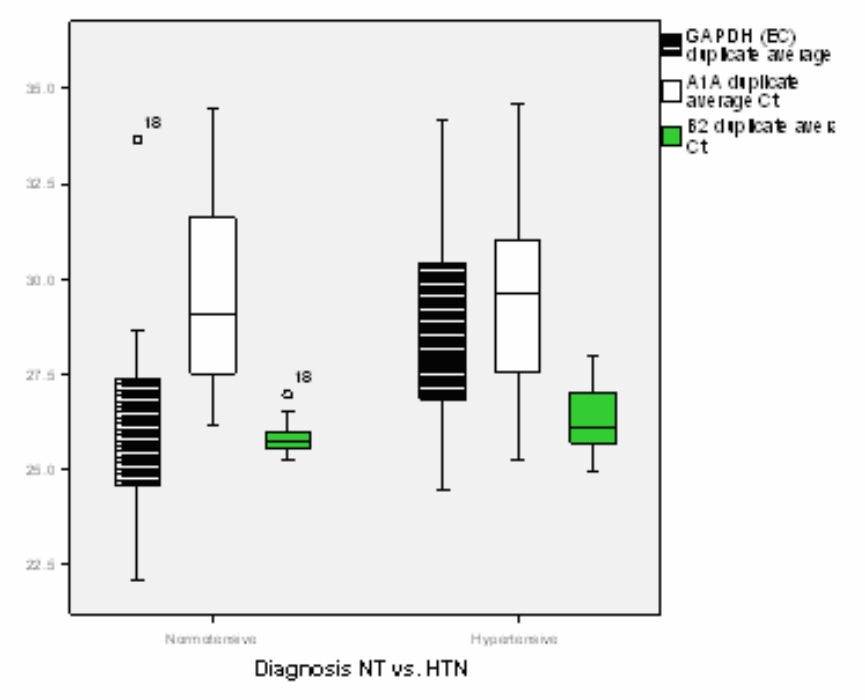


Figure 4-4. Boxplot for all gene expression raw Ct values.

Assumptions of Normality

Data were assessed for normality with skewness, kurtosis, and the Kolmogorov-Smirnof test. These measures of normality indicated that relative gene expression variables (2^-DDCt) for both α_{1A}- and β_2-ADR were non-normally distributed (skewness = 5.447, kurtosis = 32.127, $p < 0.05$; and, skewness = 4.302, kurtosis = 20.722, $p < 0.05$, for α_{1A}- and β_2-ADR, respectively). Due to these violations of normality, non-parametric tests were used for data analyses involving relative gene expression.

Analytic Results for Hypotheses

As these data were non-normally distributed, medians and inter quartile ranges (IQRs) are presented for measures of central tendency and variance. These values for the total sample are listed in Table 4-5. Further analytic results are presented by aim. In addition, amplification plots for each gene, as expressed in the TaqMan Real-Time PCR system (ABI Prism 7900) are presented in Appendix D.

Table 4-5. Gene expression medians and IQRs for total sample

Gene	Total sample $n = 41$				
	Min	*25%*	*Med*	*75%*	*max*
α_{1A}-ADR 2^-DDCt	0.004	0.336	0.63	1.766	30.484
β_2-ADR 2^-DDCt	0.009	0.105	0.32	1.000	40.224

Specific aim 1: To quantify differences in gene expression of α_{1A}- and β_2-ADR in the IMA between subjects with NT and HTN.

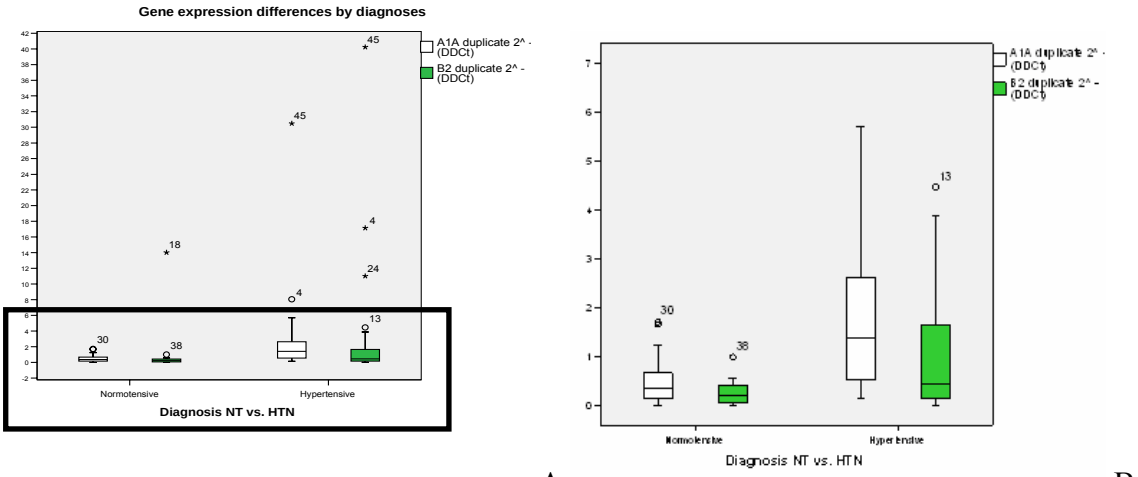
- a. To quantify relative differences in α_{1A}-ADR gene expression between study groups with NT and HTN.
- b. To quantify relative differences in β_2-ADR gene expression between study groups with NT and HTN.

A summary of the median and IQR for each gene by diagnosis is presented in Table 4-6. Figure 4-5 shows boxplots of these data. For specific aim 1, the relative differences in α_{1A}- (aim 1a) and β_2-ADR (aim 1b) gene expression between subjects with NT and

HTN was examined using the Mann-Whitney U test. For this nonparametric test, the null hypothesis is that the two variables compared have identical distributions. More specifically, it tests that the mean ranks of the 2^-DDCt values do not differ from the sum of the ranks (mean of ranks not to be confused with mean of data). The results for these tests are presented in Table 4-7. Median fold difference in gene expression of α_{1A}-ADR and β_2-ADR between subjects with NT and HTN were significant for $p < 0.05$. Fold-differences are expressed as a ratio of HTN to NT subjects.

Table 4-6. Gene expression medians and IQRs for subjects by diagnosis.

Gene	HTN $n = 24$					NT $n = 17$				
	Min	*25%*	*Med*	*75%*	*Max*	*Min*	*25%*	*Med*	*75%*	*Max*
α_{1A}-ADR 2^-DDCt	0.15	0.53	1.41	2.68	30.48	0.004	0.13	0.36	0.84	1.72
β_2-ADR 2^-DDCt	0.02	0.05	0.45	1.70	40.22	0.008	0.15	0.22	0.45	14.1



A B

Figure 4-5. Boxplots for both gene's expression by diagnosis. A) Unadjusted scale with black box showing selection for rescaling. B) Y-axis rescaled for better visualization.

Table 4-7. Median fold differences in gene expression between normotensive and hypertensive subjects and Mann-Whitney U tests.

Gene expression	Ratio of subjects with HTN:NT	
	median-fold-difference	*p*
α_{1A}-ADR 2^-DDCt	3.92	0.01*
β_2-ADR 2^-DDCt	2.05	0.02*

**alpha < 0.05*

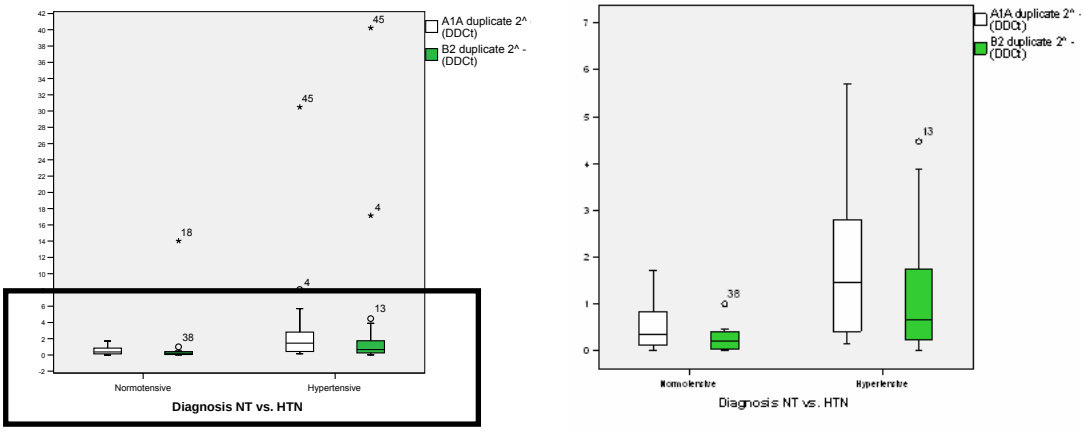
Specific aim 2: To explore relative differences in gene expression of α_{1A}-ADR and β_2-ADR in the IMA between subjects with NT and HTN by race.

- a. To explore relative differences in α_{1A}- and β_2-ADR gene expression between White/Caucasian subjects with NT and HTN.
- b. To explore relative differences in the α_{1A}- and β_2-ADR gene expression between White/Caucasians with HTN versus Black/AAs with HTN.

For aim 2a, a summary of the median and IQR for each gene in White/Caucasian subjects is presented in Table 4-8. Figure 4.6 shows boxplots of these data. To test the hypothesis that the relative fold-differences in gene expression of the α_{1A}-ADR and β_2-ADR may be due, in part, to race (Aim 2a), Mann Whitney U test was performed to compare gene expression differences between White/Caucasians with and without HTN. When Caucasian hypertensive versus normotensive subjects were compared, ranks of relative difference remained significant between median fold-differences in each gene's expression. The fold-difference is expressed as a ratio of White/Caucasian subjects with HTN to NT. These data are presented in Table 4-10.

Table 4-8. Gene expression medians, IQRs, and minimum and maximum values for White/Caucasian subjects.

Gene	White/Caucasian $n = 32$				
	Min	*25%*	*Med*	*75%*	*max*
α_{1A}-ADR 2^-DDCt					
Total	0.004	0.261	0.63	1.71	30.484
Hypertensive ($n = 17$)	0.146	0.381	1.45	3.19	30.484
Normotensive (n=15)	0.004	0.103	0.36	1.00	1.717
β_2-ADR 2^-DDCt					
Total	0.009	0.079	0.37	1.25	40.224
Hypertensive ($n = 17$)	0.021	0.223	0.66	2.82	40.224
Normotensive (n=15)	0.009	0.042	0.22	0.42	14.026



A B

Figure 4-6. Boxplots for White/Caucasian subjects, for both gene's expression by diagnosis. A) Unadjusted scale with black box showing selection for rescaling. B) Y-axis rescaled for better visualization.

Table 4-9. Median fold differences in gene expression between White/Caucasian normotensive and hypertensive subjects and Mann-Whitney U tests.

Gene expression	Ratio of subjects with HTN:NT	
	Median fold-difference	*P*
α_{1A}-ADR 2^-DDCt	4.03	0.01*
β_2-ADR 2^-DDCt	5.27	0.02*

*alpha < 0.05

For aim 2b, a summary of the median and IQR for hypertensive subjects by SIR is presented in Table 4-11. Figures 4-7 and 4-8 show boxplots of these data, by gene. For aim 2b, the Mann-Whitney U test was performed to compare Caucasian HTN versus Black/AA HTN. This comparison did not show significance, as presented in Table 4-11. This table also shows the median fold-differences in each gene's expression, expressed as a ratio of White/Caucasian HTN to Black/AA HTN subjects.

Table 4-10. Gene expression medians and IQRs for Black/AA subjects.

Gene	Hypertensives *n* = 27				
	Min	*25%*	*Med*	*75%*	*max*
α_{1A}-ADR 2^-DDCt					
White/Caucasian (*n* = 15)	0.146	0.381	1.45	3.19	30.484
Black/AA (*n* =9)	0.507	0.620	0.99	2.53	2.732
β_2-ADR 2^-DDCt					
White/Caucasian (*n* = 15)	0.021	0.223	0.66	2.82	40.224
Black/AA (*n* =9)	0.055	0.095	0.17	1.00	11.004

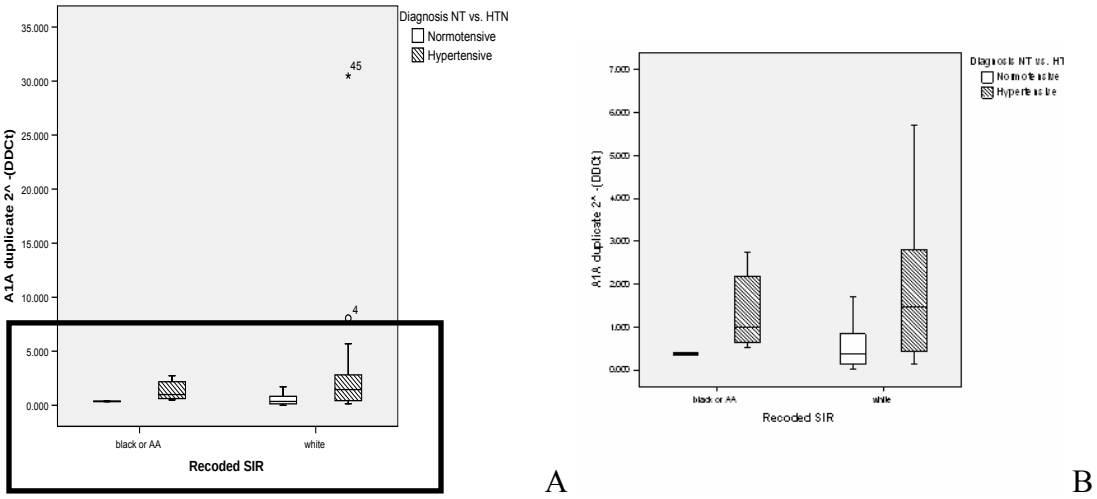


Figure 4-7a. Boxplots for α_{1A}-ADR gene expression for White/Caucasian HTN versus Black/AA HTN subjects. A) Unadjusted scale with black box showing selection for rescaling. B) Boxplot rescaled.

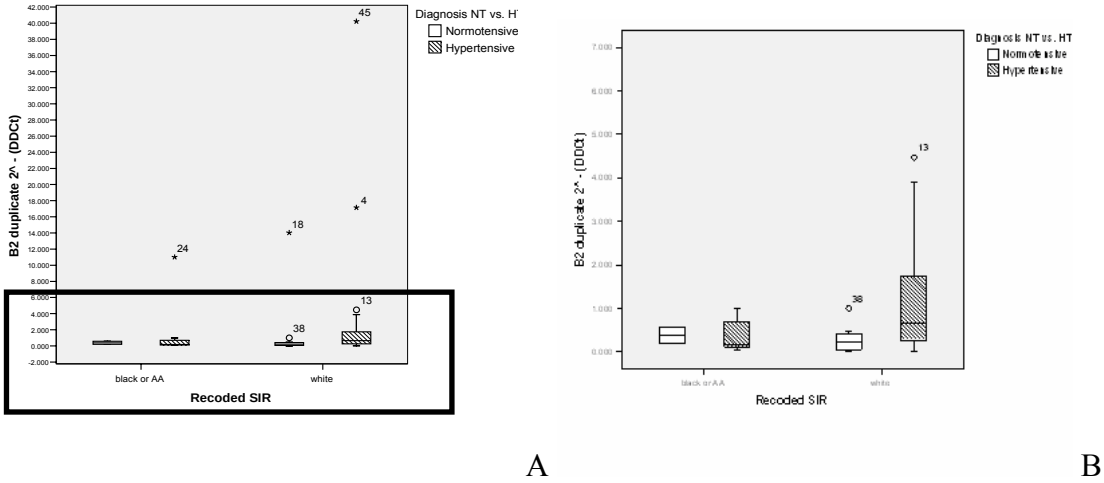


Figure 4-8. Boxplots for β_2-ADR gene expression for White/Caucasian HTN versus Black/AA HTN subjects. A) Unadjusted scale with black box showing selection for rescaling. B) Boxplot rescaled.

Table 4-11. Median fold differences in gene expression between White/Caucasian hypertensive and Black/AA hypertensive subjects and Mann-Whitney U tests.

Gene expression	Ratio of Cauc/White HTN:Black/AA HTN	
	median-fold-difference	*p*
α_{1A}-ADR 2^-DDCt	1.47	0.55
β_2-ADR 2^-DDCt	3.88	0.28

**alpha < 0.05*

Specific aim 3: To explore the relationship between level of α_{1A}- and β_2-ADR gene expression and need for post-operative positive inotropic medication administration.

Table 4-12 shows the median, IQR, minimum and maximum values for both genes by need for positive inotropes. Figure 4-9 shows the boxplots of these data. To test the hypothesis that fold-differences in gene expression exist between subjects who required post-operative positive inotrope administration and those who did not, the Mann-Whitney U test was performed (Table 4-13). Median fold-difference of α_{1A}-ADR and β_2-ADR gene expression between those who did and did not require post operative positive inotropes is also shown in Table 4-13. Fold difference is expressed as a ratio of subjects who received inotrope treatment to those who did not.

Table 4-12. Median, IQR, minimum and maximum values for α_{1A}-ADR and β_2-ADR fold difference in gene expression and need for post-operative positive inotrope medication.

Gene	Inotropes $n = 41$				
	Min	*25%*	*Med*	*75%*	*Max*
α_{1A}-ADR 2^-DDCt					
No inotropes ($n = 34$)	0.004	0.301	0.625	1.95	30.484
Yes inotropes ($n = 7$)	0.339	0.507	0.727	1.59	1.670
β_2-ADR 2^-DDCt					
No inotropes ($n = 34$)	0.009	0.089	0.273	0.93	40.224
Yes inotropes ($n = 7$)	0.021	0.248	0.451	4.47	14.026

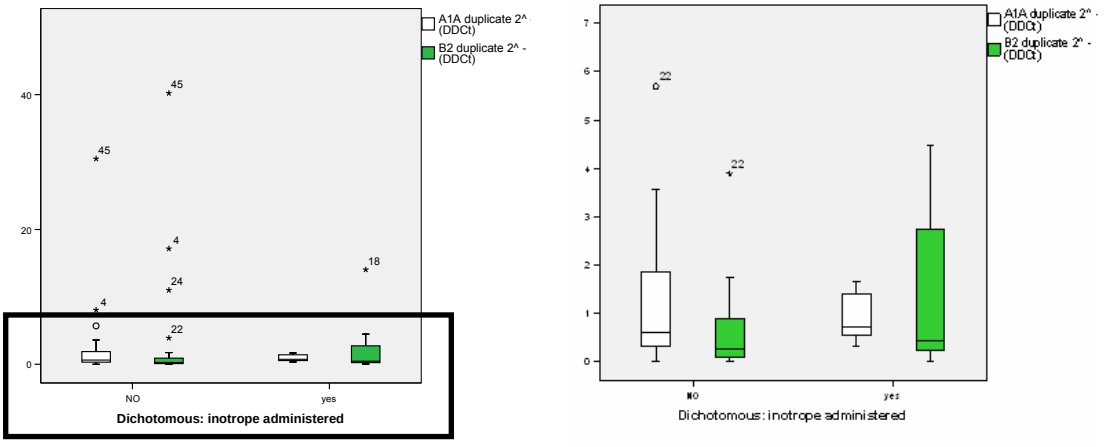


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Figure 4-9. Boxplots for both genes' expression by need for post-operative positive inotrope. A) Unadjusted scale with black box showing selection for rescaling. B) Boxplot rescaled.

Table 4-13. Fold differences in gene expression between non-inotrope and inotrope subjects and Mann-Whitney U tests.

Gene expression	Ratio of inotrope:non-inotrope	
	Median fold-difference	*p*
α_{1A}-ADR 2^-DDCt	1.18	0.73
β_2-ADR 2^-DDCt	1.67	0.36

**alpha < 0.05*

Exploratory Aims

Following statistical analyses of the three specific aims, further exploratory analyses were completed. For exploratory aim A, genotype data were examined for the α_{1A}-ADR Arg347Cys C→T, the β_2-ADR Arg16Gly G→A, and the β_2-ADR Glu27Gln C→G polymorphisms. Tables 4-14 and 4-15 indicate allele and genotype frequencies for 47 subjects who had blood collected, separated by racial/ancestral groups. Population values for the α_{1A}-ADR Arg347Cys C→T polymorphism were obtained from the Ensembl database (http://www.ensembl.org/index.html). Population estimates for European Americans were from 24 samples and estimates for African Americans were from 23 samples form the Coriell Cell repository (Ensembl, 2005). Population estimates for the β_2-ADR Arg16Gly G→A, and the β_2-ADR Glu27Gln C→G polymorphisms were obtained from the Pharmacogenetics and Pharmacogenomics Knowledge Base (Pharm GKB) database of the INVEST study (**IN**ternational **VE**rapamil SR and Trandolapril **St**udy, unpublished data), whereby 325 African American and 1,100 Cacuasian/European American subjects were gentoyped (PharmGKB, 2006)

All genotypes were determined to be in Hardy-Weinberg Equilibrium (data not shown), indicating that the gene frequencies and genotype ratios remained constant from generation to generation in a randomly-breeding population.

Table 4-14. Allele frequencies for population versus sample, by SIR/ancestry.

Gene/ Ref ID #	Codon	Amino Acid/ Allele		Population Allele Frequency		Sample Allele Frequency	
		Major	Minor	Black/AA	Cauc/ Eur-Amer	Black/AA *n* = 10	Cauc/ Eur-Amer *n* = 37
α_{1A}-1048101	347	Cys T	Arg* C	T .28 C .72	T .56 C .44	T .20 C .80	T .49 C .51
β_2-1042713	16	Gly A	Arg G	G .48 A .52	G .40 A .60	G .55 A .45	G .64 A .36
β_2-1042714	27	Gln C	Glu G	G .18 C .82	G .40 C .60	G .35 C .65	G .40 C .60

*In the AA population, Arg is the major allele

Table 4-15. Genotype frequencies for populaiton versus sample, by SIR/ancestry.

Gene/ Ref ID #	Codon	Amino Acid/ Allele		Population Genotype Frequency		Sample Genotype Frequency	
		Major	Minor	Black/AA	Cauc/ Eur-Amer	Black/AA	Cauc/ Eur-Amer
α_{1A}-1048101	347	Cys T	Arg* C	T/T .04 T/C .48 C/C .48	T/T .17 T/C .54 C/C .29	T/T .10 T/C .20 C/C .70	T/T .32 T/C .41 C/C .35
β_2-1042713	16	Gly A	Arg G	G/G .27 A/G .50 A/A .23	G/G .37 A/G .46 A/A .17	G/G .30 A/G .50 A/A .20	G/G .41 A/G .60 A/A .12
β_2-1042714	27	Gln C	Glu G	G/G .03 G/C .30 C/C .67	G/G .16 G/C .49 C/C .35	G/G .10 G/C .50 C/C .40	G/G .27 G/C .38 C/C .44

*In the AA population, Arg is the major allele.

Chi-square analyses were performed to examine the association between each of the genes by diagnosis (HTN vs. NT). When cells contained values less than 5, Fisher's Exact tests were used for nonparametric data. Table 4-16 shows these results. All Chi-square/Fisher's Exact tests were nonsignificant for $p < 0.05$.

Table 4-16. Association between genotype and diagnoses of NT and HTN for the α_{1A}-ADR and β_2-ADR genes.

Gene	NT $n = 20$		HTN $n = 27$		*p*
	Count	% within diagnosis	Count	% within diagnosis	
α_{1A}-ADR , Codon 347					0.18†
C/C	5	25.0	14	51.9	
C/T	8	40.0	8	29.6	
T/T	7	35.0	5	18.5	
β_2-ADR, Codon 16					0.67§
G/G	8	40.0	8	29.6	
G/A	10	50.0	14	51.9	
A/A	2	10.0	5	18.5	
β_2-ADR, Codon 27					0.52§
C/C	3	15.0	7	25.9	
C/G	7	35.0	11	40.7	
G/G	10	50.0	9	33.3	

† = Chi-square
§ = Fisher's Exact

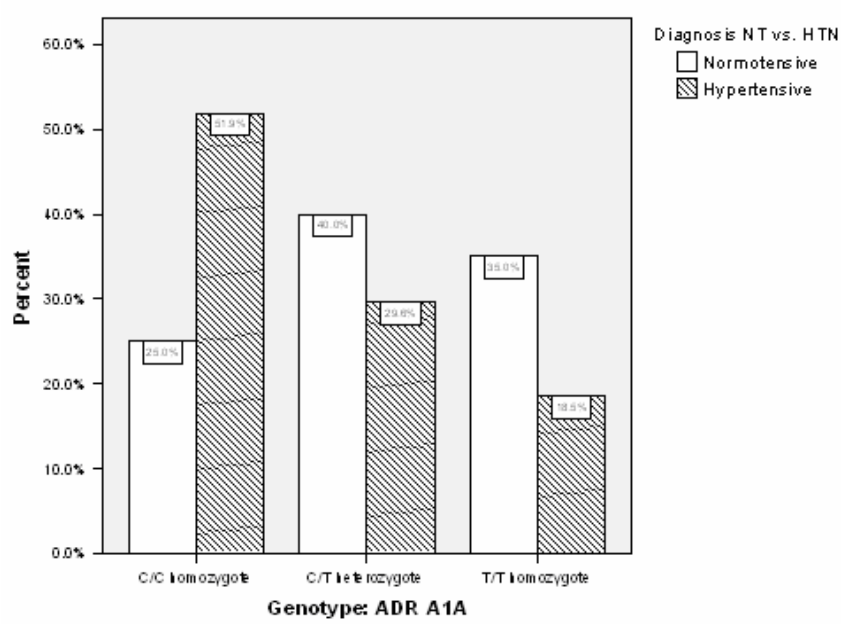


Figure 4-10. Bar chart of α_{1A}-ADR, codon 347 by diagnosis.

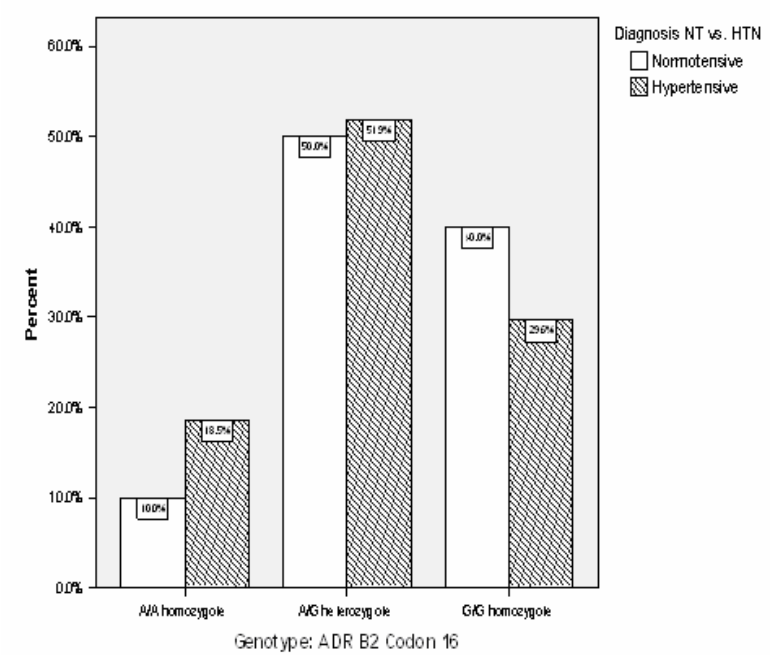


Figure 4-11. Bar chart of β_2-ADR, codon 16 by diagnosis.

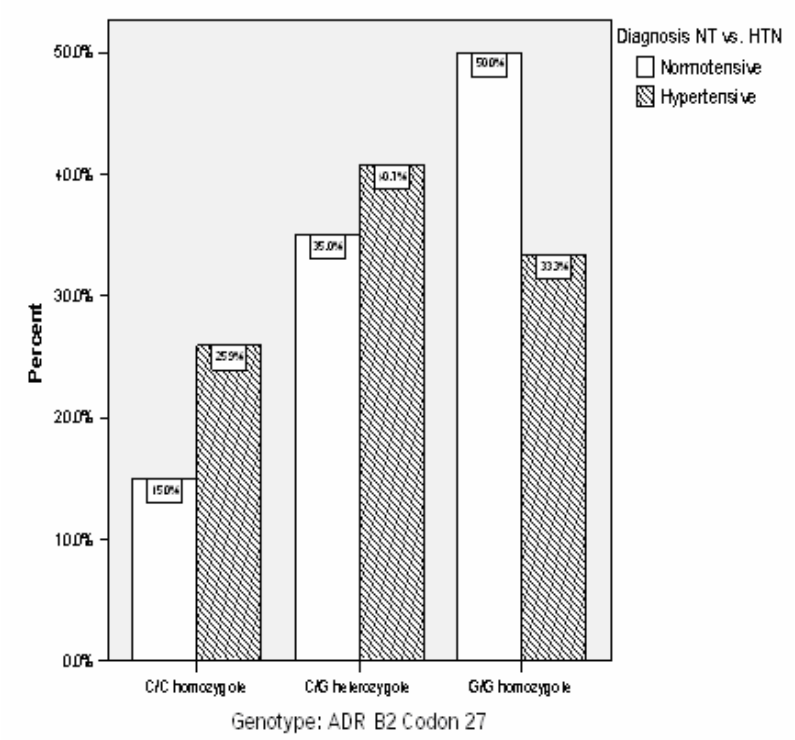


Figure 4-12. Bar chart of β_2-ADR, codon 27 by diagnosis.

To examine the effect of confounding or population stratification of race on genotype differences in HTN versus NT subjects, Fisher's exact was performed, but only on White/Caucasian subjects. Tables 4-17 show these data and indicate these associations

are not significant. Given the allele and genotype frequency differences between White/Caucasian and Black/AA subjects (for both sample and population estimates), further analyses comparing associations stratified by SIR are warranted. However, very low cell counts for the Black/AA group in this sample prevent any meaningful analyses with data analyzed by genotype. When data were analyzed by allele for each gene with Chi-square, cell counts were sufficient to examine allele by diagnosis associations for both total sample and the White/Caucasian group, but were still too low in the Black/AA group to warrant meaningful analyses. These data are presented in Tables 4-18 and 4-19.

Table 4-17. Fisher's Exact for genotype differences in White/Caucasian hypertensive vs. normotensive subjects.

Gene	White/Caucasian *n* = 37				
	NT *n* = 18		HTN *n* = 19		
Counts	Count	% within diagnosis	Count	% within diagnosis	*P*
α_{1A}-ADR, Codon 347					0.13[§]
C/C	3	16.7	9	47.4	
C/T	8	44.4	6	41.6	
T/T	7	38.9	4	21.1	
β_2-ADR, Codon 16					0.69[§]
G/G	8	44.4	6	31.6	
G/A	8	44.4	11	57.9	
A/A	2	11.1	2	10.5	
β_2-ADR, Codon 27					0.57[§]
C/C	3	16.7	6	31.6	
C/G	7	38.9	6	31.6	
G/G	8	44.4	7	36.8	

Table 4-18. Chi-square for allele counts by diagnosis for the α_{1A}-ADR and β_2-ADR genes in all subjects.

Gene	All subjects *N* = 47				
	NT *n* = 20		HTN *n* = 27		
Counts	Allele frequency	% count	Allele frequency	% count	*p*

Table 4-18 Continued.

Gene	All subjects $N = 47$				
	NT $n = 20$		HTN $n = 27$		
Counts	Allele frequency	% count	Allele frequency	% count	*p*
α_{1A}-ADR, Codon 347					0.03*
C	.45	33.0	.67	66.0	
T	.55	55.0	.33	45.0	
β_2-ADR, Codon 16					0.04
G	.65	46.0	.56	54.0	
A	.35	37.0	.44	63.0	
β_2-ADR, Codon 27					0.18
C	.33	34.0	.46	66.0	
G	.67	48.0	.54	52.0	

**alpha* < 0.05. Note: Percent count equals row count.

Table 4-19. Chi-square for alleles by diagnosis for the α_{1A}-ADR and β_2-ADR genes in White/Caucasian subjects.

Gene	White/Caucasian $n = 37$				
	NT $n = 18$		HTN $n = 19$		
Counts	Allele frequency	% count	Allele frequency	% count	*p*
α_{1A}-ADR, Codon 347					0.04*
C	.39	38.9	.63	63.1	
T	.61	61.1	.37	36.8	
β_2-ADR, Codon 16					0.58
G	.67	66.7	.61	60.5	
A	.33	33.3	.39	39.5	
β_2-ADR, Codon 27					0.33
C	.36	36.1	.47	47.4	
G	.64	63.9	.53	52.6	

**alpha* < 0.05

To explore if genotype differences were correlated with gene expression differences, Kruskal-Wallis tests were performed. This test is a nonparametric alternative to a One-Way Analysis of Variance that extends the Mann-Whitney U test to more than two groups. This is necessary, as we need to examine three groups for the genotypes. No significant differences were found for genotype by gene expression. These data are presented in Table 4-20.

Table 4-20. Kruskal–Wallis tests for genotype counts by gene expression α_{1A}-ADR and β_2-ADR genes.

Genotype X Gene expression	*n = 41*		*p*
	Count	*Median gene expression*	
α_{1A}-ADR, Codon 347 X α_{1A}-ADR 2^-DDCt			0.49
C/C	17	0.99	
C/T	13	0.58	
T/T	11	0.42	
β_2-ADR, Codon 16 X β_2-ADR 2^-DDCt			0.80
G/G	14	0.55	
A/G	21	0.67	
A/A	6	1.15	
β_2-ADR, Codon 27 x β_2-ADR 2^-DDCt			0.33
C/C	9	1.00	
C/G	14	0.63	
G/G	18	0.57	

Effect Sizes and Power Calculations

Cohen's *d* (actual effect sizes) were calculated for all aims and are presented in Table 4-21. Actual effect sizes varied by aims. The original power calculations anticipated a medium effect size, based on the literature. Therefore, these Cohen's *d* values indicate a greater sample size was needed to power these aims. With such small effect sizes, the *group* sizes would need to be greater than what was sampled to achieve a power of 80%.

Table 4-21. Power and effect sizes by aim.

	Calculations		
Aim Statistical test	**Power**	**Effect Size**	**Number needed[a]**
1a* Total sample by diagnosis: α_{1A}-ADR Mann-Whitney U	0.66	0.67	34
1b* Total sample by diagnosis: β_2-ADR Mann-Whitney U	0.35	0.35	72
2a* White/Caucasians by diagnosis: α_{1A}-ADR Mann-Whitney U	0.56	0.56	30
2a White/Caucasians by diagnosis: β_2-ADR Mann-Whitney U	0.30	0.40	64
2b Hypertensives by SIR: α_{1A}-ADR Mann-Whitney U	0.31	0.35	58

Table 4-21 Continued.

	Calculations		
Aim **Statistical test**	**Power**	**Effect Size**	**Number needed[a]**
2b Hypertensives by SIR: β_2-ADR Mann-Whitney U	0.17	0.27	100

* Indicates these tests were significant in analyses.

[a] The number needed in each group to achieve a power of 0.80. This assumes equal numbers in each group.

CHAPTER 5
DISCUSSION AND RESULTS

Introduction

All descriptive and analytic results for the proposed aims and exploratory analyses will be discussed in this chapter. Conclusions and implications for nursing as well as recommendations for future research will also be provided.

Discussion of Results

Demographics

In this study, 42.6% of all enrolled subjects were normotensive and 57.4% were hypertensive. When examining those included in the gene expression subset, the percentages were similar with 41.5% normotensive and 58.5% hypertensive subjects. These percentages indicate that there are a number of normotensive patients undergoing bypass surgery, a phenomenon surprising to some researchers. These normotensive subjects were comprised predominantly of self-identified White/Caucasians (n = 17, 85%), with only 2 subjects (10%) who self-identified as Black/AA. This study anticipated 15 subjects who were Black/AA with NT, which, based on the UF TCV Surgery department's database of patients from 2001-2002, 15 subjects would have amounted to roughly 60% of their *total* Black/AA NT population for that fiscal year. Only 8 subjects who self-identified as Black/AA and were diagnosed with HTN were recruited for the study. This made a total of 10 (21.3%) self-identified Black/AA subjects for the entire study. (This number declined by one for the gene expression subset.) The 2000 U.S. Census for Alachua County estimates the Black/AA population to be just 19.3%. Based

solely on this, this study attained a representative sample of self-identified Black/AA in Alachua County. However, the UF TCV Surgery department's database for 2001-2002 indicated approximately 100 of 389 patients who had bypass surgery were listed as Black/AA. This led us to believe that sampling 30 Black/AA subjects should not be a problem. In addition, sampling occurred at both a community-based general hospital (Shands at AGH) and a regional Veteran's medical center, which we felt would possibly lead to a greater Black/AA population from which to sample. Despite these points, the PI was unable to recruit a sufficient cohort of Black/AAs. Very few NT Black/AA patients were identified for potential recruitment. Some possible reasons for this are that many of these patients could have been emergent cases (and unable to be consented 24 hours prior to surgery, as required by IRB), many of these patients may have refused surgical intervention. Finally, *any* normotensive patients, not just normotensive AAs, may have been referred for interventional procedures such as percutaneous coronary intervention (for example, stenting, atherectomy, or balloon catheter angioplasty). This is perhaps the most plausible explanation for the reduced number of normotensive patients who undergo bypass surgery, in that they may have less severe comorbidities and are recommended to interventional cardiology rather than to thoracic surgery for bypass. Only a small number of Black/AAs (regardless of diagnosis) approached to participate in the study chose *not* to enroll. Major reasons cited for not wishing to participate were: not wanting to be "bothered with anything else" and "not feeling comfortable with the study".

Only three subjects (6.4%) self-identified as having more than one race; all three considered themselves both White/Caucasian and American Indian/Alaska Native. This number could not be compared to US Census data, as the Census does not specifically

report combinations of dual-identification. The percentage of self-identified Hispanic and non-Hispanic were 2.1% and 95.7%, respectively. Compared to US Census reports for Alachua County, approximately 5.7% report Hispanic (of any race) and 94.3% report non-Hispanic ethnicities.

Just over 21% of enrolled subjects were female and nearly 79% were male (22% and 78% female and male, respectively, in the gene expression subset). As indicated in the TCV Surgery department's database, the average percent of women undergoing CABG surgeries by the TCV surgery department is 21%. This means that for every one female undergoing the procedure by this department, there are approximately four males. Therefore, the enrolled percentage of females met the expected percentage. This indicates that this sample is representative of the population of females undergoing bypass surgery.

Interpreted collectively, the demographic data of this sample suggest it to be moderately representative of the population of bypass patients who undergo surgery in Alachua County, but is not completely representative of the entire racial, ethnic or bypass populations.

Clinical characteristics for subjects are presented in Tables 4-2 and 4-4 for enrolled and gene expression subsets, respectively. Hypertensive and normotensive groups did not significantly differ in age, height, weight, or BMI (refer to page 59). The concomitant diagnosis of T2DM was seen in 34% of the overall sample, with 2.1% (n = 1) having a diagnosis of pre-diabetes. Only 5% (n = 1) of all NT subjects were diagnosed with T2DM. Among all hypertensive subjects, 55.6% were Type 2 diabetic and 3.7% were pre-diabetic. In regards to prescribing of β-blockers, a first-line class of drugs for both coronary artery disease and hypertension (AHA, 2005; JNC VII, 2003), a surprising

percentage of subjects (34% overall) were not prescribed this medication. Twenty two percent of hypertensive and 53% of normotensive subjects were not on β-blocker medication. Of subjects with NT, 47.4% were prescribed β-blockers, but none were prescribed more than 50 mg, twice a day. Of subjects with HTN, 77.8% were prescribed this therapy with only three subjects (11.1%) taking more than 50 mg, twice a day. Table 4.2 shows that the majority of subjects who were prescribed β-blocker therapy (57.4% of the overall sample) were taking between 12.5mg to 50.0 mg, twice a day.

Gene Expression Measures of Central Tendency and Variance

When examining measures of central tendency in these data, a few things warrant consideration. First, the means are not the best representation of centrality of these data because multiple outliers skew these data and bias the means. While the mean is typically considered more stable over time (and with repeated random selection), the median is considered a middle point, an index of average position, that is not affected by skewed data with outliers (Portney & Watkins, 2000). When comparing the means and medians (data not shown), nearly every mean value is visibly inflated by the outliers and the medians appear to better represent the centrality of these data. Furthermore, evaluating the standard deviations (SDs) causes more concern. The SDs are fairly large (data not shown), especially compared to the means and medians. Since the nature of the SD is to represent the variability in the data, it is typically expressed as the spread from each end of the mean (for example, 'plus or minus'). If we were to subtract some of these SDs from their corresponding means, we would actually be left with a negative number. For example, the mean and SD for the α_{1A}-ADR relative gene expression for the total sample is 1.97 and 4.83, respectively. This would indicate a range of values from -2.86 to 6.8. From the point of view of the gene expression biological assay, it is impossible to have

negative numbers. This phenomenon occurs for nearly every set of values in this study. Therefore the SD is not the best measure of variability for these data. Taken together, this information indicates the median and IQRs are the best representation of centrality and spread in these data and are presented for these data throughout the chapters (in tables and boxplots). Furthermore, because the median-derived 2^-DDCt values are similarly less influenced by outliers, median fold-differences between groups was presented.

Discussions for Choice of GAPDH for Normalization Gene

During the initial planning phase of this project, the normalization (or, housekeeping)gene anticipated for use was cyclophilin A, as it was previously reported as a successful normalizer in arterial tissue (Lieu, Withycombe, Walker, Rong, Walzem, and Wong, et al., 2003; Trogan, Choudhury, Dansky, Rong, Breslow, & Fisher, 2002). Prior to purchasing this housekeeping gene, another review of the literature was conducted to see if any new information had been reported about this gene's use in normalization. In fact, a recent publication by Escobales and Crespo reported evidence that reactive oxygen species appeared to be mediated by a number of factors, including cyclophilin A (2005). As the reactive oxygen pathway is implicated in HTN, the use of cyclophilin A to normalize samples of hypertensive subjects made this gene inappropriate for use as a housekeeping gene in this study. Another review of the literature revealed support for the use of GAPDH in similar tissue types. Peuster, Fink, Reckers, Beerbaum, and von Schnakenburg reported consistent amplification of GAPDH among samples in a study of unstented coronary arteries in pigs (2004). Wang and Brown showed successful use of GAPDH for normalization in their study of β-ADR subtypes in atrial appendages (2001). Furthermore, preliminary analyses with a small selection of the PI's sample in single-plexed reactions yielded triplicate GAPDH values that were less than one-half a Ct from

one another and Ct values between samples that were very close. Technical experts in the UF ICBR Gene Expression Core facility viewed these preliminary data and supported the decision to use GAPDH in the final experiments. These aforementioned references and preliminary results provided the foundation for the decision to choose GAPDH as the housekeeping gene in this study.

Assessment of the Performance of GAPDH as a Normalizer

To reiterate, one of the major assumptions of performing the relative quantitation method of gene expression analyses is that the housekeeping, or normalization gene (here, GAPDH) expresses similarly across subjects and/or experimental conditions. This is typically assessed by examining the Ct values of the GAPDH wherein the Ct values are expected to show very little variability (no more than ½ -2 Cts difference) and the standard deviation of the mean should be small. If the GAPDH gene expression shows greater variability than this, it is theoretically a poor housekeeping gene for the data and is cautioned for normalization use. In this particular study, the duplicate GAPDH Ct values had a mean and standard deviation of 27.56 + 2.80 and median of 27.4 with values ranging from 22.1 to 34.2. According to Dorak (2003), the endogenous control (housekeeping gene) should be more abundant (or, have smaller Ct values) than the target genes. This was true for the α_{1A}-ADR (median 29.45, SD 2.50), but not the β2-ADR (median 26.10, SD 0.78). Possible explanations for this greater-than-expected Ct for GAPDH are poor PCR efficiency or low copy numbers (Dorak, 2003). The 12-point range of Ct values for GAPDH indicates the GAPDH did not, in fact, express consistently across subjects. While poor PCR efficiency, low copy number, and/or pipetting errors may contribute to this variation in GAPDH, another plausible explanation is the occurrence of RNA degradation. As previously stated, RNA degradation was determined

via a 2100 Bioanalyzer® (Agilent Technologies, Palo Alto, CA), and concluded that overall, the level of RNA degradation for these samples was low. However, to examine if RNA degradation may have played a role in GAPDH variation, the 18s and 28s graphical data were examined and compared to those samples that had poor triplicate Ct values for GAPDH (meaning, those with more than 2 Cts difference). Qualitatively speaking this review of the data concluded that, at least for a handful of the samples, RNA degradation could help to explain some of the variance in GAPDH. The most extreme case of this was with one sample, whereby raw Ct values were 35.9, 30.9, and 27.9 and the graphical data from the bioanalyzer indicated more RNA degradation in this particular sample as compared to other samples. Perhaps results for the GAPDH Ct values may have been less variable for some of the samples where RNA degradation was a potential issue.

Figure 4-2 shows that variation in GAPDH was not plate-specific, meaning certain 96-well plates did not show more or less variation than others. This refutes the notion that specific plates may have been outliers due to the order of preparation, time lapse between preparation and initialization of Real Time RT-PCR, or other sources of external error. In addition, a stem and leaf plot (data not shown) indicated 3 “extremes” greater than 32.9; however, these values are not greater than 2 SDs from the mean. Similar variance has been reported in the literature for GAPDH in various species and tissues. Schmid, Cohen, Henger, Irrang, Schlöndorff, and Kretzler (2003) showed variation by tissue source in their study, reporting GAPDH Ct median and standard deviation of 22.57 + 2.61 in tubulointerstitial compartments and 28.96 + 2.38 in glomeruli, both from human renal biopsies. They did not report a range of Ct values. Peuster, Fink, Reckers, Beerbaum and von Schnakenburg (2004) reported a median of 22.2 (range 19.8-26.9) of GAPDH Ct

values in their examination of porcine left coronary artery. Despite this 7-point range, they reported consistent amplification across all samples, as determined by serial dilutions of GAPDH. They used the Delta Delta Ct method, normalizing with GAPDH. Lennmyr, Terént, Svyänen, and Barbany (2005) discuss instability in GAPDH gene expression in their samples of middle cerebral artery in rats, but reported that because the changes were not statistically significant, they used the gene to normalize. Tricarico, Pinzani, Bianchi, Paglierani, Distante, and Pazzagli, et al. (2002) graphically showed raw GAPDH Cts between approximately 22-23 in human breast tissue. They correlated their target genes with the GAPDH by Spearman's rank (nonparametric), showing significance. This supported their decision not to use the GAPDH as a normalizer. The authors suggest normalization to total RNA concentration as an alternative in this type of situation. The methodology behind this solution was not delineated by the authors. The following arguments were discussed in personal communication with Y. Conley (2006) regarding this possible solution: First and foremost, if we assume that the expression of a gene is altered during a disease state (here expression of the ADR genes in HTN), then this could theoretically alter the total RNA. This would seem inappropriate, then, to normalize both diseased and non-diseased samples with a total RNA value. Whatever value is used to normalize, it must be constant. This same principle applies when considering taking the average of all GAPDH values across all samples (or even by plate) and normalizing in this fashion, making these options also undesirable.

Barber, Harmer, Coleman, and Clark (2005) performed a thorough evaluation of GAPDH as a housekeeping gene, examining its expression in 72 human tissue types. One tissue type examined was coronary artery, although the specific artery was not noted.

They reported (graphically) a mean Ct value of approximately 20, but no range. Among the 72 tissues examined, GAPDH mRNA gene expression varied 15-fold between tissue types, further supporting previously published variability. The authors also reported GAPDH Ct outliers below 13 and above 32.761; in these cases, they removed these data points. These studies highlight the variability in reports of GAPDH in different tissues and species as well as variation in how researchers handle the normalization dilemma and outliers. No studies were found that reported expected raw Ct values for GAPDH in human IMA tissue, thus providing little evidence of an expected Ct value for GAPDH in the IMA tissues used in this study.

Given the literature presented above, GAPDH was analyzed statistically for its correlation with the target genes and differences between experimental groups. There was a significant correlation between GAPDH duplicate Ct and α_{1A}-ADR duplicate Ct but not between GAPDH duplicate Ct and β2-ADR duplicate Ct (data shown on page 62). The first glance indicates that GAPDH should not be used for normalization of the α_{1A}-ADR gene expression data, but could be used for normalization of the β2-ADR. The reason for this is that a direct linear relationship should not exist between a target and housekeeping gene, theoretically speaking. The housekeeping gene should remain constant at any given value of the target gene expression. Additionally, the housekeeping gene should not show differential expression between experimental groups. In fact, the GAPDH housekeeping gene expression in this study was significantly different between hypertensive and normotensive subjects (see data page 65 and Figure 4-3). These data collectively indicate that the GAPDH used in this study did not optimally perform as a housekeeping gene. However, the data were normalized to GAPDH because no other options were available.

Raw Ct values are unable to be used for comparison, as this is an exponential value determined from a log-linear plot of PCR signal versus cycle number (Livak & Schmittgen, 2001). No other housekeeping genes were used. For this reason, interpretation of statistically significant results is extremely cautioned and further inferential analyses would be inappropriate. Boxplots of group differences and GAPDH values were shown (Figures 4-3 and 4-4) to allow for visual comparison of group data (as recommended in personal communication with N. Chegini, 2005). On a final note, use of single-plexing (loading targets and GAPDH in separate wells) instead of multiplexing (loading targets and GAPDH in same wells) at the very least, makes us confident that the values we obtained for the expression of the targets and GAPDH are more valid and reliable. This is because they were amplified separately and did not have to compete for reagents during cycling and detection. A pitfall in single-plexing is that this less accurately controls for pipetting errors.

Aims

Hypertensive subjects showed a 3.92-fold difference in relative α_{1A}-ADR gene expression compared to normotensive subjects, a difference that was statistically significant (aim 1a, Table 4-7). Examination of the median 2^-DDCt values for the α_{1A}-ADR informs us that hypertensive subjects showed nearly 4 times lower expression of the α_{1A}- ADR gene in the arterial tissue investigated, perhaps suggesting the possibility of blunted vasoconstriction in this group as compared to normotensives (note: the higher 2^-DDCt median in the hypertensive group indicates lower gene expression and thus, downregulation of the gene). It has previously been described that α_{1A}-ADR downregulation could be explained as a consequence of enhanced sympathetic tone (for example, increased vascular resistance) in HTN (Jacobs, Lenders, Willemsen & Thien,

1997; Kinugawa, Endo, Kato, Kato, Ahmmed, Omodani, 1997). This is likely the result of a negative feedback loop. This downregulation of the α_{1A}- ADR gene in hypertensive subjects most similarly supports the work of Xu, Lu, Wei, Zhang and Han (1997), who reported a decrease in α_{1A}- ADR gene expression in 12-month old systemic hypertensive rats (SHRs). Conversely, Reja and colleagues (2002) reported α_{1A}-mRNA expression was significantly greater in SHT rat tissue samples. Furthermore, Veglio and colleagues (2001) reported no difference in α_{1A}- ADR gene expression between SHRs and Wistar-Kyoto (normotensive strain) rats in either blood lymphocytes or aortas. Our results for the α_{1A}- ADR gene expression appear to be both supported by and contradictory to other reports; however, no exact study design was available for true comparison.

For the β_2-ADR gene, hypertensive subjects showed 2.05-fold difference in relative gene expression compared to normotensives, a difference that was also statistically significant (aim 1b, Table 4-7). Once again, hypertensive subjects showed reduced expression of the β_2-ADR gene in the arterial tissue compared to normotensives. No true comparisons of this data to other reports in the literature regarding β_2-ADR expression in HTN. Gaballa and colleagues (1998) reported enhanced β_2-ADR mediated vasorelaxation in large artery walls of rats after adenovirally-mediated delivery of β_2-ADRs. Similarly, Iaccarino and colleagues (2002) overexpressed β_2-ADR in SHR and WKY rats and reported enhanced β_2-ADR-mediated vasorelaxation in both rat strains, with a lessened vasodilatory effect in the hypertensive strain. Based on further aims, the authors (2002) also concluded this vasorelaxation was directly related to β_2-ADR signaling dysfunction and not endothelium-dependent nitric oxide metabolism. These studies support our data and provide a plausible explanation for the downregulation in hypertensives; that

decreased expression of β_2-ADRs implies impaired vasorelaxation in HTN. However, as explained by Bustin (2002), these values represent only steady-state mRNA levels and do not allow inference of these numbers on post-transcriptional factors. In addition, normalization with GAPDH could be causing artifact in this data, as discussed previously.

These differences in α_{1A}- and β_2-ADR relative gene expression remained significant when comparing only Caucasian NT versus HTN subjects (Aims 2a and b, Table 4-8). For the α_{1A}-ADR gene, White/Caucasian hypertensives showed 4.03-fold difference in gene expression compared to normotensives (Table 4-9). This same pattern of significantly reduced gene expression in hypertensives compared to normotensvies continued with the β_2-ADR gene; there was a 5.27-fold difference in relative gene expression in hypertensives compared to normotensive subjects. These data indicate that, in part, fold-differences in gene expression of the two genes may be related to the diagnosis of HTN in self-identified White/Caucasian subjects. To see if SIR further attributed to these differences (when diagnosis is accounted for), self-identified White/Caucasian hypertensives were compared to self-identified Black/AA hypertensives (additional aim not previously listed). For the α_{1A}-ADR, hypertensives who self-identified as Black/AAs and White/Caucasians showed 1.47 fold-difference in gene expression, and a 3.88 fold difference for β_2-ADR, with Black/AAs showing greater expression of the gene; however, these differences were not significant (see Tables 4-10 and 4-11). Given this trend of greater gene expression for both genes in the self-identified Black/AA group, larger group sizes (note only 7 subjects for Black/AAs) may have the power to detect actual differences by SIR. Additionally, the GAPDH may be causing

artifact in the data here. It is important to note that the additional between-group comparisons were planned (such as comparing Black/AA HTN versus NT subjects); however, small sample sizes (n = 7 and 2, respectively) for these groups impeded this goal.

When examining the gene expression differences by need for inotrope pharmacotherapy, no significant fold-differences were found between those who did and did not require positive inotropes in the post-operative phase of recovery and gene expression of both genes (Aims 3a and b, Tables 4-12 and 4-13). Once again, these gene expression values were influenced by normalization issues and should be cautiously interpreted.

Exploratory Aims

Sample versus population allele frequency comparisons

Some minor differences were noted in allele frequencies between sample and population estimates (refer to Table 4-14). As previously explained, allele and genotype population estimates for the α_{1A}-ADR (Arg347Cys) polymorphism were obtained from the Ensembl database (http://www.ensembl.org/index.html). No other allele or genotype frequency reports with larger samples were found for this polymorphism. Population estimates for the β_2-ADR Arg16Gly G→A, and the β_2-ADR Glu27Gln C→G polymorphisms were obtained from the INVEST study (PharmGKB, 2006), as aforementioned. It is initially important to clarify that the Arg allele is considered the major (wild-type) allele for the Black/AA population in the α_{1A}-ADR (Arg347Cys) polymorphism, but is the minor allele for the European-descent populations (White/Caucasian groups) (Small, McGraw, & Liggett, 2003). The allele frequencies in this sample are mainly comparable to the population frequency reports, with the Arg (C)

allele being slightly more frequent in the White/Caucasian sample than in the general population. However, it is important to note that these are subtle disparities, especially due to the fact that all estimates were obtained from small cohorts. For the β_2-ADR (Arg16Gly) polymorphism, allele frequency comparisons had some distinctions. The Black/AA cohort had a slightly increased frequency of the Arg (G) allele versus the population frequencies, but this is subtle considering the small sample size. For the White/Caucasian cohort, the same Arg (G) allele was much more frequent in the sample as compared to the population estimates, with the frequencies being nearly inverted. This finding is most likely due to the small sample size of the subjects in this group; a larger sample may bring the sample values closer to the population estimates. For the β_2-ADR (Gln27Glu) polymorphism,. The Glu (G) allele is much more frequent in the sample of Black/AAs than what is expected in the population estimate (35% versus 18%); however, this result is once again likely a reflection of small sample size. In the White/Caucasian group, the sample allele frequency estimates are an exact match to the population estimates indicated in the INVEST database (PharmGKB, 2006).

Sample versus population genotype frequency comparisons

Similarly, minor disparities were noted between sample and population genotype frequencies (refer to Table 4-15). For the α_{1A}-ADR (Arg347Cys) polymorphism, Black/AA homozygotes for Arg (C/C genotype) had a higher frequency in the sample than in the population (70% versus 48%). This was the likely cause of the reduction in heterozygote frequency in the sample (20% heterozygotes in sample versus 48% heterozygotes in population). Caucasians that were homozygous for both Arg/Arg (C/C genotype) and Cys/Cys (T/T genotype) were slightly inflated compared to population estimates, but were mostly comparable. Once again, these subtle differences between

sample and population frequencies are likely due to small sample sizes in both the population samples and the study samples. In regards to the β_2-ADR (Arg16Gly) genotypes, frequencies were very similar for both Black/AA and White/Caucasian sample versus population estimates. For the β_2-ADR (Gln27Glu) genotype frequencies, Black/AA heterozygotes (Gln/Glu, G/C genotype) were much more frequent in the sample than in the population (50% versus 30%). In addition, Black/AA homozygotes for the Gln (C) allele were less frequent (40% in the sample versus 67% in the population). Inversely, White/Caucasian subjects actually had a higher frequency of Gln (C) homozygotes (44% versus 35%) and lower frequency of heterozygotes (Gln/Glu, G/C genotype) (38% versus 49%) in comparison of sample versus population estimates. All of these nuances are likely due to the small number of subjects obtained in each group. A larger sample size would probably yield more analogous allele and genotype frequencies between sample and population estimates. Nonetheless, all genotypes examined were determined to be in Hardy-Weinberg equilibrium (data not shown).

None of the ADR genotypes studied were positively associated with HTN (Table 4-16). When accounting for racial differences in these associations by only analyzing White/Caucasians, statistical significance was still not achieved (see Table 4-17). With genotype association analyses, our small sample size is the most likely culprit of the insignificant findings, as previous literature has reported genotype associations for the β_2-ADR polymorphisms (McCaffery, Pogue-Geile, Ferrell, Petro, & Manuck, 2002; Li, Faulhaber, Rosenthal, Schuster, Jordan, Timmermann, and Hoehe, et al., 2001; Cockroft, Gazis, Cross, Wheatley, Dewar, and Hall, et al., 2000; Bray, Krushkal, Li, Ferrell, Kardia, Sing, and Turner, et al., 2000).

An additional exploration was made into the association between individual alleles and diagnosis of HTN, since the aforementioned genotype analyses were insignificant. Allele counts were determined by weighting the alleles based on genotype, so that heterozygotes had half of the weight of homozygotes for each allele. Chi-square analyses were conducted for all subjects with HTN versus NT by allele for each of the three polymorphisms and are presented in Table 4-18. For the α_{1A}-ADR (Arg347Cys) the C (Arg) allele is more frequent in HTN than the T (Cys) allele ($p = 0.03$), suggesting it is more important in HTN. This association remained significant for the C allele in this gene when only White/Caucasians were examined ($p = 0.04$). This may be the first report of positive association of the Arg allele of the α_{1A}-ADR (Arg347Cys) polymorphism with HTN. Only one other study reported significant association with the α_{1A}-ADR (Arg347Cys) polymorphism and HTN, but reported greater association of the Cys allele, which is inconsistent with our findings. (Jiang, Mao, Zhang, Hong, Tang, and Li, et al., 2005). Functional studies of these alleles are limited to one by Shibata and colleagues, where no difference in agonist or antagonist binding, receptor-mediated calcium signaling, or extent of receptor desensitization following agonist exposure was found between either the Arg347 or Cys347 allele of this polymorphism in transfected Chinese hamster ovary cells (Shibata, Hirasawa, Moriyama,Kawabe, Ogawa, and Tsujimoto,1996). No other ADR alleles were positively associated with HTN (see Tables 4-18 and 4-19). The lack of association between the alleles of the β_2-ADR polymorphisms at codons 16 and 27 is supported by Castellano and colleagues (2002), Herrmann and colleagues (2002), and Tomaszewski and colleagues (2002).

Finally, no significant associations were found in the analyses of genotypes by gene expression (see Table 4-20). While small sample size may have also affected these analyses, this finding could be explained by the fact that all three polymorphic variants are located in the coding region of the gene, whereby their neutral variants could impact the function of the resulting protein but not necessarily the level of gene expression. If any of the variants were located in the promoter region of the gene (see Figures 3.1 and 3.2), they may be more likely to affect RNA stability and thus possibly produce a detectable change in gene expression.

Limitations

Normalization with GAPDH

In this study of human arterial tissue samples in subjects with coronary artery disease and other comorbid diagnoses, GAPDH did not perform well as a normalizer gene even though preliminary analyses indicated it should have. At the very least, these GAPDH data support others' findings of inconsistent expression with GAPDH as a housekeeping gene (Barber, Harmer, Coleman, & Clark 2005; Tricarico, Pinzani, Bianchi, Paglierani, Distante, and Pazzagli, et al., 2002), especially in regards to human clinical samples (personal communication with N. Chegini, 2005). As no other acceptable options existed for normalization of these data, the GAPDH was used for this purpose. Interpretation of results is cautioned due to this factor.

Power

For all aims, low power presented a problem. The power analyses conducted during the planning phase of the study factored in a medium effect size, as reported in the literature (Wang & Brown, 2001). The actual effect sizes were variable and produced different power. All of these aims would have required larger sample sizes per group to

facilitate 80% power (see Table 4-21). This lack of power creates a greater possibility for Type II error for these aims, wherein we fail to reject the null hypotheses when it is in fact false. This is another limitation of this study. In regards to the sample sizes necessary to obtain this power given the variable effect sizes, some aims remain feasible, while others (examining β2-ADR by SIR) would take a tremendous amount of effort, time, and expense to recruit 200 subjects as done for this study.

Internal Validity

This study only attempted to compare subjects with and without hypertension in regards to their levels of gene expression of two specific ADR genes. As the design of this study was exploratory in nature, we are unable to infer the causes of the gene expression differences found. We can only say that differences exist. We can not even be certain of the true significance of the differences reported, as the normalization of data may be inaccurate. Therefore, the housekeeping gene, GAPDH, contributes to reducing the internal validity of this study. We are uncertain even if these differences in GAPDH expression are due to researcher error and/or subject-related genetic or environmental factors. Likewise, the PI responsibly admits to the possibility of pipetting errors in the laboratory that could affect internal validity and overall reliability of triplicate data points for Ct values. Additionally, other factors not studied may contribute to expression differences in these genes, eroding our confidence in internal validity. There are transcription factors related to recruitment and processing of relevant RNA polymerase that affect binding of transcription factors to the promoter region of the gene. Strachen & Read (1999) further explain that other regulatory regions (flanking the gene or within introns) can interact with other protein factors to amend basal levels of transcription.

Construct Validity of the Variable, Normotension

The JNC VII criteria for HTN were used to identify hypertensives in the study. This tool considers that 'absence' of the criteria for HTN constitutes NT. While this seems logical, it is important to consider some key points regarding this variable. First, some false negatives could have existed, wherein there was poor documentation of past medical history/current diagnoses, BP values, and prescribed medications that indicated a subject was not diagnosed with HTN, when in fact they were. Second, we are uncertain of which subjects who are currently normotensive will eventually develop HTN. This potential to develop HTN could set them apart both biologically and environmentally, decreasing our confidence in these subjects as being true controls. Construct validity regarding this variable is a difficult problem to address in research involving BP and HTN and should be considered when interpreting results.

External Validity

As briefly discussed previously, this sample is only moderately representative of the population. These data may only be generalized to other IMA data in which the same normalization issues occurred with similar housekeeping gene results, for similar sample characteristics. It would be irresponsible to generalize these data to clinical patients with and without HTN.

Minimal Sample Template

Another limitation of this study was the small amount of starting template. The tissue samples ranged in weight from 10 mg to 34 mg. While no more than 30 mg can be used for each mRNA extraction, multiple extractions could have been possible if more tissue were available. Having more tissue would have allowed more opportunity for optimization of reagents and, possibly, of the housekeeping gene as well. Four subjects

were excluded from gene expression analyses due to insufficient cDNA and/or "undetermined" readings in the Real Time RT-PCR process. If more template had been available, additional RT could have been performed to make more cDNA, which would have allowed additional reactions to be redone, allowing inclusion of these subjects. It is likely that some steps in the tissue preparation and RNA extraction processes could have resulted in lower yields of RNA; however, even 10 mg more tissue per subject would have provided 'backup' sources when necessary. It would be prudent to discuss the feasibility of this with the cardiothoracic surgeons for future studies.

Confounding Variables

Given that β-blockers block β-ADR receptors (and some α-ADR receptors) at the cellular level, it was hypothesized that concurrent use of β-blockers could impact gene expression and act as a confounder for gene expression of the two target genes studied. A simple correlation between the variable 'type/dose of β-blocker' and each of the target genes' expression showed no significant correlations ($R = 0.146$, $p = 0.367$ for α_{1A}-ADR; $R = 0.095$, $p = 0.562$ for β_2-ADR). As most subjects in this study were on metoprolol, a selective β_1-ADR blockade medication, confounding with the β_2-ADR receptor system is probably unlikely. On the other hand, one subject in this study was taking Labetalol, a β_2 antagonist that is non cardioselective with α_1-blocking activity. While the presence of this medication could confound the individual subject's data for both the α_{1A}-and β_2-ADR, it would unlikely affect the entirety of the data. At least one other study supports that pharmacologic β_1-ADR blockade does not affect gene expression (Wang & Brown, 2001). They (2001) utilized TaqMan gene expression analysis to study gene expression response to β_1-ADR blockade in adenylate cyclase subtypes and in β-ADR kinase within human atrium. The authors (2001) found no differences in absolute gene expression

between groups receiving pharmacologic β_1-ADR blockade, indicating that pharmaceutical β_1 blockade did not affect gene expression of the β-ADR isoforms. This study was important in examining the environmental factors that may affect expression of the ADR subtypes in human tissue. The author is aware of no literature to support α-ADR expression or protein differences as a result of β- blockade.

Based on the literature described below, the co-diagnosis of T2DM was also hypothesized to confound gene expression of the ADR target genes investigated. Recent studies have examined T2DM and vascular changes in regards to adrenergic activity. A study conducted on rats with experimentally-induced diabetes demonstrated that vasomotor responses are impaired in diabetes (Kamata, Satoh, Tanaka, & Shigenobu, 1997). However, others have reported no significant differences in response to inotropes in human diabetics versus non-diabetics (Dincer, Onay, Ari, Ozcelikay, & Altan, 1998). One group studied humans with T2DM and reported enhanced CVR in this population, and indicated a possible alteration at the receptor level as the proposed mechanism (Cipolla, Harker, & Porter, 1996). One particular study linked HTN and T2DM genetically, implicating the Arg16 allele of the β_2-adrenergic receptor gene with an increased risk for HTN in subjects with T2DM (Bengtsson, Orho-Melander, Melander, Lindblad, Ranstam, and Ranstam, et al., 2001). Despite the abundance of association and linkage studies, no expression studies were found that examined the differences in human α_{1A}-and β_2- ADR gene expression in diabetics versus non-diabetics; therefore, it can only be hypothesized that T2DM could confound ADR-specific gene expression. In this study, T2DM did not significantly correlate with either target gene expression (R = -0.052, p = 0.745 for α_{1A}-ADR; R = -0.069, p = 0.669 for β_2-ADR). The absence of a correlation

between these variables provides some confidence that T2DM was not a significant confounder of the gene expression data.

Nursing Relevance

The approach for this study and its design were borne out of the investigator's clinical experiences as a nurse and from training received at the National Institutes of Health, National Institute of Nursing Research Summer Genetics Institute. Having experience on a cardiac-telemetry unit made the investigator aware that normotensive bypass patients do, in fact, exist and could potentially serve as "normal" controls. In addition, being aware that gene expression research was at the forefront of genomic scientific inquiry, and having learned of its potential to be highly discriminatory of genomic differences in rat models, the investigator saw the potential of this methodology being informative in the clinical phenotype of HTN. This investigator saw great opportunity in approaching the clinical phenotype of HTN in a way that would create a foundation for investigating these particular genes in HTN at the genomic level in the clinical setting. This study was approached and designed perhaps differently than a pure bench researcher may have. At some level, this approach may yield more clinically applicable information to those researchers seeking knowledge about the expression of these receptors in the human phenotype of HTN.

In general, gene expression studies of this nature have the potential to inform practitioners of the impact of gene expression on disease states. In particular, studies of this nature are important in that they focus on human disease patterns and biological differences that may contribute to the overall picture of HTN. As this was a pilot study, direct clinical application to nursing or health care can not be extrapolated; however, important knowledge was generated from this study. This pilot study has shown the

feasibility of collecting human arterial tissue for the purpose of gene expression research and provided a basis for what is necessary methodologically to develop future gene expression studies in HTN. Just as pathophysiological research has informed us of disease mechanisms, gene expression research stands to take us further into understanding those pathways of disease. This type of research can be more informative than just group differences between cases and controls, but of individual patterns whereby environmental factors are accounted for in the presence of susceptibility genes. This methodology examines how the genes are expressed in a given environment. This gene by environment interaction is the pivotal crux that makes studying diseases in this way more amenable to nursing questions. The focus is not just on the genetic component, but on how the genes are expressed in the environment (a milieu created by the individual's life choices) and how certain people are more susceptible to disease than others with these genetic and environmental factors. Nurse researchers can and should consider this area of research to look at ways to benefit patients holistically through scientific research that examines both genetic and environmental factors and their interplay in health and disease. Recent advances in genomic research are leading us toward more individualized medicine, especially with microarray technology and the optimization of blood-based gene expression tools. Not only will practitioners benefit from knowledge and tools gained from this type of research for screening and treatment, patients stand to benefit from tailored health care that results from this type of research.

Practice- and Care-Related Relevance

As genetics becomes an integral part of health care and treatment considerations, nurses will be increasingly required to have more sophisticated understanding of these genetic concepts and current research. For decades, nurses have taken on roles in genetics

as patient educators, advocates, and counselors (especially with regard to prenatal testing and genetics-related reproductive issues). Now, these previous roles are becoming more sophisticated and new roles are being developed in which nurses are needed (and desired) to help translate genetics and genomics research into practice. The future of health care will require a contingent of health care providers to deliver the information accurately for appropriate use in decision-making by patients. The optimal source for these provisions is genetic counselors (GCs); however, the lack of and competitive nature of GC programs, and issues with reimbursement of GCs for their services has resulted in a small GC workforce. It is becoming increasingly apparent within the nursing profession (and among GCs) that these current GC professional issues are putting nurses in the position to assume GC-like roles; to provide patients with knowledge, interpretation, counseling, and assistance in decision-making in regards to genetic information, screening, testing, and participation in research. Furthermore, as the pace of biotechnology exponentially accelerates, so does the incorporation of biotechnology into practice and care. Nurses must be active in continuing education regarding genetics and genomics and be evermore aware of the research in these fields that will likely affect practice in the near future.

With specific regard to research, nursing research in genetics through interdisciplinary teams is a charge of the NIH Roadmap (Huerta, Farber, Wilder, Kleinman, Grady, and Schwartz, et al., 2005) and is supported for individual research endeavors by the National Institutes of Health (Jenkins, Grady, & Collins, 2005), National Institute of Nursing Research (Grady & Collins, 2003; Sigmon, Grady, & Amende, 1997), and the International Society of Nurses in Genetics (ISONG; organization goals, http://www.isong.org/about/index.cfm, 2005). Loescher and Merkle

(2005) published a review of recently-funded NINR studies using genetic and/or genomic methods where nurses were primary investigators. At least thirteen nurse-driven studies were cited in which advanced biological, genetic and/or genomic methods were utilized, from linkage analyses to microarray, reinforcing the notion that where there is funding occurring, there is desire, need, and support for the research. Lashley, a geneticist and nursing leader in genetics, lists 'disease markers' and 'disease mechanisms' as two of the top research directions for nursing research in genetics (2001). Dr. Janet Williams, another nurse leader in genetics, developed the first NIH-funded postdoctoral program in Nursing Genetics at the University of Iowa, where scientific research that uses biological, molecular, and genetic methods and technology is fostered and encouraged among nurse scientists in the program. In 2000, Hill published her research on comprehensive HTN care in young urban black men, a study that integrated genetic science, clinical interventions, and patient outcomes. Feetham (2000) summarized Hill's recommendation that "nursing research must address research questions and hypotheses that integrate genetic science and issues if nursing practice is to be based on science that is current, relevant, and leveraged effectively" (p. 258).

Nurse-Directed Research and Qualitative Findings

Historically, nurses have recognized the importance of the patient as a whole. This philosophy has transcended practice and has permeated nurse-directed research. During the completion of this study, the PI had a number of experiences with potential and enrolled subjects that reflected the notion that it is both prudent and important to maintain a philosophy of holism when conducting clinically-based research. These next paragraphs present some of the qualitative findings realized during the completion of the study.

This study involved recruitment and consent of subjects who are preparing to undergo a moderate- to high-risk surgical cardiac intervention. This brought about some valuable interactions between the researcher and potential/enrolled subjects. Some of these interactions should be highlighted in order to gain the most out of the research experience. For one, while some participants expressed apprehension about enrolling in genetics studies, particularly among Black/AAs, a majority of the participants in this study reported a strong desire to contribute to research for HTN and genetics. Subject concerns regarding the study revolved around protection of data from insurers, protection of DNA and tissue, and having any unwanted 3rd party from obtaining the information.

A majority of potential subjects and participants expressed feeling stressed about their upcoming bypass surgery. These expressions often occurred during the recruitment and consenting process. The researcher maintained awareness of this during these procedures and often decided to postpone discussion of the study with potential subjects in order to talk about their fears and concerns. Many concerns stemmed from fear of death or complications, being out of work for surgery and recovery, feeling like a burden to family members, and concerns with insurance and payment of hospital bills. Once patient education was provided in regards to the surgery, the researcher felt more comfortable proposing participation in the study. The researcher made it clear that potential subjects were not, *in any way*, obligated to participate given the 'counseling'. To emphasize this, the researcher provided additional time for their review of the study materials prior to consenting subjects.

Case Study

One particular participant stands out in regards to apprehension in participating in the study. He was a self-identified Black/AA male, 44 years old. He was concerned with

the aims involving race and voiced mistrust of research. A conversation regarding the studies' background and significance of HTN with regards to race occurred. After further discussion, he explained that his sensitivity to the issue stemmed from feeling like a victim of racism in the hospital. He was also feeling a great deal of stress from financial insecurity, a poor support system, fear of poor recovery, and lack of resources after discharge. In addition, he felt that he would continue to receive less than standard-of-care because of his race and financial status. The researcher initiated an in-depth discussion about his feelings and concerns and provided him with the name and contact information for the hospital's patient advocate/liaison. The researcher felt that continuing with the recruitment of this subject for the study would be unethical; however, the subject expressed interest. The ethical issues with obligation of reciprocation were explained and the researcher expressed not wanting to add any further undue stress in his life. The subject requested an opportunity to review the paperwork until the next day. The following day, he expressed sincere desire to participate, stating [paraphrasing] that he wasn't sure if he would ever be able to contribute to science and that if he could, he would want it to be something that would possibly contribute to people in his population with similar health problems. This patient was enrolled in the study.

Summary

These interactions provided the researcher with a great deal of insight into the qualitative issues surrounding subject participation in research and the stress invoked by patients facing bypass surgery. These interactions may not be entirely unique to nursing, but are important to nursing research and in the developing of future research involving genetics and bypass patients. The case study particularly highlights the importance of nurses' being well-versed on issues of genetics and race in research and practice.

Recommendations for Future Research

First and foremost, it would be most prudent to test and optimize more than one housekeeping gene when relying on an endogenous control for normalization. When financially feasible, a housekeeping gene "plate" can be purchased (such as the one sold by Applied Biosystems), where a panel of common housekeeping genes can be tested with the sample of interest in order to select the one that meets all of the criteria for a proper endogenous control.

Second, attempting to obtain larger tissue samples would likely lead to greater success in optimizing the reactions, perhaps even for multi-plexing (which reduces costs and saves template). Other non-bypass arterial tissue sources should also be considered, as not every bypass patient requires a bypass using the IMA.

Future studies that examine these genes and their differential expression in HTN could be much more informative if actual BP values could be obtained. While there are numerous issues with the collection and use of BP (including, but not limited to, measurement issues, interindividual variability in values, circadian variation, the White-coat phenomenon, and most importantly, the confounding of BP values by concomitant use of antihypertensive medications), having a continuous variable by which to compare the gene expression, rather than a simple dichotomous variable, could lead to more informative and inferential statistical analyses (where the aforementioned limitations could be allayed). For example, one could possibly predict gene expression levels based on BP levels with regression analyses.

Future studies that examine the impact of self-identified race should *very* carefully consider the current debate in this area. If a greater (and more representative) sample were obtained, there would be sufficient power to analyze other health-related variables

linked to race such as indicators of socioeconomic status (wealth, income, poverty level, access to health care), education, and indices of racism. Moreover, the use of ancestry informative markers to account for the biological variance in geographic ancestry could improve further inquiries and provide a more quantitative measure of ancestral origins, rather than with the indirect self-report of race as a proxy.

Qualitative inquiries that focused on a) the nature of participation in research involving race and/or genetics; and b) perceived stress by patients preparing for bypass surgery could be important. This information could ameliorate studies of this nature in the future.

Finally, future studies should examine more HTN candidate genes and additional environmental factors so that more complex questions can be answered that may lead to better prevention, screening, and treatment of HTN through the use of biomarkers. Eventually such information can inform practitioners of individualized biophysiological patterns and treatment. The PI recognizes that the feasibility of using human tissue samples for future clinical screening would be an impractical endeavor. The use of blood-based gene expression methods is now being refined and will likely be adapted for clinical screening tools. The use of tissue in research, however, still stands to provide the best direct measurement of gene expression and should continue to be utilized in disease-based, gene expression research.

Conclusions

A great deal of literature has supported the potential for gene expression to unlock many of the mysteries of complex diseases. This is likely due to the nature of gene expression being affected by both genetic and environmental factors. While animal

models are invaluable in this line of research, gene expression studies that extend to human models of disease will serve us greatly in truly understanding human disease.

To the author's knowledge, this study is the first to explore α_{1A}- and β_2-ADR gene expression differences in human arterial tissue between normotensive and hypertensive adults. This study demonstrated that collecting, preserving, and preparing human tissue for the purpose of performing Real Time RT-PCR for relative gene expression was possible and moderately feasible, given some training and adequate funding. This study outlines some of the technical challenges in performing these types of analyses but shows the potential to optimize this process for more internally valid results. This study also provided estimates of effect sizes for use in future research. In addition, this study obtained valuable qualitative information regarding subject recruitment, consent, perceived stress, and concerns regarding participation in research involving genetics and race. All statistical findings from this study should be cautiously interpreted. To summarize: 1) Significant fold-differences in α_{1A}- and β_2-ADR gene expression were found between people with and without HTN; 2) These differences remained significant when examining only self-identified White/Caucasian hypertensive versus normotensive subjects; 3) Significant differences were not found between self-identified White/Caucasians and self-identified Black/AAs with HTN; 4) The need for post-operative positive inotrope medication did not significantly correlate to either α_{1A}- or β_2-ADR gene expression; 5) ADR genotypes were not significantly associated with the diagnosis of HTN; 6) The C (Arg) allele of the α_{1A}- ADR 347 polymorphism was significantly associated with HTN; 7) There was no significant relationship between ADR genotypes and ADR gene expression; 8) Unsuccessful normalization with GAPDH

in this study supports other findings and contributes largely to the lack of confidence in results. Replication of this research with careful consideration of the points discussed would lead to important information about the nature of clinical differences in ADR gene expression in the disease process of HTN.

APPENDIX A
SUBJECT ENROLLMENT/DEMOGRAPHIC FORM

Name _____ Date _____

Phone _____

A. Age : _____ Date of Birth:____/____/____

B. Sex : 1. Male 2. Female

C. ETHNICITY
Do you consider yourself to be Hispanic or Latino? (See definition below.) Select one or more.

- ☐ ***Hispanic or Latino*** (A person of Mexican, Puerto Rican, Cuban, South or Central American, or other Spanish culture or origin, regardless of race.)
- ☐ ***Not Hispanic or Latino***

D. RACE
What race do you consider yourself to be? Select **one or more** of the following.

- ☐ ***American Indian or Alaska Native.*** A person having origins in any of the original peoples of North, Central, or South America, and who maintains tribal affiliation or community attachment.
- ☐ ***Asian.*** A person having origins in any of the original peoples of the Far East, Southeast Asia, or the Indian subcontinent, including, for example, Cambodia, China, India, Japan, Korea, Malaysia, Pakistan, the Philippine Islands, Thailand, and Vietnam. (Note: Individuals from the Philippine Islands have been recorded as Pacific Islanders in previous data collection strategies.)
- ☐ ***Black or African American.*** A person having origins in any of the black racial groups of Africa. Terms such as "Haitian" or "Negro" can be used in addition to "Black" or African American."
- ☐ ***Native Hawaiian or Other Pacific Islander.*** A person having origins in any of the original peoples of Hawaii, Guam, Samoa, or other Pacific Islands.
- ☐ ***White.*** A person having origins in any of the original peoples of Europe, the Middle East, or North Africa.
- ☐ Check here if you do not wish to provide some or all of the above information.

E. Your highest level of completed education is:
____Less than 9th grade in high school
____Less than 12th grade in high school
____High school diploma
____GED
____Some college or technical training
____Associate degree
____Bachelor's degree
____Master's degree
____Doctoral degree (M.D., Ph.D., or J.D.)

F. Body Height: _____ ft Weight: _____ lbs

G. Did you smoke? YES NO
If yes, do you smoke now? YES NO
How long have you (or did you) smoke? _____ years

H. Do you drink alcohol? YES NO
If yes, how often do you drink alcohol? _____
How many drinks do you usually have in one week? _____

I. Do you exercise? YES NO
What types of activities do you do when you exercise?_____
How many times during the week do you usually exercise? _____

J. Marital status: (please circle)

Married Single Divorced Separated Other

K. Please circle the size of your family unit (number of people in household) and circle what your approximate yearly household income is (this information is confidential).

Size of Family Unit	**< 200%**	**200-400%**	**> 400%**
1	$0 – 17,999	$18,000 – 34,999	$35,000 +
2	$0 – 23,999	$24,000 – 46,999	$47,000 +
3	$0 – 29,999	$30,000 – 58,999	$59,000 +
4	$0 – 35,999	$36,000 – 70,999	$71,000 +
5	$0 – 41,999	$42,000 – 82,999	$83,000 +
6	$0 – 47,999	$48,000 – 94,999	$95,000 +
7	$0 – 53,999	$54,000 – 106,999	$107,000 +
8	$0 – 59,999	$60,000 – 118,999	$119, 000 +

Past Medical History

_____
_____
_____
_____

Medications
Prescribed:

_____
_____
_____

Over the counter:

_____
_____
_____

Herbal Remedies

_____
_____

N. Would you like me to let your primary care provider know that you are participating in this study? If so, please provide your PCP's name and address.

PCP Name _____
Address _____
_____
Phone _____

APPENDIX B
UF IRB-01 INFORMED CONSENT FORM

IRB# 33-2004

Informed Consent to Participate in Research and Authorization for Collection, Use, and Disclosure of Protected Health Information

You are being asked to take part in a research study. This form provides you with information about the study and seeks your authorization for the collection, use and disclosure of your protected health information necessary for the study. The Principal Investigator (the person in charge of this research) or a representative of the Principal Investigator will also describe this study to you and answer all of your questions. Your participation is entirely voluntary. Before you decide whether or not to take part, read the information below and ask questions about anything you do not understand. If you choose not to participate in this study you will not be penalized or lose any benefits to which you would otherwise be entitled.

1. Name of Participant ("Study Subject")

_____

2. Title of Research Study

Alpha 1A- and Beta 2-Adrenoceptor Gene Expression Differences In Hypertensive and Normotensive Persons By Race
Subtitle 1: Race, HTN, and Vascular Adrenoceptor Gene Expression

3. Principal Investigator and Telephone Number(s)

Jennifer R. Dungan, MSN, ARNP
PI Office: (352) 273-6512
PI Cell: (352) 256-7487
Co PI: Ann Horgas, RN, PhD
Co-PI Office: (352) 273-6318

4. Source of Funding or Other Material Support

Material Support: University of Florida
Funding: American Heart Association, # 0415124B (partial)
NIH/National Institutes of Nursing Research, # 1 F31 NR009148-01
Sigma Theta Tau, Alpha Theta Chapter (no grant #)

5. What is the purpose of this research study?

The purpose of this research study is to look at differences in the gene expression of two genes (the alpha 1A- and beta 2-adrenergic receptor genes) between persons with and without high blood pressure. Gene expression refers to the way your genes work. This is done by looking at the messenger ribonucleic acid (mRNA) levels found in your tissue sample. These levels tell us if the gene is functioning too much or not enough. By collecting a tissue sample from you and others in the study, we may be able to determine if function of certain genes is important in high blood pressure.

6. What will be done if you take part in this research study?

If you decide to take part in this research study, three things will happen. First, we will collect a teaspoon of blood from a vein in one of your arms and store it in a locked freezer. This blood will be used to genotype—or characterize—certain genes that are important in cardiovascular disease and genes that may be important in statistically determining similarity in groups within the population. Second, the PI will be given a piece of tissue from your chest that is normally thrown away during your coronary artery bypass surgery. This piece of tissue is leftover from surgery, and will come from an artery near your heart. This piece of tissue will be analyzed for gene expression of two genes. Once the tissue is analyzed, it will be thrown away. Third, the PI will review your medical chart to see if you received a certain type of heart medication. If you did receive the type of medication, the PI will collect information about your response to the medication. NOTE: You will not purposefully be given this drug as a result of being in the study.

Small amounts of your DNA may be sent to outside laboratories for this and future analyses. Any future analyses of your DNA would be for research purposes only. Tests for clinical markers that would affect your clinical care (for example, testing for a specific disease) will not be performed without your signed informed consent.

The choice to let Jennifer Dungan draw and keep your blood for doing research is entirely up to you. No matter what you decide to do, it will not affect your care. If you decide that your blood can be kept for research but you later change your mind, tell Jennifer Dungan who will remove and destroy any of your blood she still has. Otherwise, the samples may be kept up to 15 years, until they are used up, or until Jennifer Dungan decides to destroy them.

Please review the following four statements carefully. If you disagree with any of these statements, you should not participate in the study.

1. I understand that my samples will be stored for up to 15 years, coded to protect my identity, and that my identity will not be disclosed to anyone without my permission, except when required by law.

2. I understand that some excess blood may be kept by Jennifer Dungan for use in future research to learn about, prevent, treat, or cure hypertension and cardiovascular disorders.

3. I understand that my blood (but not tissue) may be used for research to answer other medical questions that are not necessarily related to hypertension and cardiovascular disorders.

4. I understand that Jennifer Dungan (or someone she chooses) can contact me in the future to ask me to take part in more research.

If you have any questions now or at any time during the study, you may contact the Principal Investigator listed in #3 of this form.

7. If you choose to participate in this study, how long will you be expected to participate in the research?

The active participation in the study to collect blood and tissue is approximately 1 hour. Passive participation, which involves reviewing your chart after surgery, is expected to take one month or less. Following chart review, your participation is complete in the study.

8. How many people are expected to participate in this research?

For this study, we expect to have between 60-72 participants. We aim to have at least 30 people with high blood pressure and 30 people with normal blood pressure enrolled in the study.

9. What are the possible discomforts and risks?

The only potential risk is that associated with drawing blood. The risks of drawing blood from a vein include discomfort at the site of puncture; possible bruising and swelling around the puncture site; rarely an infection; and, uncommonly, faintness from the procedure.

This study may include risks that are unknown at this time.

Participation in more than one research study or project may further increase the risks to you. Please inform the Principal Investigator (listed in #3 of this consent form) or the person reviewing this consent with you before enrolling in this or any other research study or project.

Throughout the study, the researchers will notify you of new information that may become available and might affect your decision to remain in the study.

If you wish to discuss the information above or any discomforts you may experience, you may ask questions now or call the Principal Investigator or contact person listed on the front page of this form.

10a. What are the possible benefits to you?

There are no possible benefits to you if you participate in the study. You will not be compensated for the study. You will also not be given any information regarding the results of this study.

10b. What are the possible benefits to others?

Even though the research that is done on your tissue will not be used to help you, it may be helpful to others with high blood pressure in the future. Because of this study, it may be possible in the future to determine the importance of gene expression in high blood pressure.

11. If you choose to take part in this research study, will it cost you anything?

There will be no cost to your for any specimens that are collected and stored, or any other materials used in this research project.

Costs for routine medical care procedures that are not being done only for the study will be charged to you or your insurance. These costs may not be charged if you are a veteran and you are being treated at the North Florida/South Georgia Veterans Health System (NF/SG VHS).

12. Will you receive compensation for taking part in this research study?

No, you will not receive compensation for taking part in this study.

13. What if you are injured because of the study?

If you experience an injury that is directly caused by this study, only professional consultative care that you receive at the University of Florida Health Science Center will be provided without charge. However, hospital expenses will have to be paid by you or your insurance provider No other compensation is offered. Please contact the Principal Investigator listed in Item 3 of this form if you experience an injury or have any questions about any discomforts that you experience while participating in this study.

14. What other options or treatments are available if you do not want to be in this study?

Participation in this study is entirely voluntary. You are free to refuse to be in this study. If you refuse to participate, it will not effect the treatment to which you are otherwise entitled.

15a. Can you withdraw from this research study?

You are free to withdraw your consent and to stop participating in this research study at any time. If you do withdraw your consent, there will be no penalty, and you will not lose any benefits you are entitled to.

If you decide to withdraw your consent to participate in this research study for any reason, you should contact Jennifer R. Dungan at (352) 273-6512.

If you have any questions regarding your rights as a research subject, you may phone the Institutional Review Board (IRB) office at (352) 846-1494.

15b. If you withdraw, can information about you still be used and/or collected?

If you decide to withdraw from the study, data already collected may be used in the completion of the study's analysis, but no further data will be collected and/or used.

15c. Can the Principal Investigator withdraw you from this research study?

You may be withdrawn from the study without your consent for the following reasons:

It is discovered that you no longer meet the inclusion/exclusion criteria.

16. If you agree to participate in this research study, the Principal Investigator will create, collect, and use private information about you and your health. Once this information is collected, how will it be kept secret (confidential) in order to protect your privacy?

Information collected about you and your health (called protected health information), will be stored in locked filing cabinets or in computers with security passwords. Only certain people have the legal right to review these research records, and they will protect the secrecy (confidentiality) of these records as much as the law allows. These people include the researchers for this study, certain University of Florida officials, the hospital or clinic (if any) involved in this research, and the Institutional Review Board (IRB; an IRB is a group of people who are responsible for looking after the rights and welfare of people taking part in research). Otherwise your research records will not be released without your permission unless required by law or a court order.
Results of genetic testing will not be included in your medical record and will be kept in a secure electronic database that is available only to the study investigators. Your records will not be released to any person or agency unless you provide written consent to Jennifer Dungan requesting the release of the results of the study.

Jennifer Dungan will be responsible for making sure that any stored blood sample is protected in the specimen bank and that your medical information is kept confidential. Your sample will not be stored with your name or other identifying information, but instead will be given a code number to protect your identity. These samples and this code number will only be given to researchers whose research is approved by an Institutional Review Board (IRB). (An IRB is a group of people who are responsible for looking after the rights and welfare of people taking part in research.) The researchers will not be told who you are or given any identifying information about you. Because the nature and value of any future research cannot be known at this time, any results obtained from using your tissue will not be given to you or your doctor.

If the results of this research are published or presented at scientific meetings, your identity will not be disclosed.

If you participate in this research study, the researchers will collect, use, and share your protected health information with others. Items 17 to 26 below describe how this information will be collected, used, and shared.

17. If you agree to participate in this research study, what protected health information about you may be collected, used and shared with others?

To determine your eligibility for the study and as part of your participation in the study, your protected health information may be collected, used, and shared with others to determine if you can participate in the study, and then as part of your participation in the study. This information can be gathered from you or your past, current or future health records, from procedures such as physical examinations, x-rays, blood or urine tests or from other procedures or tests. This information will be created by participating in study procedures, or from your study visits and telephone calls. More specifically, the following information may be collected, used, and shared with others:

Complete past medical history, current medications, race, age, and records of previous blood pressures obtained during physical examinations to determine eligibility criteria.

Also, as part of the demographic information obtained from you to be used in data analysis, the following information will be obtained: name, age, phone number, race, ethnicity, current medications, records of previous blood pressure, income, education, job, marriage status, number of people in your household, first degree relative information and information about your smoking, drinking, and exercise habits.

If you agree to be in this research study, it is possible that some of the information collected might be copied into a "limited data set" to be used for other research purposes. If so, the limited data set may only include information that does not directly identify you. For example, the limited data set cannot include your name, address, telephone number, social security number, or any other photographs, numbers, codes, or so forth that link you to the information in the limited data set. If used, limited data sets have legal agreements to protect your identity and confidentiality and privacy.

18. For what study-related purposes will your protected health information be collected, used, and shared with others?

Your protected health information may be collected, used, and shared with others to make sure you can participate in the research, through your participation in the research, and to evaluate the results of the research study. More specifically, your protected health information may be collected, used, and shared with others for the following study-related purpose(s):

1) to determine the impact of gene expression on high blood pressure and response to certain medications

19. Who will be allowed to collect, use, and share your protected health information?

Your protected health information may be collected, used, and shared with others by:

- the study Principal Investigator Jennifer R. Dungan and her staff
- other professionals at the University of Florida or Shands Hospital that provide study-related treatment or procedures
- the University of Florida Institutional Review Board

20. Once collected or used, who may your protected health information be shared with?

Your protected health information may be shared with:

- the study sponsors: The American Heart Association, The National Institutes of Nursing Research (of the National Institutes of Health) and Sigma Theta Tau, Alpha Theta Chapter
- United States and foreign governmental agencies who are responsible for overseeing research, such as the Food and Drug Administration, the Department of Health and Human Services, and the Office of Human Research Protections
- Government agencies who are responsible for overseeing public health concerns such as the Centers for Disease Control and Federal, State and local health departments
- Malcom Randall VA Medical center (Gainesville)

21. If you agree to participate in this research, how long will your protected health information be used and shared with others?

Your protected health information will be used until the end of the study. Thereafter, your protected health information will be stripped from the research database and the database containing the unidentifiable information will be kept under the protection of the principal investigator forever.

22. Why are you being asked to allow the collection, use and sharing of your protected health information?

Under a new Federal Law, researchers cannot collect, use, or share with others any of your protected health information for research unless you allow them to by signing this consent and authorization.

23. Are you required to sign this consent and authorization and allow the researchers to collect, use and share with others your protected health information?

No, and your refusal to sign will not affect your treatment, payment, enrollment, or eligibility for any benefits outside this research study. *However, you cannot participate in this research unless you allow the collection, use and sharing of your protected health information by signing this consent/authorization.*

24. Can you review or copy your protected health information that has been collected, used or shared with others under this authorization?

You have the right to review and copy your protected health information. However, you will not be allowed to do so until after the study is finished.

25. Is there a risk that your protected health information could be given to others beyond your authorization?

Yes. There is a risk that information received by authorized persons could be given to others beyond your authorization and not covered by the law.

26. Can you revoke (cancel) your authorization for collection, use and sharing with others of your protected health information?

Yes. You can revoke your authorization at any time before, during, or after your participation in the research. If you revoke, no new information will be collected about you. However, information that was already collected may still be used and shared with others if the researchers have relied on it to complete and protect the validity of the research. You can revoke your authorization by giving a written request with your signature on it to the Principal Investigator.

27. How will the researcher(s) benefit from your being in this study?

In general, presenting research results helps the career of a scientist. Therefore, the Principal Investigator may benefit if the results of this study are presented at scientific meetings or in scientific journals.

28. Signatures

As a representative of this study, I have explained to the participant the purpose, the procedures, the possible benefits, and the risks of this research study; the alternatives to being in the study; and how the participant's protected health information will be collected, used, and shared with others:

_____

Signature of Person Obtaining Consent and Authorization Date

You have been informed about this study's purpose, procedures, possible benefits, and risks; the alternatives to being in the study; and how your protected health information will be collected, used and shared with others. You have received a copy of this Form. You have been given the opportunity to ask questions before you sign, and you have been told that you can ask other questions at any time.

You voluntarily agree to participate in this study. You hereby authorize the collection, use and sharing of your protected health information as described in sections 17-26 above. By signing this form, you are not waiving any of your legal rights.

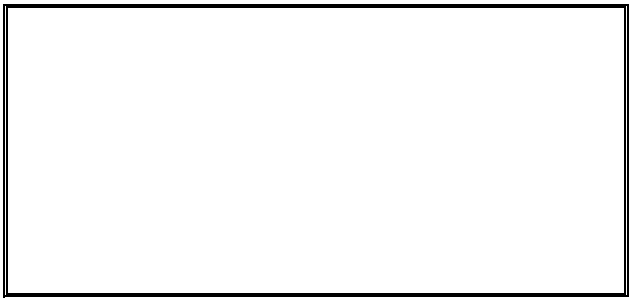
_____

Signature of Person Consenting and Authorizing Date

APPENDIX C
VA SCI INFORMED CONSENT FORM

IRB # 33-2004

Informed Consent to Participate in Research and Authorization for Collection, Use, and Disclosure of Protected Health Information



You are being asked to take part in a research study. This form provides you with information about the study and seeks your authorization for the collection, use and sharing of your protected health information necessary for the study. The Principal Investigator (the person in charge of this research) or a representative of the Principal Investigator will also describe this study to you and answer all of your questions. Your participation is entirely voluntary. Before you decide whether or not to take part, read the information below and ask questions about anything you do not understand. If you choose not to participate in this study you will not be penalized or lose any benefits to which you would otherwise be entitled.

1. Name of Participant ("Study Subject")

_____

2. Title of Research Study

Alpha 1A- and Beta 2-Adrenoceptor Gene Expression Differences In Hypertensive and Normotensive Persons By Race
Subtitle 1: Race, HTN, and Vascular Adrenoceptor Gene Expression

3. Principal Investigator and Telephone Number(s)

Jennifer R. Dungan, MSN, ARNP
PI Office: (352) 273-6512
PI Cell: (352) 256-7487
Co PI: Ann Horgas, RN, PhD (352) 273-6318
Sub-PI (VA PI): Philip J. Hess, M.D. (352) 413-0143 (pager)

4. Source of Funding or Other Material Support

Material Support: University of Florida
Funding: American Heart Association, # 0415124B (partial)
NIH/National Institutes of Nursing Research, # 1 F31 NR009148-01
Sigma Theta Tau, Alpha Theta Chapter (no grant #)

5. What is the purpose of this research study?

The purpose of this research study is to look at differences in the gene expression of two genes (the alpha 1A- and beta 2-adrenergic receptor genes) between persons with and without high blood pressure. Gene expression refers to the way your genes work. This is done by looking at the messenger ribonucleic acid (mRNA) levels found in your tissue sample. These levels tell us if the gene is functioning too much or not enough. By collecting a tissue sample from you and others in the study, we may be able to determine if function of certain genes is important in high blood pressure.

6. What will be done if you take part in this research study?

If you decide to take part in this research study, three things will happen. First, we will collect a teaspoon of blood from a vein in one of your arms and store it in a locked freezer. This blood will be used to genotype—or characterize—certain genes that are important in cardiovascular disease and genes that may be important in statistically determining similarity in groups within the population. Second, the PI will be given a piece of tissue from your chest that is normally thrown away during your coronary artery bypass surgery. This piece of tissue is leftover from surgery, and will come from an artery near your heart. This piece of tissue will be analyzed for gene expression of two genes. Once the tissue is analyzed, it will be thrown away. Third, the PI will review your medical chart to see if you received a certain type of heart medication. If you did receive the type of medication, the PI will collect information about your response to the medication. NOTE: You will not purposefully be given this drug as a result of being in the study. The data we analyze and use from this study (not the tissue) may be used in future studies that address similar questions. You may choose not to allow us to use your data in future studies

Small amounts of your DNA may be sent to outside laboratories for this and future analyses. Any future analyses of your DNA would be for research purposes only. Tests for clinical markers that would affect your clinical care (for example, testing for a specific disease) will not be performed without your signed informed consent.

The choice to let Jennifer Dungan draw and keep your blood for doing research is entirely up to you. No matter what you decide to do, it will not affect your care. If you decide that your blood can be kept for research but you later change your mind, tell Jennifer Dungan who will remove and destroy any of your blood she still has. Otherwise, the samples may be kept up to 15 years, until they are used up, or until Jennifer Dungan decides to destroy them.

Please review the following four statements carefully. If you disagree with any of these statements, you should not participate in the study.

5. I understand that my samples will be stored for up to 15 years, coded to protect my identity, and that my identity will not be disclosed to anyone without my permission, except when required by law.

6. I understand that some excess blood may be kept by Jennifer Dungan for use in future research to learn about, prevent, treat, or cure hypertension and cardiovascular disorders.

7. I understand that my blood (but not tissue) may be used for research to answer other medical questions that are not necessarily related to hypertension and cardiovascular disorders.

8. I understand that Jennifer Dungan (or someone she chooses) can contact me in the future to ask me to take part in more research.

If you have any questions now or at any time during the study, you may contact the Principal Investigator listed in #3 of this form.

7. If you choose to participate in this study, how long will you be expected to participate in the research?

The active participation in the study to collect blood and tissue is approximately 1 hour. Passive participation, which involves reviewing your chart after surgery, is expected to take one month or less. Following chart review, your participation is complete in the study.

8. How many people are expected to participate in this research?

For this study, we expect to have between 60-72 participants. We aim to have at least 30 people with high blood pressure and 30 people with normal blood pressure enrolled in the study.

9. What are the possible discomforts and risks?

The only potential risk is that associated with drawing blood. The risks of drawing blood from a vein include discomfort at the site of puncture; possible bruising and swelling around the puncture site; rarely an infection; and, uncommonly, faintness from the procedure.

This study may include risks that are unknown at this time.

Participation in more than one research study or project may further increase the risks to you. Please inform the Principal Investigator (listed in #3 of this consent form) or the person reviewing this consent with you before enrolling in this or any other research study or project.

Throughout the study, the researchers will notify you of new information that may become available and might affect your decision to remain in the study.

If you wish to discuss the information above or any discomforts you may experience, you may ask questions now or call the Principal Investigator or contact person listed on the front page of this form.

10a. What are the possible benefits to you?

There are no possible benefits to you if you participate in the study. You will not be compensated for the study. You will also not be given any information regarding the results of this study.

10b. What are the possible benefits to others?

Even though the research that is done on your tissue will not be used to help you, it may be helpful to others with high blood pressure in the future. Because of this study, it may be possible in the future to determine the importance of gene expression in high blood pressure.

11. If you choose to take part in this research study, will it cost you anything?

There will be no cost to your for any specimens that are collected and stored, or any other materials used in this research project.

Costs for routine medical care procedures that are not being done only for the study will be charged to you or your insurance. These costs may not be charged if you are a veteran and you are being treated at the North Florida/South Georgia Veterans Health System (NF/SG VHS).

12. Will you receive compensation for taking part in this research study?

No, you will not receive compensation for taking part in this study.

13. What if you are injured because of the study?

If you experience an injury that is directly caused by this study, only professional consultative care that you receive at the University of Florida Health Science Center will be provided without charge. However, hospital expenses will have to be paid by you or your insurance provider No other compensation is offered. Please contact the Principal Investigator listed in Item 3 of this form if you experience an injury or have any questions about any discomforts that you experience while participating in this study.

You will not have to pay hospital expenses if you are being treated at the North Florida/South Georgia Veterans Health System (NF/SG VHS) and experience any physical injury during participation in a Veterans Health System-approved study.

14. What other options or treatments are available if you do not want to be in this study?

Participation in this study is entirely voluntary. You are free to refuse to be in this study. If you refuse to participate, it will not effect the treatment to which you are otherwise entitled.

15a. Can you withdraw from this research study?

You are free to withdraw your consent and to stop participating in this research study at any time. If you do withdraw your consent, there will be no penalty, and you will not lose any benefits you are entitled to.

If you decide to withdraw your consent to participate in this research study for any reason, you should contact Jennifer R. Dungan at (352) 273-6512.

If you have any questions regarding your rights as a research subject, you may phone the Institutional Review Board (IRB) office at (352) 846-1494.

15b. If you withdraw, can information about you still be used and/or collected?

If you decide to withdraw from the study, data already collected may be used in the completion of the study's analysis, but no further data will be collected and/or used.

15c. Can the Principal Investigator withdraw you from this research study?

You may be withdrawn from the study without your consent for the following reasons:

It is discovered that you no longer meet the inclusion/exclusion criteria.

16. If you agree to participate in this research study, the Principal Investigator will create, collect, and use private information about you and your health. Once this information is collected, how will it be kept secret (confidential) in order to protect your privacy?

Information collected about you and your health (called protected health information), will be stored in locked filing cabinets or in computers with security passwords. Only certain people have the legal right to review these research records, and they will protect the secrecy (confidentiality) of these records as much as the law allows. These people include the researchers for this study, certain University of Florida officials, the hospital or clinic (if any) involved in this research, and the Institutional Review Board (IRB; an IRB is a group of people who are responsible for looking after the rights and welfare of people taking part in research). Otherwise your research records will not be released without your permission unless required by law or a court order.

Results of genetic testing will not be included in your medical record and will be kept in a secure electronic database that is available only to the study investigators. Your records will not be released to any person or agency unless you provide written consent to Jennifer Dungan requesting the release of the results of the study.

Jennifer Dungan will be responsible for making sure that any stored blood sample is protected in the specimen bank and that your medical information is kept confidential. Your sample will not be stored with your name or other identifying information, but instead will be given a code number to protect your identity. These samples and this code number will only be given to researchers whose research is approved by an Institutional Review Board (IRB). (An IRB is a group of people who are responsible for looking after the rights and welfare of people taking part in research.) The researchers will not be told who you are or given any identifying information about you. Because the nature and value of any future research cannot be known at this time, any results obtained from using your tissue will not be given to you or your doctor.

If the results of this research are published or presented at scientific meetings, your identity will not be disclosed.

If you participate in this research study, the researchers will collect, use, and share your protected health information with others. Items 17 to 26 below describe how this information will be collected, used, and shared.

17. If you agree to participate in this research study, what protected health information about you may be collected, used and shared with others?

To determine your eligibility for the study and as part of your participation in the study, your protected health information may be collected, used, and shared with others to determine if you can participate in the study, and then as part of your participation in the study. This information can be gathered from you or your past, current or future health records, from procedures such as physical examinations, x-rays, blood or urine tests or from other procedures or tests. This information will be created by participating in study procedures, or from your study visits and telephone calls. More specifically, the following information may be collected, used, and shared with others:

Complete past medical history, current medications, race, age, and records of previous blood pressures obtained during physical examinations to determine eligibility criteria.

Also, as part of the demographic information obtained from you to be used in data analysis, the following information will be obtained: name, age, phone number, race, ethnicity, current medications, records of previous blood pressure, income, education, job, marriage status, number of people in your household, first degree relative information and information about your smoking, drinking, and exercise habits.

If you agree to be in this research study, it is possible that some of the information collected might be copied into a "limited data set" to be used for other research purposes. If so, the limited data set may only include information that does not directly identify you. For example, the limited data set cannot include your name, address, telephone number, social security number, or any other photographs, numbers, codes, or so forth that link you to the information in the limited data set. If used, limited data sets have legal agreements to protect your identity and confidentiality and privacy.

18. For what study-related purposes will your protected health information be collected, used, and shared with others?

Your protected health information may be collected, used, and shared with others to make sure you can participate in the research, through your participation in the research, and to evaluate the results of the research study. More specifically, your protected health information may be collected, used, and shared with others for the following study-related purpose(s):

1) to determine the impact of gene expression on high blood pressure and response to certain medications

19. Who will be allowed to collect, use, and share your protected health information?

Your protected health information may be collected, used, and shared with others by:

- the study Principal Investigator Jennifer R. Dungan and her staff
- the University of Florida Institutional Review Board
- Malcom Randall VA Medical center (Gainesville)

20. Once collected or used, who may your protected health information be shared with?

Your protected health information may be shared with:

- the study sponsors: The American Heart Association, The National Institutes of Nursing Research (of the National Institutes of Health) and Sigma Theta Tau, Alpha Theta Chapter
- United States and foreign governmental agencies who are responsible for overseeing research, such as the Food and Drug Administration, the Department of Health and Human Services, and the Office of Human Research Protections
- Government agencies who are responsible for overseeing public health concerns such as the Centers for Disease Control and Federal, State and local health departments
- other professionals at the University of Florida or Shands Hospital that provide study-related treatment or procedures
- Malcom Randall VA Medical center (Gainesville)

21. If you agree to participate in this research, how long will your protected health information be used and shared with others?

Your protected health information will be used until the end of the study. Thereafter, your protected health information will be stripped from the research database and the database containing the unidentifiable information will be kept under the protection of the principal investigator forever.

22. Why are you being asked to allow the collection, use and sharing of your protected health information?

Under a new Federal Law, researchers cannot collect, use, or share with others any of your protected health information for research unless you allow them to by signing this consent and authorization.

23. Are you required to sign this consent and authorization and allow the researchers to collect, use and share with others your protected health information?

No, and your refusal to sign will not affect your treatment, payment, enrollment, or eligibility for any benefits outside this research study. *However, you cannot participate in this research unless you allow the collection, use and sharing of your protected health information by signing this consent/authorization.*

24. Can you review or copy your protected health information that has been collected, used or shared with others under this authorization?

You have the right to review and copy your protected health information. However, you will not be allowed to do so until after the study is finished.

25. Is there a risk that your protected health information could be given to others beyond your authorization?

Yes. There is a risk that information received by authorized persons could be given to others beyond your authorization and not covered by the law.

26. Can you revoke (cancel) your authorization for collection, use and sharing with others of your protected health information?

Yes. You can revoke your authorization at any time before, during, or after your participation in the research. If you revoke, no new information will be collected about you. However, information that was already collected may still be used and shared with others if the researchers have relied on it to complete and protect the validity of the research. You can revoke your authorization by giving a written request with your signature on it to the Principal Investigator.

27. How will the researcher(s) benefit from your being in this study?

In general, presenting research results helps the career of a scientist. Therefore, the Principal Investigator may benefit if the results of this study are presented at scientific meetings or in scientific journals.

28. Signatures

As a representative of this study, I have explained to the participant the purpose, the procedures, the possible benefits, and the risks of this research study; the alternatives to being in the study; and how the participant's protected health information will be collected, used, and shared with others:

_____

Signature of Person Obtaining Consent and Authorization Date

You have been informed about this study's purpose, procedures, possible benefits, and risks; the alternatives to being in the study; and how your protected health information will be collected, used and shared with others. You have received a copy of this Form. You have been given the opportunity to ask questions before you sign, and you have been told that you can ask other questions at any time.

You voluntarily agree to participate in this study. You hereby authorize the collection, use and sharing of your protected health information as described in sections 17-26 above. By signing this form, you are not waiving any of your legal rights.

_____

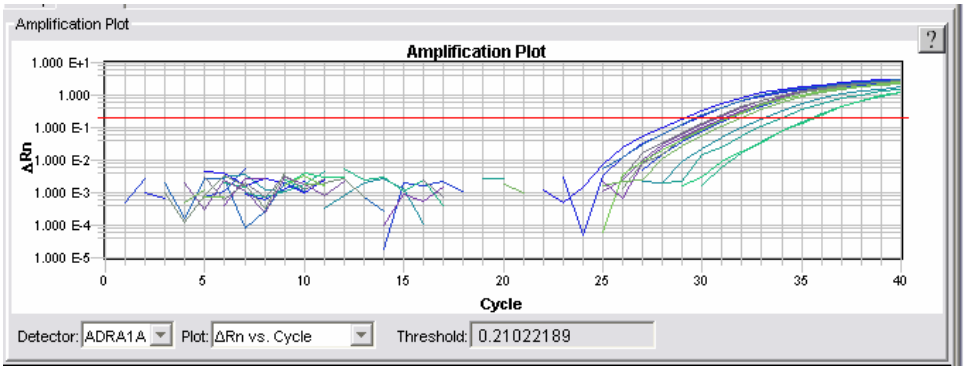
Signature of Person Consenting and Authorizing Date

VA regulations require a witness for all of the signatures provided above.

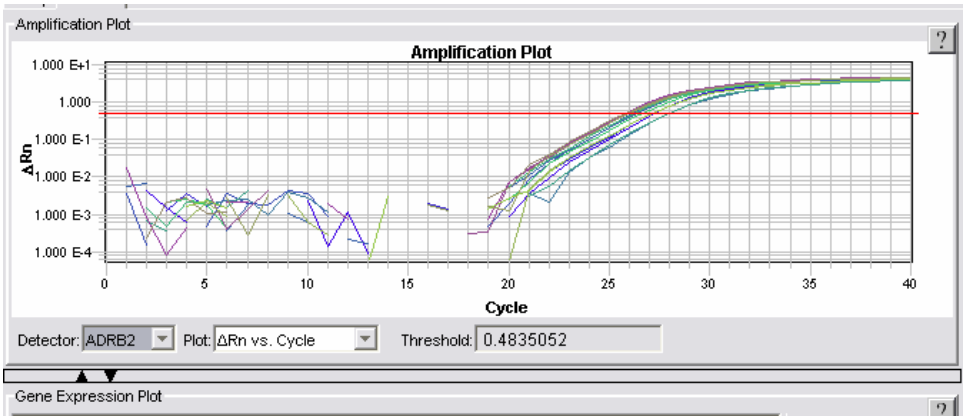
_____

Signature of Witness Date

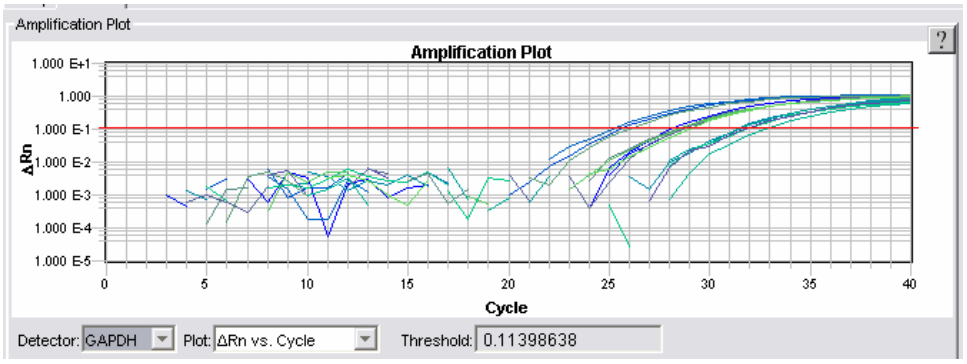
APPENDIX D
TAQMAN REAL-TIME PCR AMPLIFICATION PLOTS



A



B



C

Figure D-1. TaqMan Real-time amplification plot for each gene. A) $\alpha 1_A$-ADR gene. B) β_2-ADR gene. C) GAPDH normalizer gene. Note: Red line indicates threshold line and plots are for duplicate analyses, adjusted to the chosen calibrator and normalizer.

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BIOGRAPHICAL SKETCH

Jennifer Dungan was born Jennifer Rene' Elizabeth Chodzinski in Melbourne, Florida. Having an interest in the medical field early, she became a certified nursing assistant through the dual-enrollment program at the community college and worked in two sub-acute/nursing home facilities in Melbourne. She completed her bachelor's degree in nursing at the University of Florida in 2001.

During her undergraduate education, she was one of three nursing students chosen to participate in the UF University Scholars Undergraduate Research Program, where she was mentored by Carolyn Yucha, then Associate Dean for Research in Nursing. Always having an interest in cardiovascular disease, she focused her mini-research project on a non-pharmacological intervention to reduce blood pressure in adults, incorporating biofeedback as a research tool. She continued this project for her Honors project in nursing, and was able to present her research findings as a student at local, regional, and national conferences. She graduated with high honors and was the recipient of the UF College of Nursing Research Award.

Following graduation, she worked full-time on the Cardiac-Telemetry Unit at Shands at Alachua General Hospital (AGH) and was accepted into the UF College of Nursing's Accelerated Bachelor's to PhD Program the following Fall semester. During this rigorous program, she continued to work part-time at AGH and as a research assistant in the UF CON Office for Research Support. She completed her master's in adult health nursing in December 2002. Her personal family history of cardiac disease

and her experiences on the cardiac-telemetry unit led her to become interested in the genetics of cardiovascular disease. In the summer of 2003, she was one of twenty nurses accepted into the National Institutes of Health (NIH), National Institute of Nursing Research's (NINR) Summer Genetics Institute (SGI). Through this intense summer program, she obtained didactic and laboratory training in molecular and clinical genetics, and developed her proposal for her dissertation study. This program provided her with her minor in genetics, as granted by Georgetown University in affiliation with the NIH-NINR SGI program.

Upon her return in the Fall, she worked with her advisor, Carolyn Yucha's R01 grant, "Prediction of Biofeedback Success," where she genotyped subject samples and examined adrenergic receptor genotypes as predictors of biofeedback success in lowering blood pressure in adults with hypertension. For her presentation on this project, she won Best Student Poster at the annual meeting of the Association of Applied Psychophysiology and Biofeedback. This continued to foster her goals in genetics research in cardiovascular disease.

Ms. Dungan secured partial funding for her dissertation study from the American Heart Association. She was then awarded the Ruth L. Kirschstein National Research Service Award from the NIH-NINR, which provided additional training and funding for her study. In addition, Jennifer obtained a small research grant from the Alpha Theta Chapter of Sigma Theta Tau International Nursing Honor Society.

Ms. Dungan's long-term career goal is to develop a successful, well-funded research program in translational research involving cardiovascular genetics. Her underlying objective is to better serve patients through science. Her particular area of

interest is hypertension and how susceptibility genes and environmental factors interplay to facilitate poor outcomes. After defending her dissertation in February, she will be entering a Postdoctoral Fellowship in Aging at the Duke University Center for the Study of Aging and Human Development. She will focus her postdoctoral training in the areas of gerontology, genomics, genetics, and cardiovascular disease.