GENETIC AND PHARMACOGENETIC ASSOCIATIONS WITH HEART FAILURE PATIENT SURVIVAL

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ABSTRACT

JASMINE A. TALAMEH: Genetic and Pharmacogenetic Associations with Heart Failure Patient Survival
(Under the direction of J. Herbert Patterson)

Heart failure (HF) is an enormous public health problem. Survival and beta-blocker response rates in HF patients are highly variable and cannot be accurately predicted by clinical characteristics alone. The sympathetic nervous system (SNS) and the renin-angiotensin-aldosterone system (RAAS) dually contribute to HF pathophysiology, and inhibition of these systems by beta-blockers, on average, significantly prolongs HF patient survival. Common, functional genetic variants affect the activity of the SNS and RAAS, but their association with HF patient outcomes has not been fully characterized. Therefore the collective objective of this doctoral dissertation research was to determine the association of common, functional genetic variants in the SNS and RAAS with HF patient survival and beta-blocker survival benefit. Eleven variants from nine genes in the SNS and RAAS were genotyped in 722 HF patients with fluorescent, electrophoretic, and mass spectrometric methods. No variants were independently associated with HF patient survival, but *ADBR1* Ser49Gly was significantly associated with beta-blocker survival benefit. Beta-blocker use at baseline was associated with a statistically significant 46% reduction in mortality in Ser49-homozygotes and a non-significant 38% increase in Gly49-carriers. Simple and internally-weighted genetic risk scores were used to assess the additive association of the

SNS and RAAS variants with HF patient survival and beta-blocker survival benefit. The genetic risk scores were not associated with either outcome, did not add to the predictability of clinical risk factors, or reclassify HF patients into new mortality risk categories. A recursive partitioning data mining method was used to detect gene-gene interactions associated with HF patient survival and beta-blocker survival benefit. No gene-gene interactions were associated with outcome in all of the patients or specifically the African-Americans, but in the non-African-Americans *ADRB1* Ser49-Arg389/ Gly49-Arg389 diplotype interacted with *AGTR1* A1166C. In the patients aged less than 60 and treated with beta-blockers, the mortality rate was approximately 3-fold higher if patients had the *ADRB1* Ser49-Arg389/Gly49-Arg389 diplotype and carried *AGTR1* 1166C. The findings in this dissertation research have profound clinical implications. HF patients with a genetic predisposition for high mortality risk or beta-blocker ineffectiveness could be targeted for closer clinical monitoring and/or additional/alternative pharmacologic therapies.

To Matt, family, friends, and faculty who have supported me.

For those living with heart failure, those we have lost to heart failure,

and those who love someone with heart failure.

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LIST OF ABBREVIATIONS

AA	African-American
Arg	arginine
ACE	angiotensin-converting enzyme
ACE	gene for the angiotensin-converting enzyme
ADRA2C	gene for the alpha-2C adrenergic receptor
ADRB1	gene for the beta-1 adrenergic receptor
ADRB2	gene for the beta-2 adrenergic receptor
ADRB3	gene for the beta-3 adrenergic receptor
AGT	gene for angiotensinogen
AGTR1	gene for the angiotensin II receptor type 1
ARB	angiotensin receptor blocker
AUC	area under the curve
BB	beta-blocker
BDKRB2	bradykinin receptor type B2
bp	base pair
BP	blood pressure
bpm	beats per minute
C	cytosine
CART	classification and regression tree
CI	confidence interval
CYP11B2	gene for cytochrome P450, family 11, subfamily B, polypeptide 2

dbSNP	database of single nucleotide polymorphisms
dl	deciliters
DM	diabetes mellitus
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EPI	epinephrine
FDR	false discovery rate
G	guanine
GFR	glomerular filtration rate
Gly	glycine
GRK5	gene for G protein-coupled receptor kinase 5
GRS	genetic risk score
GWAS	genome wide association study
HF	heart failure
HR	hazard ratio
HTN	hypertension
HWE	Hardy-Weinberg equilibrium
Hx	history
indel	insertion/deletion polymorphism
IDCM	idiopathic dilated cardiomyopathy
LDL	low-density lipoprotein
LVAD	left ventricular assist device

LVEF	left ventricular ejection fraction
M	methionine
MAF	minor allele frequency
MALDI-TOF	matrix assisted laser desorption and ionization time-of-flight
Met	methionine
MI	myocardial infarction
min	minute(s)
mg	milligram(s)
ml	milliliter(s)
mmHg	millimeters of mercury
MS	mass spectrometry
n	sample size
NCBI	National Center for Biotechnology Information
NE	norepinephrine
NOS	gene for nitric oxide synthase
NYHA	New York Heart Association
RAAS	renin-angiotensin-aldosterone system
REN	gene for renin
RFLP	restriction fragment length polymorphism
ROC	receiver operating characteristic
rsID	reference sequence identifier
SAP	shrimp alkaline phosphatase
SCr	serum creatinine

sd	standard deviation
Ser	serine
SLC6A2	gene for solute carrier family 6 member 2
SNP	single nucleotide polymorphism
SNS	sympathetic nervous system
Thr	Threonine
UNITE	Unified Investigators to Evaluate Heart Failure
UTR	untranslated region
WEKA	Waikato Environment for Knowledge Analysis

CHAPTER I:
INTRODUCTION

Summary

Heart failure (HF) is an enormous public health problem. Hyperactivity of the sympathetic nervous system (SNS) and renin-angiotensin-aldosterone system (RAAS) plays a major role in HF pathophysiology. Direct pharmacologic inhibition of the SNS and indirect inhibition of the RAAS with beta-blockers significantly improves survival in patients with HF, on average, but the individual patient responses to beta-blockade widely vary. Genetic variation, affecting the functional activity of the SNS and RAAS, may be a possible explanation for variation in HF patient survival and beta-blocker response. The aims of this dissertation research were to determine if genetic variants in the SNS and RAAS, individually, additively, or with interactions between, are associated with survival and beta-blocker survival benefit in patients with HF.

The Problem of Heart Failure

HF is an enormous public health problem, with immense cost, incidence, prevalence, morbidity, and mortality. In 2010, HF cost the United States an estimated $39.2 billion (1), and in 2015 the projected cost is $44.6 billion (2). Although only 13% of Medicare beneficiaries have HF, they accounted for 37% of all Medicare spending and 50% of Medicare inpatient costs (3). The development of HF is very common. The lifetime risk for developing HF is 20% at age 40, and an estimated 5.7 million Americans

have HF (2). This burden of HF is expected to increase as the population ages, acute mortality from myocardial infarction (MI) declines, and the survival of patients with HF is prolonged. HF continues to have high morbidity and mortality rates. One out of four HF patients admitted to the hospital will be readmitted within 30 days (4). Overall, 20% of HF patients will die within one year, and 50% will die within five years (2). For end-stage HF, the one-year mortality rate with optimal medical management is 75%, which is higher than many types of cancer (5). Heart transplant is the only cure for HF, which improves end-stage HF one-year survival to approximately 88% and five-year survival to approximately 75% (6). However in 2010, there were only 2,406 hearts donated (6), and the low donation rate has remained constant with no substantive increase anticipated in the near future (6).

Despite these striking statistics, the treatment of HF has significantly improved over the past few decades, owing to the introduction of effective pharmacologic and mechanical device therapies. Several classes of drugs have been developed that improve HF symptoms and/or survival (7), but this comes at the expense of increasing the number of drugs that HF patients take chronically. For example, Medicare beneficiaries with HF have an average of 61 prescriptions written in one year; the typical beneficiary had 29 (3). The resulting polypharmacy can lead to problems including economic burden, patient adherence, and drug interactions (8). Left ventricular assist devices (LVAD) are becoming more common as either bridge-to-transplant or destination therapies; however the survival rates with LVAD are not as great as with heart transplant: 74% at one year and 55% at two years (9). The use of LVAD results in serious adverse events,

predominately caused by infection, bleeding, neurologic dysfunction, and device malfunction (10).

The current therapies for HF do not distinguish among the complex types of HF, in which there are many potential etiologies, diverse clinical features, and numerous clinical subsets. For example, patients with HF caused by MI, chemotherapy, or no known identifiable cause (i.e. idiopathic dilated cardiomyopathy which could possibly be viral or inherited) are generally treated the same (7), even though the source and manifestation of myocardial damage are grossly different. Also, there is not unequivocal evidence for pharmacotherapies in patients with HF and preserved left ventricular ejection fraction (LVEF). Although the syndromes under the moniker of HF are diverse, a final common pathway among the heterogeneous HF patient population is neurohormonal activation. The major neurohormonal systems that are activated in HF are the sympathetic nervous system (SNS) and renin-angiotensin-aldosterone (RAAS) system.

The Sympathetic Nervous System

The purpose of the SNS, as part of the autonomic nervous system, is to elicit the fight-or-flight response. In a healthy person, the SNS responds to stress with a wide variety of physiologic responses such as vasoconstriction, cardiac inotropy and chronotropy, and release of renin from the kidney. Sympathetic outflow to the heart and peripheral circulation is regulated by cardiovascular reflexes originating from aortic and carotid baroreceptors, cardiopulmonary baroreceptors, and peripheral chemoreceptors (11). The SNS mediates cardiovascular action via four pathways: 1) norepinephrine (NE)

release at the sinus and atrioventricular nodes and left ventricle; 2) epinephrine (EPI) released in the circulation by the adrenal cortex; 3) local release of EPI and NE in the peripheral vessels; and 4) circulating NE which can act in multiple locations (12). Both NE and EPI exert their biological actions via activation of nine different adrenergic receptor subtypes: alpha-1 (1A, 1B, and 1D), alpha-2 (2A, 2B, and 2C), and beta (1, 2, and 3). Adrenergic receptors are members of the super-family of seven transmembrane receptors that signal primarily via interaction with heterotrimeric G proteins. These healthy SNS responses may have evolved to compensate for non-specific, short-term loss of blood volume and/or pressure. However in HF, cardiac output is persistently affected, for example due to myocardial infarction, ventricular hypertrophy, or idiopathic dilated cardiomyopathy. Thus, HF results in long-term activation of the SNS, which in turn leads to abnormalities in SNS function and adverse consequences (Figure 1).

SNS hyperactivity in HF. In HF, the SNS is hyperactive with the goal of maintaining cardiac output. Plasma NE concentration is significantly higher in patients with HF compared to healthy controls (13). Despite the reduced organ blood flows caused by HF that can elevate plasma NE concentration, methods have determined that the increased plasma NE concentration in HF is due to both increased release and decreased clearance of NE from plasma (14). The exaggerated release of NE in HF at rest is similar in magnitude to the release of NE in healthy persons during exercise (15). Increased central sympathetic outflow has also been demonstrated in HF by direct measurement of sympathetic nerve activity using microneurography (16). Several possible mechanisms can explain SNS hyperactivity in HF, such as abnormalities in cardiovascular reflexes and circulating and central hormones. The sympatho-inhibitory

cardiovascular reflexes, such as the arterial baroreceptor reflex, are significantly suppressed (17), whereas the sympatho-excitatory reflexes, such as the cardiac sympathetic afferent reflex (18), are augmented. Angiotensin II levels are also increased in HF, which can facilitate sympathetic neurotransmission via several mechanisms described in detail below (See: Interaction with SNS).

Consequences of SNS hyperactivity in HF. SNS hyperactivity in HF has adverse consequences at molecular, physiologic, and clinical levels. There are multiple alterations in the beta-adrenergic receptor signaling pathway, including down-regulation of receptors (decreased beta receptor density) (19), desensitization via uncoupling from Gs (stimulatory G protein) (20), and an increase in Gi (inhibitory G protein) (21). Notably, receptor down-regulation in HF is specific to beta-1 receptors, and beta-2 receptor density remains unchanged (22). Down-regulation and desensitization of beta-receptors is thought to be a protective adaptation in HF because persistent sympathetic stimulation is toxic to the cardiac myocyte (23). Persistent SNS activation also leads to the pathophysiologic cardiac remodeling process, in which the heart goes through maladaptive changes in size, shape, and function after an injury (24). The degree of SNS hyperactivity is also correlated with hemodynamic abnormalities (25), symptoms (26), and survival (27) in patients with HF (25).

The Renin-Angiotensin-Aldosterone System

The RAAS is both a circulating and local hormonal system with the purpose of maintaining blood pressure and fluid homeostasis. When blood pressure or volume is lowered in a healthy person, renin is secreted from the juxtaglomerular cells of the kidney

into the circulation, which converts angiotensinogen (synthesized in the liver) to angiotensin I. Angiotensin I is converted to angiotensin II by the angiotensin-converting enzyme. Angiotensin II elicits the majority of its actions via the angiotensin II type 1 receptor, which includes vasoconstriction, sodium and water retention, and aldosterone secretion. Notably, the release of renin is the rate-limiting step in the RAAS activation (28).

RAAS hyperactivity in HF. Like the SNS, the RAAS is hyperactive in HF to compensate for the persistent decrease in cardiac output, which has been demonstrated in experimental HF (29) and HF patients (30,31) by increased plasma levels of renin, angiotensin II, and aldosterone. Also like the SNS, there are several mechanisms that can account for the loss of the counter-regulatory balance in the RAAS. Baroreceptor dysfunction is a common mechanism for RAAS and SNS hyperactivity. In a healthy person, under- and over-filling of the vasculature initiates afferent signals from various sensory receptors (e.g. atrial and arterial baroreceptors), aiming ultimately to restore perfusion pressures with sodium and water retention or induce natriuresis to relieve circulatory congestion. However in HF, there are disturbances in the afferent signaling from volume-sensing sites resulting in blunted natriuresis in the face of venous congestion and elevated cardiac filling pressures (32). In addition, the suppression of renin release and renal excretion responses to natriuretic peptide are attenuated in HF (33).

Consequences of RAAS hyperactivity in HF. Similar to the SNS, RAAS hyperactivity in HF has adverse consequences at molecular, physiologic, and clinical levels. Angiotensin II plays a critical role in pathophysiologic cardiac remodeling via

several mechanisms: increasing DNA, protein, and collagen synthesis in cardiac fibroblasts (34,35); mediating stretch-induced hypertrophy (36); and cardiac myocyte necrosis (37). Therefore it is not surprising that RAAS activity is associated with left ventricular dysfunction (13). Other physiologic consequences of RAAS hyperactivity in HF include peripheral vasoconstriction, sodium retention, and hence, circulatory congestion. RAAS activity is associated with the progression to HF from asymptomatic left ventricular dysfunction (13) and HF morbidity and mortality (38-40).

Interaction with SNS. Although discussed separately, the SNS and RAAS mutually facilitate each other's hyperactivity (Figure 2). Specifically, angiotensin II can facilitate sympathetic neurotransmission via several mechanisms: stimulatory action on sympathetic ganglia (41); increasing neurotransmitter release at sympathetic nerve endings (42); preventing NE uptake at sympathetic nerve terminals (43); centrally stimulating angiotensin II type 1 receptors in the brain (44); increasing central sympathetic nerve activity (45); the release of catecholamines from the adrenal medulla (46); facilitation of NE release from sympathetic nerve terminals (47); and modulation of baroreflex control of heart rate (48). In turn, SNS stimulation of beta-1 adrenergic receptors in the kidney results in renin release (49).

Beta-Blockers in Heart Failure

Beta-blockers are one of the greatest advances in HF therapy, resulting in a 35% reduction in mortality when added to angiotensin-converting enzyme (ACE) inhibitors. Although the use of beta-blockers in HF was once contraindicated because of their negative inotropic effects, multiple large, placebo-controlled clinical trials demonstrate

significant reductions in morbidity and mortality with the use of beta-blockers in HF (50,51). The long-term benefits of inhibition of the adverse effects of SNS hyperactivity greatly outweigh the negative inotropic effects of beta-blockers. Metoprolol CR/XL, carvedilol, and bisoprolol are the three beta-blockers that significantly reduced mortality in large HF trials, and hence, are recommended for HF in the treatment guidelines (7). Despite the success of these three beta-blockers, a class effect cannot be assumed because bucindolol failed to significantly reduce mortality in a large clinical trial (52). Bucindolol is a non-selective β-1and β-2 adrenergic receptor antagonist, but bucindolol also has the unique pharmacologic property of marked sympatholysis. Metoprolol and bisoprolol are selective β-1 receptor antagonists, whereas carvedilol also blocks β-2 and α-1. Although, the pharmacologic differences between metoprolol, bisoprolol, and carvedilol have not translated into differences in efficacy.

Importantly, these results from large beta-blocker trials demonstrate an average benefit, but the individual patient responses to beta-blockers vary. For example, long-term optimal dosing of beta-blockers fails to improve LVEF over 5% in as many as 43% of HF patients (53). In a randomized, double-blind trial of metoprolol versus carvedilol in 150 HF patients, the 95% CI for change in LVEF was -8.2% to +22.6% for metoprolol and -11.1% to +32.9% for carvedilol (54). In a study of 171 chronic HF patients treated with metoprolol or carvedilol for 9 to 12 months, only 22% of patients had an increase in LVEF $\geq$ 15% (55). Controversy exists over whether there is racial and regional variation in beta-blocker efficacy. The large beta-blocker clinical trials enrolled mostly Caucasian men. In a meta-analysis of the large beta-blocker trials, the point estimate for reduction in mortality in African-Americans (AA) (RR = 0.67) was similar to Caucasians (RR =

0.63), but the estimate was not statistically significant in 545 AAs (56). Less than half of the patients enrolled in the large beta-blocker trials were from the United States, and the relative risk reduction for each beta-blocker was of smaller magnitude and not statistically significant in Americans compared to the rest of the world (57).

Genetic Variation within the SNS and RAAS

Genetic variation is differences in DNA sequences between individuals and populations, and it can take on a variety of forms, frequencies, and functions. The most common form of genetic variation is the single nucleotide polymorphism (SNP), which occurs every 100 to 300 base-pairs along the 3 billion base-pair human genome. Other common forms of genetic variation are insertion/deletions (indels) and copy number variations (CNVs). Genetic variation can be common, i.e. occurring in greater than 5% of the general population, or an extremely rare mutation. Most genetic variation is believed to be random mutation and have neutral effect, but some genetic variation could have profound effect on phenotype, in which a single genetic variant is sufficient to cause disease. The functional effect of genetic variation on phenotype can be easily seen for some human phenotypic traits, such as eye, hair, and skin color, but genetic variation also affects traits that are not readily visible, such as the SNS and RAAS (58,59).

Indeed, there is a large amount of variation within genes relevant to the SNS and RAAS, including receptors, enzymes, neurotransmitters, and hormones. For example, in the National Center for Biotechnology Information (NCBI) and National Library of Medicine Database of Single Nucleotide Polymorphisms (dbSNP) (60), there are 112 variants reported within the human beta-1 adrenergic receptor gene (*ADRB1*), and 536

variants reported within the angiotensin-converting enzyme gene (*ACE*). There are both uncommon and common genetic variants identified within the SNS and RAAS. For example, an indel in the alpha-2C adrenergic receptor gene (*ADRA2C*) occurs in only 4% of Caucasians, while an indel in *ACE* occurs in 44% of Caucasians. Importantly, the frequencies of genetic variants in the SNS and RAAS can differ between races. For comparison, the same *ADRA2C* indel that is 4% frequent in Caucasians is ten times more common in AAs (frequency 43%), but the *ACE* indel has nearly identical frequency in Caucasians (44%) and AAs (43%). This genetic variation in the SNS and RAAS is not merely random mutation; these genetic variants can have profound effects on protein function or expression. For example, the *ACE* indel mentioned above explains 50% of the variation in serum ACE levels (61). Another example is that the beta-1 adrenergic receptor has agonist-stimulated activity that is three times higher with an arginine at amino acid 389 compared to glycine (62).

Data on how the functional genetic variation within the SNS and RAAS can translate into effects on the HF clinical phenotype is developing. Because the SNS and RAAS are integral for HF development, progression, and pharmacotherapy, it is logical that functional genetic variants could affect the HF clinical phenotype at any or all of those stages (See references 63 and 64, Appendices I & II, for review articles). For example, a glycine at amino acid 49 in the beta-1 adrenergic receptor, which results in increased receptor down-regulation compared to a serine, resulted in an odds ratio of 14.7 for the risk of developing idiopathic dilated cardiomyopathy in a small study (65). In patients with established HF, a serine at amino acid 49 in the beta-1 adrenergic receptor

was associated with decreased survival (adjusted risk ratio 2.03) (66). This same genetic variant was also associated with survival benefit of beta-blockers in HF patients (67).

The relationship between SNS and RAAS genetic variation and HF clinical outcomes, such as survival and beta-blocker response, has not been fully characterized. The genetic and pharmacogenetic association literature for HF is still in very early stages and subject to several limitations. For example, many of the previous HF genetic and pharmacogenetic studies were low power due to short follow-up (most < 5 years) and/or small sample size (most n < 400), which could lead to falsely negative results. Positive associations from small, single-center HF genetic and pharmacogenetic studies could be false due to selection bias or chance, and those associations have not yet been replicated in larger, multicenter HF patient cohorts to rule out those possibilities. Most of the previous studies only tested one to three variants; therefore they are unable to determine the association of multiple variant combinations or interactions. Previous studies are also limited by retrospective design, the exclusion of HF patients with preserved ejection fraction, and poor or no representation of AAs. Retrospective studies were often not initially designed for genetic and pharmacogenetic research, and hence they often lack power. The inclusion of HF patients with preserved ejection fraction in pharmacogenetic studies is important because of differences in the HF phenotype and drug response compared to HF patients with reduced ejection fraction. Including AAs in genetic and pharmacogenetic HF studies is important because, as exemplified above, there are racial discrepancies in allele frequencies.

Specific limitations of the HF pharmacogenetic literature include 100% beta-blocker treatment rates, only intermediate phenotypic endpoints, lack of beta-blocker

dose analysis, lack of specific beta-blocker analysis, and the analysis of the non-FDA approved beta-blocker bucindolol. Falsely negative or the reverse pharmacogenetic associations could stem from 100% beta-blocker treatment rates present in some of the previous HF pharmacogenetic literature. Hypothetical scenarios demonstrating the need for an untreated portion of patients in pharmacogenetic studies are shown in Figure 3. Many previous HF pharmacogenetic studies only evaluated intermediate phenotypes such as ventricular remodeling and not clinical outcomes such as survival, and ventricular remodeling is not a perfect surrogate for beta-blocker survival benefit. Many previous HF pharmacogenetic studies also lacked beta-blocker dose, which is important because genetic effects could vary by dose. For example, *ADRB1* Ser49-homozygous patients treated with a high dose of beta-blockade had a greater survival benefit as compared with a low dose. Whereas *ADRB1* Gly49-carriers had a similar survival rate regardless of beta-blocker dose (67). Many previous HF pharmacogenetic studies lacked specific beta-blocker data or did not test for beta-blocker specific interactions. However *in vitro* data suggests that pharmacogenetic interactions may be beta-blocker specific (68), which may translate into clinical differences. The most robust HF pharmacogenetic data comes from sub-studies of the Beta-Blocker Evaluation of Survival Trial (BEST) (52). However the beta-blocker tested in BEST is bucindolol, which has unique pharmacologic properties including marked sympatholysis (69), and bucindolol did not significantly reduce mortality like other FDA-approved beta-blockers (52). Therefore the pharmacogenetic data on bucindolol may not be applicable to metoprolol CR/XL, carvedilol, or bisoprolol.

Because of the many limitations of the previous HF genetic and pharmacogenetic literature, more definitive and comprehensive research is needed to characterize the

relationship between SNS and RAAS genetic variants and HF survival and beta-blocker survival benefit. This dissertation research addresses the limitations described above by using a well-powered, HF patient cohort and the novel application of advanced analytical methods to determine the individual (Specific Aim I), additive (Specific Aim II), and interactive (Specific Aim III) association of multiple SNS and RAAS variants with HF patient survival and beta-blocker survival benefit.

Perspective

HF is an enormous public health problem. Although there have been great advances in the therapy for HF in the past few decades, morbidity and mortality still remain high. The progression to death and response to a cornerstone of HF pharmacotherapy, beta-blockade, are highly variable among HF patients. Two objectives are critical in abating the HF epidemic, and this dissertation research takes steps towards achieving these objectives: 1) improve the use of current HF therapies and 2) improve the identification of HF patients with high risk of death. Because the SNS and RAAS are integral in HF pathophysiology, and genetic variation affects these systems, the aims of this dissertation research were to determine if genetic variants in the SNS and RAAS, individually (Specific Aim I), additively (Specific Aim II), or with interactions between (Specific Aim III), are associated with survival and beta-blocker response in patients with HF. This dissertation research could have profound clinical implications, as it could lead to more tools for identifying high risk HF patients and HF patients that are most likely to respond to beta-blockers.

Specific Aims

I. Determine if functionally annotated genetic variants within the SNS & RAAS are independently associated with survival and beta-blocker response in patients with HF. Hypothesis: Genetic variants causing increased activity of the SNS or RAAS *in vitro* and *in vivo* will be independently associated with higher risk of all-cause mortality in patients with HF, but patients with the higher activity genotypes will have a greater reduction in mortality with beta-blockers.

II. Determine if a genetic risk score, composed of a panel of functionally annotated SNS & RAAS genetic variants, is associated with survival and beta-blocker response in patients with HF. Hypothesis: The SNS & RAAS genetic variants individually will have a modest association with survival and beta-blocker response, but patients possessing a combination of high activity variants will have additive risk for all-cause mortality and beta-blocker response.

III. Determine if gene-gene interactions among functionally annotated SNS & RAAS genetic variants are associated with survival and beta-blocker response in HF patients. Hypothesis: Because gene-gene interactions are a ubiquitous component of complex diseases such as HF, and the SNS and RAAS dually contribute to HF pathophysiology, gene-gene interactions between variants in these systems will be associated with HF patient survival and beta-blocker response.

Figure legends

Figure 1. A comparison of healthy SNS activation (left half of figure) and in HF (right half of figure) is shown. When a decrease in cardiac output occurs in an otherwise healthy person, the SNS is activated, resulting in increased inotropy, chronotropy, vasoconstriction, and the release of renin. Once cardiac output is restored, the SNS resumes baseline activity. However in a person with HF, cardiac output is only maintained with continuous SNS activation, which leads to adverse consequences such as cardiac myocyte apoptosis, disrupted adrenergic receptor signaling, and cardiac remodeling.

Figure 2. The interaction between the SNS and RAAS is shown. Sites labeled AII are sites in which angiotensin II facilitates the SNS, and those labeled SNS are sites in which the SNS facilitates the RAAS.

Figure 3. Hypothetical scenarios demonstrating the need for an untreated portion of patients in pharmacogenetic studies. Values are hypothetical mortality rates in *ADRB1* Ser49-homozygous (Ser49/Ser49) or Gly49-carrying (Gly49-car) patients either treated with beta-blockers (+BB) or not (-BB) and the reality and appearance if 100% of patients were treated with beta-blockers in the study.

Figure 1. Comparison of healthy SNS activation and in heart failure.

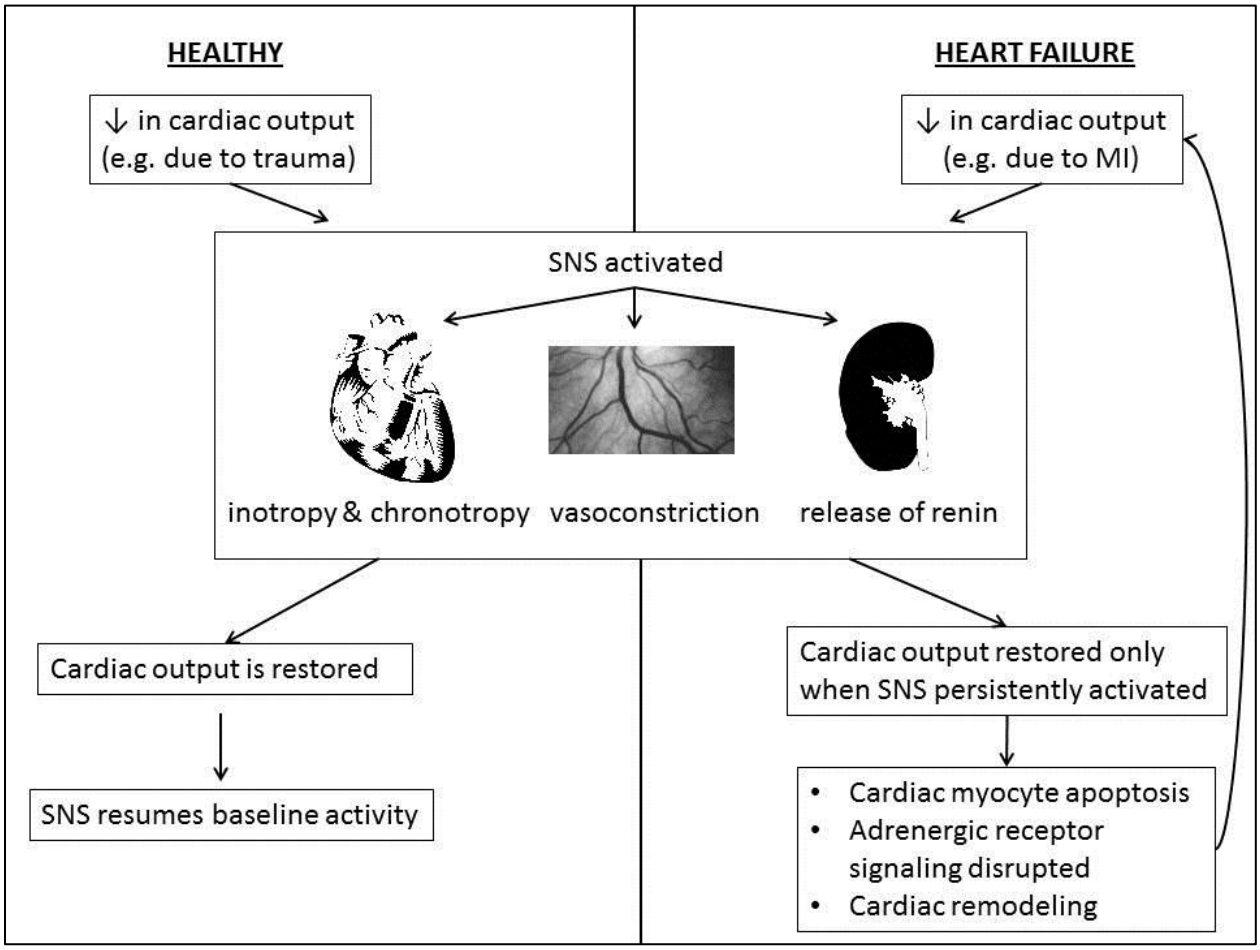


Figure 2. Interaction between SNS and RAAS.

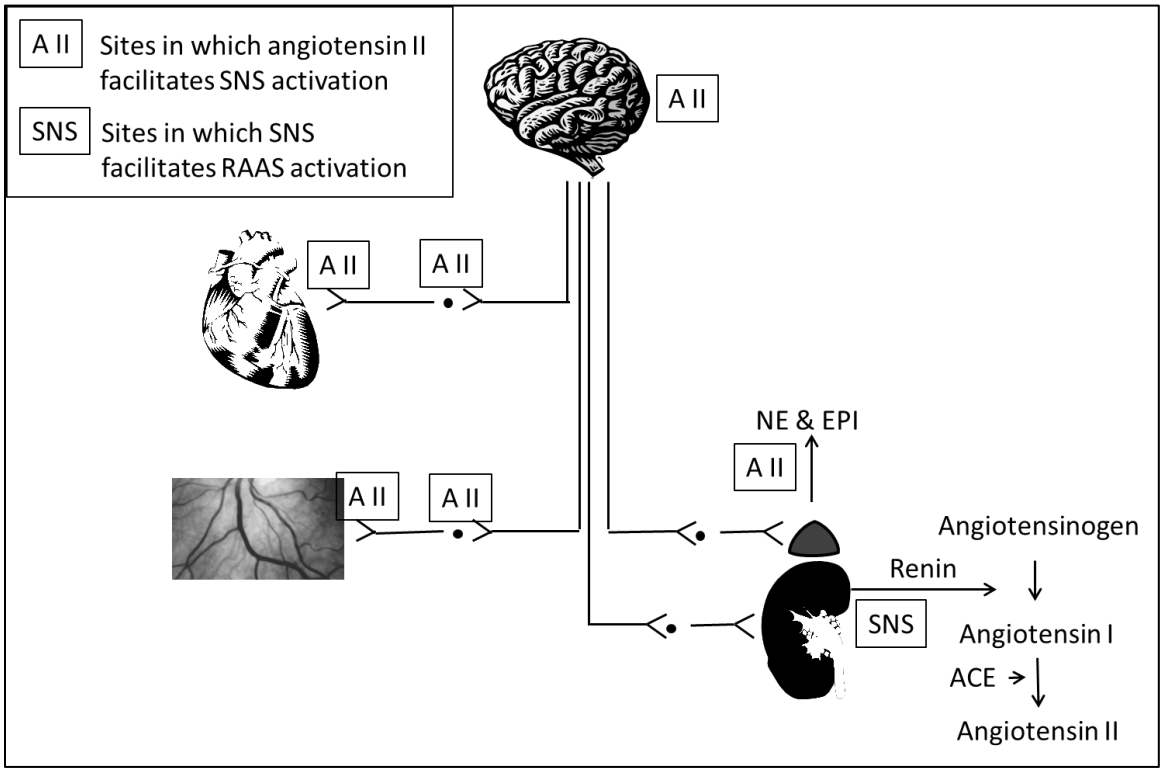
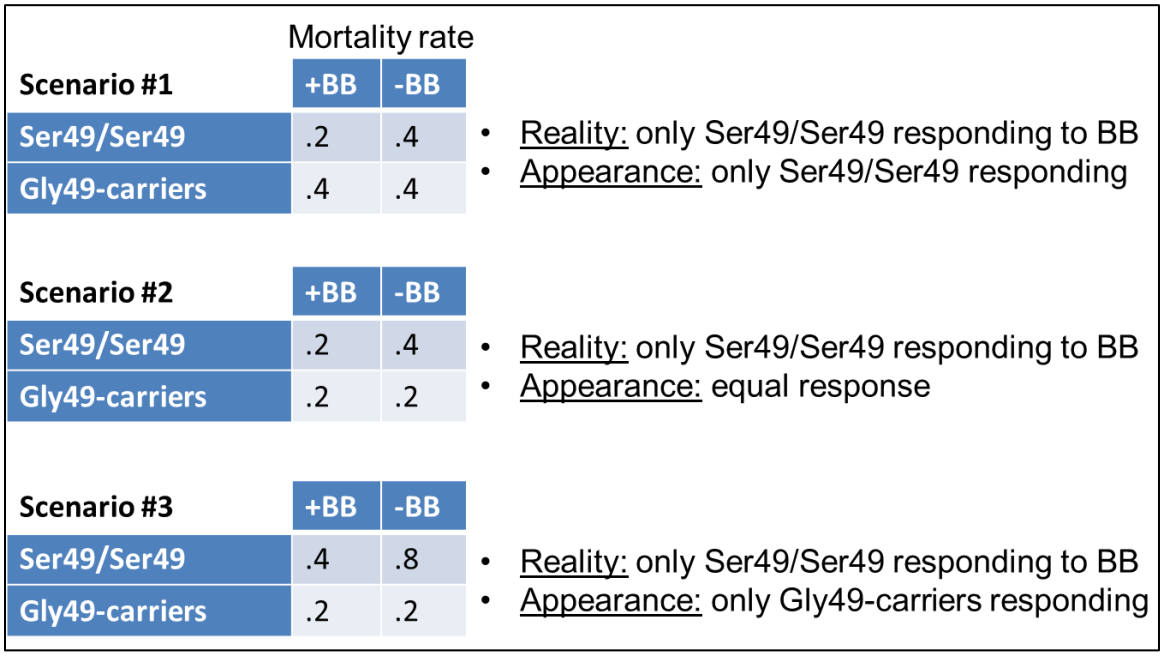


Figure 3. Hypothetical scenarios demonstrating need for untreated patients in pharmacogenetic studies.



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CHAPTER II:
IDENTIFICATION OF CANDIDATE GENETIC VARIANTS

Summary

Chapter I introduced the severity of the HF public health problem and the roles that the SNS and RAAS play in HF pathophysiology and clinical outcomes. The goal of this chapter was to identify candidate genetic variants within the SNS and RAAS that have *in vitro* and *in vivo* evidence to support an association with HF survival and beta-blocker response. A final list of eleven candidate genetic variants was identified in the literature and will be used for analyses. A comparison of the candidate gene approach to other genetic association approaches was introduced, and the limitations of the candidate gene approach used herein were discussed.

Introduction

Because there are approximately three million differences in DNA sequences between individuals, deciding which genetic variants to test and how many to test is an extreme challenge for investigators. Genetic association study designs fall on an enormous spectrum. On one extreme, an investigator may test a single genetic variant. On the other extreme, an investigator may scan the entire genome. When an investigator decides to only test one or a few variants, this is referred to as a "candidate gene approach" because the investigator has a strong *a priori* hypothesis for the best candidates to find an association. The *a prıori* hypothesis for the candidate genetic

variants is ideally based on substantial pre-existing evidence for an association with the trait (e.g. molecular, physiologic, pre-clinical, or clinical evidence). When pre-existing evidence for particular candidate genes is lacking, an investigator may test all of the common variation in the genome. This is referred to as a genome-wide association study (GWAS), which has been referred to as "hypothesis-free," or the broad hypothesis that there are variants somewhere within the genome that will be associated with the trait. Many genetic association study designs fall somewhere in the middle of the two extremes, with intermediate numbers of genetic variants tested for a general hypothesis. For example, if the trait is drug response, the hypothesis could be that any genetic variation within pharmacokinetic genes (e.g. drug metabolizing enzymes and transporters) will be associated with drug response. This hypothesis is narrower than testing the entire genome, but it also does not make the hypothesis for specific variants. Instead of 1 variant or 3 million variants, this hypothesis falls in the middle range with a few thousand variants.

Of course each end of the genetic association spectrum (i.e. candidate gene association versus GWAS), has its advantages and disadvantages. When only selecting a few genetic variants, obviously genetic association from other genes will be missed. The primary limitation of candidate gene association studies is that they are limited to known or hypothesized biologic relevance. However there may be yet undiscovered genes important for a trait, which would be detected in the GWAS. For example, in another common, complex disease, age-related macular degeneration, candidate gene studies failed to find any genetic variants that accounted for a large proportion of the overall prevalence, but GWAS did (1). However a disadvantage of testing many genetic variants

is the potential for false positives. For example, if testing 1 million variants, and using an alpha = 0.05, the investigator may find 50,000 variants associated with the trait by chance alone. Therefore to control for the potential false positives, stringent levels for statistical significance must be set, and therefore very large sample sizes are required to have the power to detect true associations. In this sense, candidate gene studies have the advantage of being flexible and having power to test few variants. Another advantage of candidate gene studies is coverage of uncommon or rare variants. GWAS typically only cover common genetic variation (minor allele frequency [MAF] > 5%). Cost is also a significant factor when deciding on the number of variants to test, as genotyping costs and sample sizes must increase with the number of variants. GWAS are typically hypothesis generating and therefore require validation samples as well. In addition, the data generated in GWAS are computationally intensive and often require more elaborate IT infrastructure and statistical support than a candidate gene study.

The candidate gene approach was chosen for this dissertation research for several reasons. The sample size of the HF patient cohort (n = 720) was not amenable for GWAS, which requires thousands of patients for power to meet the stringent statistical significance thresholds. And also the lower cost of only genotyping a few variants was another reason. There is also good data in the literature to support an *a priori* hypothesis for certain variants. Specifically, it is well-established that the SNS and RAAS are involved with HF pathophysiology and pharmacology. There are known genetic variants in these systems that are known to affect these systems at molecular, physiologic, and clinical levels, and hence a "hypothesis-free" GWAS approach was not required.

The rationale for candidate variant selection is as follows: Because the SNS and RAAS are so closely associated with HF pathophysiology and pharmacology (See Chapter I: Introduction), variants affecting those systems were chosen. Variants also known to affect gene expression or function were chosen because they are more likely to translate into clinically relevant outcomes. Much variation in the genome is thought to be random mutation and have a neutral effect; this variation would not be expected to translate into clinically meaningful outcomes. Because the best candidates for an association with HF clinical outcomes (survival and beta-blocker response) wanted to be chosen, variants that had effects further translating from molecular effects to physiologic or clinical outcomes (e.g. ventricular remodeling or beta-blocker response) were chosen. Only common variants (e.g. MAF >5% in Caucasian-Americans or AAs) were chosen due to statistical power and generalizability of the results to large numbers of patients. Herein the methods for finding candidate genetic variants that meet these criteria were described and the literature supporting the candidate variant list was summarized.

Methods

Selection criteria. Candidate genetic variants must be part of the SNS or RAAS, have a MAF of greater than 5% in Caucasian-Americans or AAs, affect gene expression or function, and are associated with HF patient clinical outcomes or relevant physiologic processes (i.e. ventricular remodeling). When linkage disequilibrium exists, the true functional variant was chosen if known.

Search strategy. Candidate genetic variants were identified in the PubMed database from 1966 to May 2009 by combining the following search terms: heart failure,

sympathetic adrenergic system, renin-angiotensin-aldosterone system, genetic, polymorphism, beta-blocker, pharmacogenetic, survival, and left ventricular ejection fraction. Once variants were identified to have an association with HF relevant outcomes such as drug response, ventricular remodeling, or survival, then functional and pre-clinical data was searched for using the specific variant name or rsID (reference sequence identifier). Variant population frequencies were found in published literature, the HapMap database (2), or NCBI (3). Studies were limited to those published in English.

Results

Twelve candidate variants met the selection criteria. However one candidate variant, M235T in the gene for angiotensinogen (*AGT*), was excluded because it was found to be in complete linkage disequilibrium with G-6A in *AGT,* which was later determined to be the causal functional variant (4). Table 1 summarizes the identification and location information for the candidate genetic variants. Table 2 summarizes the MAF of the variants in populations of Caucasian and African descent. Tables 3 & 4 summarize the gene function within SNS or RAAS, respectively, and the molecular and clinical HF phenotypes of the variants.

Discussion

Eleven candidate genetic variants in the SNS and RAAS were chosen for this dissertation research based on candidate frequency and molecular/clinical phenotypes. It is important to point out the limitations of this candidate gene approach. Given the complexity of the SNS and RAAS, it is somewhat surprising that only 11 candidate

genetic variants met the selection criteria. Therefore important genetic variation in these systems may be missed in this dissertation research simply because they have not been previously studied. This also highlights the fact that genetic variation in systems other than the SNS and RAAS, such as inflammatory, that affect the HF phenotype are also not being tested as part of this dissertation research. Importantly, when looking at beta-blocker response in HF, no pharmacokinetic (PK) genes were selected for this dissertation. However the few studies testing for an association between PK genetic variants and beta-blocker response have not found an association with pharmacodynamics (5,6). Publication bias, in which the effects of a variant may be false positive or exaggerated (7), is also an important consideration because the variant selection was based on previously published literature. The genetic and pharmacogenetic literature for HF is still in very early stages, as there is not a great deal of literature available, and the current literature covers only a few variants in small sample sizes. Of course the candidate gene approach is not the only method for finding genetic association. An alternative method would be to use tag SNPs to cover all common genetic variation in the SNS or RAAS genes, instead of just picking only one or two variants. Of course GWAS is another alternative, because in a complex disease such as HF, many genetic variants are probably involved. However, a large HF patient population would be required. The strengths of the 11 candidate genetic variants for the dissertation research are low cost, low computational demand, improved statistical power, frequency, and substantial background *in vitro* and *in vivo* data.

Table 1. Identification and location information of candidate genetic variants

Gene	rsID	Nucleotide substitution	Amino acid Substitution	Chromosome	Chromosome position (GRCh37.p5 assembly)	Variant type
ADRB1	rs1801252	A1231G	Ser49Gly	10	115804036	missense SNP
ADRB1	rs1801253	C1251G	Arg389Gly	10	115805056	missense SNP
ADRB2	rs1042713	A285G	Arg16Gly	5	148206440	missense SNP
ADRB2	rs1042714	C318G	Gln27Glu	5	148206473	missense SNP
ACE	rs1799752	287-bp deletion	n/a	17	61565890	intronic indel
ADRA2C	rs61767072	12-bp deletion	322GlyAla-GlyPro325	4	3769297	frameshift indel
GRK5	rs17098707	A355T	Gln41Leu	10	121086097	missense SNP
AGT	rs5051	G-6A	n/a	1	230849872	5’ UTR SNP
AGTR1	rs5186	A1166C	n/a	3	148459988	3’ UTR SNP
CYP11B2	rs1799998	C-344T	n/a	8	143999600	5’ UTR SNP
BDKRB2	n/a	9-bp deletion	n/a	14	n/a	non-coding exon indel

Table 2. Minor allele frequencies (MAF) of candidate genetic variants

Gene	rsID	Minor allele	MAF Caucasian	MAF African
ADRB1	rs1801252	G	0.17	0.25
ADRB1	rs1801253	G	0.27	0.38
ADRB2	rs1042713	A	0.40	0.50
ADRB2	rs1042714	G	0.42	0.20
ACE	rs1799752	Ins	0.44	0.43
ADRA2C	rs61767072	Del	0.04	0.43
GRK5	rs17098707	T	0.02	0.24
AGT	rs5051	A	0.42	0.82
AGTR1	rs5186	C	0.25	0.05
CYP11B2	rs1799998	C	0.43	0.29
BDKRB2	n/a	Del	0.50	0.40

Table 3. Gene function and molecular and clinical phenotypes of candidate SNS genetic variants

SNS				
Gene	Gene function	Variant (common notation)	Molecular phenotype	Clinical phenotype
ADRB1	Mediates cardiac inotropy and chronotropy	Ser49Gly	Gly49 ↑ desensitization (8,9)	Gly49 ↑ survival (9)
		Arg389Gly	Arg389 ↑ function (10)	Arg389 ↓ survival (11)
ADRB2	Mediates cardiac inotropy and chronotropy	Gly16Arg	Gly16 ↑ desensitization (12,13)	Gly 16 ↑ survival (13)
		Gln27Glu	Glu27 desensitization resistant (12,13)	Gln27 ↓ risk of worsening HF (14)
ADRA2C	Pre-synaptic auto-inhibition of NE release	Codon 322-325 Ins/Del	Del ↓ function (15)	Del ↓ survival (16)
GRK5	Desensitization of beta-adrenergic receptors	Gln41Leu	Leu41 ↑ desensitization (17)	Leu41 ↑ survival (17)

Table 4. Gene function and molecular and clinical phenotypes of candidate RAAS genetic variants

RAAS				
Gene	Gene function	Variant (common notation)	Molecular phenotype	Clinical phenotype
ACE	Conversion of angiotensin I to angiotensin II	Intron 16 Ins/Del	Del ↑ plasma ACE (18)	Del ↓ survival (19)
AGT	Substrate for renin, converted into angiotensin I	G-6A	-6A ↑ transcription rate (4)	-6A ↓ survival (20)
AGTR1	Mediates major CV effects of angiotensin II	A1166C	1166C ↑ sensitivity (21)	1166C ↓ survival (22)
CYP11B2	Synthesizes aldosterone	T-344C	-344C ↑ plasma aldosterone (23-25)	-344C ↓ survival (24)
BDKRB2	Mediates CV actions of bradykinin	Exon 1 Ins/Del	Ins ↓ transcription rate (26,27)	Ins ↑ LV growth (27)

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CHAPTER III:
CHARACTERIZATION OF CANDIDATE GENETIC VARIANTS

Summary

The previous chapter covered the methods and results for the candidate gene search. This chapter describes genotyping technologies and the methods used for genotyping the eleven selected candidates. Three platforms were used: TaqMan® fluorescent allelic discrimination, QIAxcel® capillary electrophoresis, and Sequenom® MALDI-TOF mass spectrometry. These methods were accurate and reproducible when subject to a series of quality control measures except for the *ADRA2C* indel, which significantly deviated from Hardy-Weinberg Equilibrium (HWE). Preferential amplification of the *ADRA2C* deletion allele must have occurred, and therefore analyses of the *ADRA2C* indel must be performed as insertion-homozygous versus deletion-carrier and not as the three individual genotypes.

Introduction

Rapid advances in genomic technology have yielded a panoply of genotyping methods to choose from. However with the large amount of genotyping methods available comes a host of factors to consider when choosing the optimal method (Table 5). Genotyping technologies can be broadly classified into an allele discrimination step and then the allele detection step (1). Almost all genotyping methods require an initial

PCR amplification step, and then allele discrimination is commonly achieved using primer extension, hybridization, ligation, or enzymatic cleavage. Primer extension relies on the highly accurate DNA polymerase enzyme to incorporate allele-specific nucleotides, and hence it is very reliable. Assay designs are simple, fast, flexible, customizable, multiplex, and widely commercially available (e.g. Sequenom® MassEXTEND™). Hybridization techniques rely on the thermal stability of perfectly complementary DNA probes at the variant loci. Therefore the major disadvantages of this technique are non-specific binding to other loci in the genome or cross-hybridization. The advantage of hybridization techniques is increased throughput because of the lack of an enzymatic reaction requirement. Ligation relies on the high accuracy of the DNA ligase enzyme to join two oligonucleotides only when there is perfect complementarity with the DNA template. With appropriate tags and divergent oligonucleotides, ligation methods can be successfully scaled up to high throughput. Enzymatic cleavage methods rely on the specificity of certain enzymes for certain DNA sequences. For example, restriction fragment length polymorphism (RFLP) utilizes restriction enzymes that cleave double-stranded DNA at unique sites consistently. RFLP is one of the earliest genotyping methods, but it has limited throughput and multiplex capacity. Notably, allele discrimination could involve a combination of any of the above approaches (e.g. TaqMan® uses a combination of hybridization and 5' nuclease activity of DNA polymerase).

Allelic detection methods generally fall under four broad categories: gel electrophoresis, mass-based, fluorescence, and chemiluminescence (1). Gel electrophoresis is the longest used detection method, but traditional agarose gel

electrophoresis is low resolution, labor-demanding, and time-demanding. More recently these disadvantages have been overcome by capillary gel electrophoresis (e.g. Qiaxcel®) (2). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a commonly used mass-based technique for detecting oligonucleotides. A major advantage of MALDI-TOF MS is that a mixture of oligonucleotides can be rapidly separated and accurately analyzed simultaneously. This method is commonly used for detecting products of primer extension reactions (e.g. Sequenom®). The genotype for each allele-specific extension product is determined by its unique mass. A major limitation of this method is reduced peak resolution due to similar masses of the extension products. Fluorescence is a commonly used detection method because it is simple, fast, and accurate. TaqMan® is an example of fluorescent detection, in which probes with a quencher on one end and the fluorescent dye on the other end are used. When the probes are intact, the fluorescent signal is quenched. However, the fluorescent signal is emitted when the fluorescent dye is cleaved from the quencher by a DNA polymerase with 5' exonuclease activity. The alleles have specific fluorescent dyes which reveal genotype. Chemiluminescence utilizes a cascade of enzymatic reactions that generate light. This method is fast, automatable, and has a high signal-to-noise ratio. A method that uses chemiluminescence is Pyrosequencing™. When a template nucleotide is complementary to the nucleotide being extended, DNA polymerase incorporates the nucleotide initiating the cascade of reactions to emit light. A disadvantage of this method is the lack of multiplex capacity.

A combination of three platforms was necessary for genotyping the 11 candidate variants identified in Chapter II: Identification of Candidate Genetic Variants. Because of

availability, cost efficiency, customizability, flexibility, and multiplex capacity, a Sequenom® assay genotyping all 11 variants simultaneously was initially assessed for compatibility. The Sequenom® assay design only allowed 6 of 11 variants into the assay design, presumably because of the inability to design extension products with clearly distinguishable masses. The remaining five variants were rs1042713 (*ADRB2* Arg16Gly), rs1799998 (*CYP11B2* T-344C), and the three indels (*BDKRB2* 9-bp, *ADRA2C* 12-bp, and *ACE* 287-bp). TaqMan® was chosen for the two remaining SNPs due to cost efficiency, flexibility, ease-of-use, availability, and reliability. The indels were genotyped using Qiaxcel® capillary electrophoresis due to availability, cost-efficiency, ease-of-use, and rapidity. Qiaxcel® capillary electrophoresis is very compatible with genotyping indels because the PCR products are separated with high resolution (down to 2 bp indel) and the genotypes are determined based on the size of the PCR products.

Methods

DNA samples. The DNA samples on which genotyping was performed are described in detail in Chapter IV. Briefly, the Unified Investigators to Evaluate Heart Failure (UNITE-HF) began enrollment of HF patients into the UNITE-HF registry from U.S. outpatient HF specialty clinics in 2000. All patients had a history of HF defined as dyspnea on exertion or edema due to cardiac cause. Patients were enrolled without regard to LVEF, symptoms, or new/return patient status. Patients were only excluded if death due to comorbidity was expected within one year. Patients in the UNITE-HF registry that also consented to provide a DNA sample composed the UNITE-DNA cohort studied herein.

Quality control. To ensure genotyping accuracy, the following quality control measures were performed: one negative control per 96-well plate (i.e. water used in place of DNA); all samples without an initial genotype call were re-analyzed; comparison of UNITE-DNA allele frequencies to published frequencies of the same race; repeat genotyping of 10% of samples, randomly chosen and verification of concordance between calls; visual inspection of all genotype calls by one individual; visual inspection of genotype calls for 10% of samples selected at random by a separate individual; the χ^2 test for deviation from the Hardy-Weinberg equilibrium (HWE); blinding to patient characteristics and outcome; comparison of genotype calls for at least two samples of each genotype to calls from an independent laboratory.

DNA sample preparation. Peripheral blood (25 mL) was drawn from each patient using ethylenediaminetetraacetic acid tubes as an anticoagulant. Genomic DNA was extracted from peripheral blood samples using standard methods (3). The stock DNA concentration was determined using the Quant-iT™ PicoGreen® dsDNA kit (Invitrogen, Molecular Probes, OR, USA). Working stock DNA (1 ng/μL) was made by normalizing aliquots of stock DNA in molecular-grade water (Mediatech, VA, USA) using a Biomek® 3000 laboratory automated workstation (Beckman Coulter, CA, USA).

TaqMan® fluorescent allele discrimination. Genomic DNA was amplified in a 10 μL volume using TaqMan® genotyping assay kits (C_8896484_10 for rs1799998 [*CYP11B2* T-344C] and C_2084764_20 for rs1042713 [*ADRB2* Arg16Gly], Applied Biosystems, Foster City, CA, USA) on a DNA Engine Tetrad® 2 Peltier thermal cycler (BioRad, CA, USA). Briefly, the reaction mixture contained 2 μL of genomic DNA (1ng/μL), 0.5 μl TaqMan® probes (10x), 5 μL GTXpress Master Mix (2x), and 2.5 μL of

molecular grade water. The reaction mixture was incubated at 95^{o}C for 20 seconds for DNA polymerase activation followed by 39 cycles of 95^{o}C for 15 seconds for denaturation and 60^{o}C for 60 seconds for annealing/extension. Allelic discrimination was performed on the post-PCR product with a CFX96^{TM} (Bio-Rad, CA, USA) with allelic discrimination software supplied by the manufacturer.

QIAxcel® capillary electrophoresis for *BDKRB2* 9-bp indel. For PCR amplification of the *BDKRB2* 9-bp indel, the following forward and reverse primers were used: 5'- GCCCTTGAAAGATGAGCTG -3' and 5'-AACTCCCCACGACCACAG -3' based on the exon 1 9 bp insertion/deletion polymorphism in *BDKRB2* reported by Braun et al (4). The expected PCR product size with the insertion allele was 275 bp and the deletion allele was 266 bp. The PCR mixture contained 10 µL of HotStarTaq® Plus Master Mix (2X) (Qiagen, Hilden, Germany), 4 µL of Q solution (5X) (Qiagen), 2 µL of each primer (10 µM), and 2 µL of working stock DNA (1 ng/µL) in a final volume of 20 µL. A DNA Engine Tetrad® 2 Peltier thermal cycler (BioRad, Hercules, CA, USA) was used with the following conditions based on Braun et al (4): initial incubation at 95°C for 5 minutes followed by 40 amplification cycles (1 minute at 94°C, 1 minute at 53°C, and 1 minute at 72°C) and a final elongation at 72°C for 10 minutes.

High-resolution capillary electrophoresis was performed using a QIAxcel® DNA high-resolution gel cartridge (Qiagen) on a QIAxcel system (Qiagen), as per the manufacturer's instructions. A QX DNA Size Marker (Qiagen) with 17 fragment sizes ranging in size from 25 to 450 bp was used to size PCR products. A QX Alignment Marker (Qiagen), which consisted of 15 bp and 500 bp fragments, was injected onto the cartridge with each sample. The 0M700 method in the BioCalculator® software (Qiagen)

was used for all analyses; this corresponds to a 10 second sample injection time at 5 kV, and 700 second separation time at 3 kV. The QIAxcel® system injected less than 0.1 μL of 20 μL PCR products onto the cartridge for analysis. The retention time of the PCR fragments relative to the 15 bp and 500 bp QX Alignment Marker fragments was calculated using the BioCalculator® software (Qiagen). The PCR product sizes were then determined by comparing the retention time with the QX DNA Size Marker. The BioCalculator® software produces a digital gel image and an electropherogram for fragment analysis.

QIAxcel® capillary electrophoresis for *ADRA2C* 12-bp indel. For the *ADRA2C* 12-bp indel, the following forward and reverse primers were used (5): 5'-GTGGAGCCGGACGAGAGC - 3' and 5' – GGCGCGACAGGAAGAACTC – 3'. The expected PCR product size with the insertion allele was 232 bp and the deletion allele was 220 bp. The PCR mixture was the same as for the *BDRKB2* 9-bp indel. A DNA Engine Tetrad® 2 Peltier thermal cycler (BioRad, Hercules, CA, USA) was used with the following conditions: initial incubation at 95°C for 5 minutes followed by 44 amplification cycles (1 minute at 94°C, 1 minute at 60°C, and 1 minute at 72°C) and a final elongation at 72°C for 10 minutes. The PCR product was run on the QIAxcel capillary electrophoresis system similar to the *BDKRB2* 9-bp indel, except the 0H700 method in the BioCalculator® software (Qiagen) was used for all analyses; this corresponds to a 20 second sample injection time at 2 kV, and 700 second separation time at 3 kV.

QIAxcel® capillary electrophoresis for *ACE* 287-bp indel. Two stages of PCR amplification were required for genotyping the *ACE* 287-bp indel due to known

preferential amplification of the deletion allele (6). The first stage of PCR used the following forward and reverse primers: 5' - CTGGAGACCACTCCCATCCTTTCT - '3 and 5' - GATGTGGCCATCACATTCGTCAGAT -3'. The expected first-stage PCR product size with the deletion allele was 191 bp and the insertion allele was 478 bp. The first-stage PCR mixture contained 10 µL of AmpliTaq Gold® PCR Master Mix (Applied Biosystems, Foster City, CA), 0.5 µL of each primer (10 µM), 1 µL of working stock DNA (1 ng/µL), and 8 µL of molecular grade water in a final volume of 20 µL. A DNA Engine Tetrad® 2 Peltier thermal cycler (BioRad, Hercules, CA, USA) was used with the following conditions: initial incubation at 93°C for 20 minutes followed by 50 amplification cycles (30 seconds at 94°C, 30 seconds at 58°C, and 30 seconds at 72°C) and a final elongation at 72°C for 5 minutes.

Unlike the much smaller *BDKRB2* 9-bp and *ADRA2C* 12-bp indels, the *ACE* 287-bp indel did not require the use of a high resolution QIAxcel cartridge. Instead, a QIAxcel® DNA screening cartridge (Qiagen) was used. A QX DNA Size Marker (Qiagen) with 11 fragment sizes ranging in size from 50 to 800 bp was used to size PCR products. A QX Alignment Marker (Qiagen), which consisted of 15 bp and 1000 bp fragments, was injected onto the cartridge with each sample. The AL320 method in the BioCalculator® software (Qiagen) was used for all analyses; this corresponds to a 20 second sample injection time at 8 kV, and 320 second separation time at 6 kV. The QIAxcel® system injected less than 0.1 µL of 20 µL PCR products onto a cartridge for analysis. The retention time (and hence bp size) of the PCR fragments relative to the 15 bp and 1000 bp QX Alignment Marker fragments was calculated using the BioCalculator® software (Qiagen).

All patients who were genotyped as deletion homozygotes in the first stage were subject to a second, independent PCR amplification with forward and reverse primers that recognize an insertion-specific sequence (7): 5' – TGGGACCACAGCGCCCGCCACTAC – 3' and 5' – TCGCCAGCCCTCCCATGCCCATAA – '3. The reaction yields a 335-bp amplicon only in the presence of an insertion allele, and no product in samples homozygous for deletion. The PCR mixture and thermal cycling conditions were the same as for the *ADRA2C* 12-bp indel, except for a 68^{o}C annealing temperature. The second-stage PCR products were similarly run to the first-stage PCR except the AH320 method in the BioCalculator® software (Qiagen) was used for all analyses; this corresponds to a 20 second sample injection time at 2 kV, and 320 second separation time at 6 kV.

Sequenom® MALDI-TOF mass spectrometry. The multiplex SNP assay was designed using Sequenom® iPLEX® Assay Design software version 3.1. The primers used are shown in Table 6. All reagents used were from the Sequenom® Complete Genotyping Reagent Set (Sequenom, Inc., San Diego, CA, USA). Briefly, the PCR reaction mixture consisted of 0.5 µL PCR buffer, 0.4 µL magnesium chloride, 0.1 µL dNTPs, 0.2 µL Taq polymerase, 1.0 µL forward and reverse primer mix (0.5 µM), 1.8 µL molecular grade water, and 1.0 µL genomic DNA (10ng/µL) to a final volume of 5.0 µL. The thermal cycling conditions were as follows: 94^{o}C for 2 minutes, followed by 45 cycles of 94^{o}C for 30 seconds, 56^{o}C for 30 seconds, 72^{o}C for 1 minute, with a final extension at 72^{o}C for 5 minutes. After PCR amplification, the PCR product is incubated with shrimp alkaline phosphatase (SAP) enzyme to neutralize any free dNTPs. The SAP reaction mixture consists of 0.3 µL SAP enzyme, 0.17 µL SAP buffer, and 1.53 µL

molecular grade water to create a final volume of 2.0 µL added to the PCR product. The SAP reaction mixture + PCR product is incubated at 37°C for 40 minutes followed by 85°C for 5 minutes. After the SAP reaction is the extension reaction. The extension reaction mixture contains 0.2 µL extension buffer, 0.2 µL termination mix, 0.084 µL extension primer (7-14 µM), 0.041 µL extension enzyme, and 0.755 µL molecular grade water to create a final volume of 2.0 µL which is added to the PCR product. The thermal cycling conditions for the extension reaction are as follows: 94°C for 30 seconds followed by 40 cycles of 94°C for 5 seconds, 52°C for 5 seconds, 80°C for 5 seconds, and a final extension at 72°C for 3 minutes. After the extension reaction, salt adducts are removed by adding 25 µL of molecular grade water and 6 mg of resin to each PCR product. PCR product is then spotted onto a 384 SpectroCHIP using a MassARRAY® nanodispenser and analyzed using MALDI-TOF MS platform (Sequenom, Inc., San Diego, CA, USA).

Linkage disequilibrium. Linkage disequilibrium, calculated as r^2 and D', was determined using SAS® version 9.2 (SAS Institute, Inc., Cary, NC, USA). Haplotypes for the *ADRB1* and *ADRB2* variants were estimated using the open access software PHASE version 2.1.1 (University of Washington, Seattle, WA, USA) (8,9).

Results

All genotyping assays were reliable (call rates greater than 99%), accurate (100% concordance with genotypes from an independent laboratory, similar allele frequencies to the literature, and within HWE), and reproducible (100% concordance of subsequent run for 10% randomly chosen samples), except for the *ADRA2C* 12-bp indel as shown in

Tables 7 and 8. Like the *ACE* indel, it is likely that preferential amplification of the deletion allele must have been occurring because the number of expected heterozygotes was much higher than the observed number (Table 9). Therefore (assumed) heterozygous samples were incorrectly genotyped as deletion homozygotes. Linkage disequilibrium, diplotypes identified, and frequencies by race for the *ADRB1* and *ADRB2* genetic variants are shown in Table 10.

Discussion

The genotyping methods for the 11 candidate genetic variants were reliable, accurate, and reproducible, except for the *ADRA2C* 12-bp indel that significantly deviated from HWE. HWE is the preservation of allele and genotype distributions from generation to generation. Considering a diallelic locus with alleles A and B and population frequencies of 1-q and q respectively, the probabilities for the three possible genotypes (AA, AB, and BB) will follow the Hardy-Weinberg law: $(1 - q)^2$, $2q(1 - q)$, and q^2 (10). Reporting violations from HWE in genetic association literature is extremely important because the conclusions made from the genetic association study could be false. Violations from HWE signals important problems, errors, or peculiarities in the dataset and may explain failed replication of genetic associations in subsequent studies. Failure to test and report HWE is common in the literature, even in high profile genetics journals (11), and this has led to the call for retraction of at least one genetic association publication (12).

HWE depends on a series of assumptions about the population: the population is infinitely large, no new mutation, no selection, no migration, and random mating.

Therefore violations of HWE can be due to 1) violations of these assumptions, 2) chance, or 3) genotyping errors. All of the HWE assumptions cannot be directly verified in UNITE-DNA, although they seem very reasonable. UNITE-DNA is not infinitely large, but the population is large enough that a chance deviation from HWE due to sampling bias is highly unlikely (13). Additionally, the *ADRA2C* indel has been known for years, and hence it is not a new mutation. However selection bias may play a role because the *ADRA2C* indel has been previously associated with mortality in HF patients (14,15), but the data are conflicting and not confirmed herein (see Chapter V). Migration is an unlikely issue because the patients are 21st century Americans, where no large migration into or out of the U.S. has occurred for centuries. Migration effects were also not evident for any of the other 10 variants, and the patients come from geographically diverse areas across the U.S. The selection of mates is rarely random; individuals may preferentially select one another because of physical and behavioral characteristics that are influenced by genetics. However most traits that contribute to non-random mating are controlled by many loci.

Because UNITE-DNA generally meets the assumptions of HWE, that leaves chance and genotyping errors to explain the HWE violation for the *ADRA2C* 12-bp indel. Because the p-values for HWE in both the Caucasian and AA patients were less than 0.0001, the result is not due to chance. Therefore, the most likely explanation for the violation of the *ADRA2C* 12-bp indel from HWE would be genotyping error. The *ADRA2C* 12-bp indel passed all genotyping quality control checks until the final HWE check. However looking at the expected and observed genotype frequencies and electropherograms, preferential amplification, like the *ACE* indel, must have been

occurring. The following are potential mechanisms for preferential amplification (16): 1) The two alleles in a heterozygous sample denature at different temperatures, and the allele that denatures less efficiently will not be detected. 2) Differential priming, in which the primer anneals more efficiently to one of the alleles. This is possibly due to a SNP that is in linkage disequilibrium with the allele of interest that lies underneath the primer annealing site. However, there are no reported SNPs under the primers used for *ADRA2C* indel genotyping. 3) Under conditions of limiting enzyme, as is the case with high concentrations of PCR product, the probability of a complete primer extension may be greater for the shorter products.

There are several different approaches to reduce or manage preferential amplification. If any of the three mechanisms above are suspected, the following adjustments could be made: 1) Use a denaturing temperature at which both alleles are completely denatured. 2) Ensure that there are no polymorphisms underlying the primer annealing sites. 3) Use high concentrations of DNA polymerase. If these adjustments do not work, two-stage PCR could be used like in the *ACE* indel assay. The first stage of PCR uses primers that flank the entire indel, and all samples that are genotyped as deletion homozygotes in the first stage of PCR are subject to a second stage of PCR. The second stage of PCR uses insertion-specific primers, so that a PCR product is only amplified in the presence of an insertion allele. Hence, those samples with detectable PCR products are truly heterozygotes. The simplest way to cope with preferential amplification, and the method chosen herein, is to analyze the data as insertion-homozygotes versus deletion-carriers. Although this method does not require any further labor, the disadvantage is the inability to detect differences between heterozygotes and

deletion homozygotes. This disadvantage is minimal in the Caucasian UNITE-DNA patients because the deletion frequency is so low (MAF = .04) there would be very few deletion homozygotes, and therefore very little power to analyze that genotype group. However the *ADRA2C* deletion is much more common in AAs (MAF = 0.4); therefore a large proportion of the AA patients are expected to be deletion homozygotes, but we are unable to analyze them separately.

Table 5. Factors to consider when selecting genotyping methods.

Factor	Description
Throughput	The number of samples that can be genotyped per unit of time
Reliability	The number of samples with successful genotype calls
Reproducibility	Yielding identical genotype calls in subsequent runs
Accuracy	The method provides the true genotype call, usually assessed by genotyping the same variant with different methods or by an independent laboratory
Availability	The equipment is already present in the laboratory or part of the campus resource or core genotyping facility
Cost-efficiency	The method is productive relative to the cost
Ease-of-use	Less time for technician training and fewer errors made
Number of samples	Large sample sizes may require high throughput methods
Number of variants	Large numbers of variants may require microarray chip technology
Type of variants	Some genotyping platforms are incompatible or unreliable with indels or CNVs
Multiplex capacity	Multiple variants can be genotyped in a single reaction
Technical support	Availability of manufacturer for trouble-shooting system failures
Automation capacity	To minimize human error
Turn-around-time	Some genotype calls, such as for making therapeutic decisions, require a rapid return of results
Customization	The ability to design a unique assay to meet the investigator's needs
Flexibility	The ability to genotype many different variants using the same equipment and reagents
Quality of DNA available	Some genotyping methods are more robust when the DNA quality is poor
Amount of DNA available	Some genotyping methods require only minute amounts of DNA when DNA is scarce

Table 6. Primers used in Sequenom® genotyping assay.

rsID	Forward	Reverse	Extension
rs1042714	ACGTTGGATGACATGACG ATGCCCATGCC	ACGTTGGATGAGCGCCTTC TTGCTGGCAC	ACACCTCGTCCCTT T
rs1801253	ACGTTGGATGCCTTCAAC CCCATCATCTAC	ACGTTGGATGAGCCCTGC GCGCGCAGCAGA	CGCAAGGCCTTCC AG
rs17098707	ACGTTGGATGAGCTTACC TATGGTCCTTCG	ACGTTGGATGAGCGCAAA GGGAAAAGCAAG	TCGGAGGTCTTCA CAC
rs5051	ACGTTGGATGTGTAGTAC CCAGAACAACGG	ACGTTGGATGAGCCTGGG AACAGCTCCATC	ACGGCAGCTTCTT CCCC
rs1801252	ACGTTGGATGGTCGCCGC CCGCCTCGTT	ACGTTGGATGATGAGCGC CATCAGCAGAC	TTCTGCCTCCCGCC AGCGAA
rs5186	ACGTTGGATGCCACATAA TGCATTTTCTCC	ACGTTGGATGAGAACATT CCTCTGCAGCAC	TCAATTCTGAAAA GTAGCTAA

Table 7. Concordance and call rates for 11 candidate variants.

Gene	Variant (Minor Allele)	Method	Concordance with independent lab	Concordance with 10% random repeat	Call rate
ADRB1	Ser49Gly (Gly)	Sequenom	100%	100%	99.5%
	Arg389Gly(Gly)	Sequenom	100%	100%	99.8%
ADRB2	Gly16Arg(Arg)	TaqMan	100%	100%	99.5%
	Gln27Glu(Glu)	Sequenom	100%	100%	99.8%
ACE	Ins/Del (Ins)	QIAxcel	100%	100%	99.2%
ADRA2C	Ins/Del (Del)	QIAxcel	100%	100%	99.5%
GRK5	Gln41Leu(Leu)	Sequenom	100%	100%	99.8%
AGT	G-6A (A)	Sequenom	100%	100%	99.4%
AGTR1	1166C(C)	Sequenom	100%	100%	99.8%
CYP11B2	T-344C (T)	TaqMan	100%	100%	99.8%
BDKRB2	Ins/Del (Ins)	QIAxcel	100%	100%	99.3%

Table 8. Allele frequencies in the literature and UNITE-DNA and HWE p-value by race for 11 candidate variants.

Gene	Variant (Minor Allele)	UNITE-DNA	Published Cauc	UNITE-DNA Cauc	Caucasian HWE p-value*	Published AA	UNITE-DNA AA	AA HWE pvalue*
ADRB1	Ser49Gly (Gly)	0.18	0.17	0.14	0.2287	0.25	0.25	0.0504
	Arg389Gly (Gly)	0.31	0.27	0.25	0.4071	0.38	0.41	0.1160
ADRB2	Gly16Arg (Arg)	0.42	0.40	0.36	0.0940	0.50	0.52	0.4502
	Gln27Glu (Glu)	0.33	0.42	0.43	0.9714	0.20	0.18	0.1463
ACE	Ins/Del (Ins)	0.43	0.44	0.43	0.0307	0.43	0.42	0.8386
ADRA2C	Ins/Del (Del)	0.24	0.04	0.09	<.0001	0.43	0.50	<.0001
GRK5	Gln41Leu (Leu)	0.09	0.02	0.01	0.0011	0.24	0.21	0.1958
AGT	G-6A (A)	0.59	0.39	0.42	0.3467	0.83	0.85	0.1384
AGTR1	1166C (C)	0.20	0.28	0.29	0.1222	0.05	0.07	0.7322
CYP11B2	T-344C (C)	0.36	0.43	0.46	0.4654	0.15	0.20	0.0548
BDKRB2	Ins/Del (Del)	0.47	0.50	0.49	0.4793	0.40	0.45	0.1164

Cauc = Caucasian; *Bonferroni-corrected alpha = .002

Table 9. Expected and observed genotype distributions for the *ADRA2C* indel by race.

ADRA2C genotype Caucasian	Expected	Observed
Insertion homozygotes	361	373
Heterozygotes	69	45
Deletion homozygotes	3	15
ADRA2C genotype AA	Expected	Observed
Insertion homozygotes	67	91
Heterozygotes	134	86
Deletion homozygotes	66	90

Table 10. Linkage disequilibrium, diplotypes identified, and frequencies by race for the *ADRB1* and *ADRB2* genetic variants.

ADRB1 linkage disequilibrium	White patients (n = 429)	Black patients (n = 270)
r^2	0.24	0.47
D’	1.00	1.00
ADRB1 haplotypes		
Ser49/Arg389	519 (60%)	187 (35%)
Ser49/Gly389	215 (25%)	219 (41%)
Gly49/Arg389	124 (14%)	134 (25%)
ADRB2 linkage disequilibrium	White patients (n = 432)	Black patients (n = 269)
r^2	0.65	0.48
D’	1.00	1.00
ADRB2 haplotypes		
Gly16/Glu27	370 (43%)	94 (17%)
Gly16/Gln27	184 (21%)	163 (30%)
Arg16/Gln27	310 (36%)	281 (52%)

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CHAPTER IV:
CLINICAL FACTORS ASSOCIATED WITH HF PATIENT SURVIVAL AND BETA-BLOCKER RESPONSE

Summary

The previous chapter described the genotyping methods and results for the 11 candidate genetic variants. The objectives for this chapter were to characterize the UNITE-DNA patient population and determine the clinical characteristics associated with HF survival and beta-blocker (BB) survival benefit. UNITE-DNA has good representation of groups that have been under-represented in the landmark HF clinical trials (e.g. women and AA) and a large BB untreated portion of patients, giving power to test for statistical interactions with BB treatment. Baseline age, diabetes, NYHA class, systolic BP, LVEF, and GFR were independently associated with HF survival. BB use at baseline was significantly associated with an approximate 30% lower mortality after rigorous control for clinical covariates, and no clinical characteristics were significantly associated with BB survival benefit.

Introduction

The survival of patients with HF is highly variable, with one-year survival rates ranging from 93% (1) to 25% (2). The ability to accurately estimate the risk of death in patients with HF is extremely important, enabling informed decisions by providers, patients, and patients' families on medications, devices, transplantation, and end-of-life

care. Many clinical and demographic factors have been associated with survival in HF patients: gender (3), race (4), age (5,6), NYHA class (6), etiology (6), diabetes (5,6), history of hypertension (6), heart rate (1), BP (7), LVEF (5), and renal function (8). Several models combining these clinical risk factors have been developed in various HF patient settings with variable accuracy (1,9,10). One of the most commonly used and accurate mortality risk prediction models is the Seattle HF model (11). The Seattle HF model consists of easily obtainable characteristics relating to clinical status, therapy (pharmacological as well as devices), and laboratory parameters, and the overall receiver operating characteristic area under the curve was 0.729 (95% CI 0.714-0.744). Evidence is emerging for genetic risk factors in HF mortality (12-20) (for details see Chapter II: Identification of Candidate Genetic Variants), but it is unclear whether the addition of genetic factors to established clinical models improves risk prediction.

Like survival, BB response in patients with HF is also highly variable (for detailed examples see Chapter I: Introduction). However, there are no established clinical or demographic characteristics that predict BB efficacy in HF. There is some evidence for gender (21), racial (21), regional (22), baseline BP (23), and etiologic differences (23) in BB response, but currently the HF treatment guidelines recommend that all patients with an LVEF < 40% be treated with the same target doses of BB (24). Moreover, no large-scale studies have demonstrated that BB improves outcomes in HF patients with preserved LVEF (LVEF > 40%) (24). Also like survival, evidence is emerging for genetic determinants of BB response in HF (Appendices I & II) (25,26).

Robust modeling of the clinical characteristics associated with HF survival and BB response is not only important for patients, but it is also critically important for

genetic and pharmacogenetic research. Over- or under-estimation of the true genetic or pharmacogenetic association with outcome can result, depending on the direction and magnitude of relationships between the clinical covariates and between the clinical covariates and the genetic variant. Population stratification, which is systematic differences in allele frequencies between subpopulations due to different ancestry, is a major source of confounding in genetic association studies. Confounding can be prevented using a randomized study design, but when this is not possible (such as in the non-randomized, observational UNITE-DNA registry described herein) strategies to control for the confounding factors must be used. Three general approaches can be used to control for confounding factors when there are a small number of genetic variants genotyped (27): 1) stratify the sample according to the confounding factors so that the comparison groups are relatively homogenous, 2) use regression modeling adjustment so that the association between the genetic variant and outcome can be assessed at fixed values of the covariates, or 3) use propensity score matching to balance the comparison groups (28). Regression adjustment is the most commonly used method presumably because of its simplicity. All three methods are introduced in this chapter, and each method will be used when testing the genetic and pharmacogenetic associations in this dissertation research. Specifically, stratification by race will be used in all analyses because of the differences in allele frequencies between races. The clinical models that will be used for regression adjustment of genetic and pharmacogenetic associations are developed in this chapter. And because UNITE-DNA was not randomized to BB treatment, propensity matching was used to balance known covariates in the BB untreated and BB treated patients. Overall, the objectives for this chapter are to 1)

thoroughly describe the UNITE-DNA patient population overall and in relevant strata, 2) determine the independent clinical factors associated with survival in UNITE-DNA, 3) determine the independent association of BB treatment with survival in UNITE-DNA, and 4) determine the clinical factors associated with BB response in UNITE-DNA.

Methods

Registry. The Unified Investigators to Evaluate Heart Failure (UNITE-HF) network of dedicated and like-minded investigators was founded in 1999 by the UNC Heart Failure Program, Dr. Kirkwood F. Adams, Jr. PI, to promote clinical registry, biomarker, and genomic research in HF (29). The list of study sites and investigators that contributed subjects for this research is shown in Table 11, and UNC-Chapel Hill has served as the network coordinating center since its founding. The network began enrollment of HF patients into the prospective, multicenter, and observational UNITE-HF registry in early 2000. Patients that enrolled into the UNITE-HF registry that also consented to give a DNA sample composed the UNITE-DNA sample described herein. The inclusion criteria were broad: All patients had a history of HF defined as dyspnea on exertion or edema due to cardiac cause. New or return adult outpatients seen in HF specialty clinics were eligible. There was no specific LVEF requirement and patients could be asymptomatic at enrollment. The exclusion criteria were minimal: Patients were excluded if death due to comorbidity was expected within one year. At baseline, the following data were collected on a common form set: demographics, pertinent past medical history including ventricular function and HF signs/symptoms, detailed information on cardiovascular medications including specific type and dose of BB. Race

was self-reported. During follow-up, vital status was collected initially from the study sites, then periodically by Social Security Death Index. Forms were faxed to the UNITE-HF Coordinating Center and processed to a final SAS analysis dataset using standard methods for data entry and query for data verification. All statistical analyses were performed in Statistical Analysis Software (SAS) version 9.2 (Cary, NC, USA).

Baseline analysis. Continuous baseline characteristics were summarized using mean and standard deviation, and categorical baseline characteristics were summarized using counts and percentages. Baseline characteristics were compared by race (AA versus Non-AA), BB treatment (on BB versus off BB at baseline), etiology (ischemic versus non-ischemic), and vital status (currently alive versus died during follow-up) using chi-square, Fisher's exact, or student's t-test as appropriate. Race was not known for two subjects so they were excluded from race-stratified analyses, and etiology was not known for 16 subjects so they were excluded from etiology-stratified analyses.

Survival analysis. To assess the association of clinical characteristics with the primary endpoint, all-cause mortality, a series of univariate proportional hazards regression models were fit, one model for each of 11 pre-defined clinical factors: gender, race, age, etiology (ischemic vs. non-ischemic), history of hypertension, history of diabetes, NYHA class, systolic blood pressure, resting heart rate, LVEF, and GFR. A significance level of $p < 0.10$ was prospectively defined for inclusion of clinical factors in the multivariable analysis. Given n = 722, alpha = 0.1, and 8.9 years of follow-up, there was 80% power to detect a hazard ratio of 1.19 for a dichotomous variable in the univariate analysis. Final inclusion in the multivariable model was determined using stepwise selection with a significance level of $p < 0.05$. The assumption of proportional

hazards was confirmed by testing time-dependent covariates in the model (e.g. variable*time interaction term). The influence of missing data was assessed by repeating analyses with simple imputation for missing data (replacement of missing values with mean value of variable from UNITE-DNA overall).

BB response analysis. To assess the association between BB treatment and the primary endpoint, all-cause mortality, BB treatment (yes versus no at baseline), BB dose (in 25mg metoprolol equivalents), and specific BBs (metoprolol tartrate or succinate and carvedilol) were tested in the multivariable clinical model derived from UNITE-DNA described above (the "reduced model"). For more conservative estimates of the BB treatment association with survival, BB treatment was also tested in models using all 11 candidate variables (the "full model") and the multivariable clinical model derived from the overall UNITE-HF registry using the same methods as UNITE-DNA described above (n = 1304; the "UNITE-HF registry model," which includes all candidate variables in the full model except heart rate and history of hypertension). Potential non-linearity of BB dose response was tested using polynomial terms (e.g. BB dose*BB dose) in the multivariable model.

Because UNITE-DNA is not a BB randomized clinical trial, any significant results for BB response were subject to propensity matching (30). Propensity matching was based on the probability of UNITE-DNA patients being treated with BB based on the 11 candidate variables in the full clinical model plus anti-RAAS treatment. Patients treated and un-treated with BB were matched 1:1 using a greedy 8→1 matching algorithm (31). Because sample size is decreased with propensity matching, the propensity score was also used as a covariate in modeling the entire UNITE-DNA cohort.

Because propensity matching only balances groups based on known covariates, a sensitivity analysis was performed to evaluate the effect of a possible unknown or unmeasured confounder (32,33). HRs for BB versus no BB adjusted for an unknown binary confounder were derived assuming different hazards and distributions of the unmeasured confounder in the two treatments. To determine if any of the 11 candidate clinical variables are associated with BB response, a multiplicative interaction term (clinical variable*BB) was introduced in the full clinical model. A statistically significant interaction was defined as alpha = 0.1/11 = 0.0091.

Results

Baseline characteristics and follow-up. The UNITE-DNA cohort consists of 722 patients. The mean length of follow-up for patients that are still living is 8.9 years, and the number of events for the primary endpoint, all-cause mortality, was 336 (47%). Baseline characteristics are shown in Tables 12, 13, 14 and 15 in UNITE-DNA overall and stratified by race, BB treatment status, etiology, and vital status, respectively. In UNITE-DNA overall, there was good representation of women (37%), AAs (38%), wide age range (57 ± 13 years), wide LVEF range (32% ± 15%), and characteristics that are typical of a community HF patient population. The BB treatment rate was low (67%), but anti-RAAS treatment was high (ACE inhibitor or ARB = 90%). The baseline characteristics for the propensity-matched dataset (n = 406) are well balanced between BB-treated (n= 203) and BB-untreated (n = 203) patients as shown in Table 16, and they are similar to the entire UNITE-DNA dataset (n = 722). The baseline characteristics

compared between the patients that remained in the propensity-matched dataset and the patients without matches, and hence excluded from the dataset, are shown in Table 17.

Survival analysis. The Kaplan-Meier survival curve for UNITE-DNA is shown in Figure 4. The results for the univariate and final multivariable model are shown in Tables 18 and 19. Six variables remained in the final multivariable clinical model: age, history of diabetes, NYHA class, systolic blood pressure, LVEF, and GFR. There were 669 patients with complete data for these six covariates for the survival analysis. However, the results were similar when using imputed mean data for any missing covariates, and thus, including all UNITE-DNA patients in the survival analysis. The proportional hazards assumption was met for the clinical variables.

BB response analysis. BB treatment was significantly associated with an approximately 30% reduction in mortality (Figure 5), and the results were similar regardless of the adjustment model used (reduced model [HR = 0.72; 95% CI = 0.57-0.90; p = 0.0046] vs. full model [HR = 0.69; 95% CI = 0.54-0.88; p = 0.0031] vs. UNITE-HF registry model [HR = 0.68; 95% CI = 0.54-0.86; p = 0.0012]), in the propensity-matched dataset (HR = 0.74; 95% CI = 0.56-0.98; p = 0.0346), and in the propensity-adjusted model (HR = 0.73; 95% CI = 0.57-0.93; p = 0.0180). An unknown binary confounder with a HR = 2 would have to be present in 40% more of the BB untreated patients than the BB treated patients to render the BB association non-significant. There was no statistically significant interaction between BB and any of the candidate clinical variables (Table 20). BB dose was also associated with a reduction in mortality (Figure 6), although it only reached nominal significance (reduced model for every 25mg increase in metoprolol equivalent dose [HR = 0.96; 95% CI = 0.92-0.99; p =

0.0240] vs. full model [HR = 0.95; 95% CI = 0.92-0.99; p = 0.0161] vs. UNITE-HF registry model [HR = 0.95; 95% CI = 0.91-0.99; p = 0.0077]), in the propensity-matched dataset (HR = 0.95; 95% CI = 0.90-0.1.01; p = 0.0732), and in the propensity-adjusted model (HR = 0.96; 95% CI = 0.93-0.1.00; p = 0.0476). Polynomial terms for non-linear dose response were not statistically significant, indicating that dose response is approximately linear on the ln(HR) scale. The majority of patients were on metoprolol (tartrate 21%; succinate 20%) or carvedilol (51%). The reduced model adjusted results were similar for the specific BBs: metoprolol (tartrate or succinate) HR = 0.71; 95% CI = 0.52-0.96; p = 0.0270; and carvedilol HR = 0.74; 95% CI = 0.56-0.98; p = 0.0357. The results were similar when using imputed mean data for any missing covariates, and thus, including all UNITE-DNA patients in the BB response analysis. The proportional hazards assumption was met for the BB treatment variables.

Discussion

Objective #1: Thoroughly describe the UNITE-DNA patient population overall and relevant strata. Among published genetic and pharmacogenetic studies, UNITE-DNA is one of the largest with the longest follow-up. The UNITE-DNA sample also has the advantages of a high event rate, geographical diversity, and a large proportion of BB untreated patients. Although it is very unfortunate that there is a high mortality rate in UNITE-DNA, for research purposes it permits more power to test for associations with all-cause mortality. And although low BB treatment rate would appear

to be a poor characteristic of this patient sample, it is actually an advantage because it provides power to test for statistical interactions with BB treatment. The low BB treatment rate is most likely a reflection of the time of enrollment relative to the publication of the major HF BB clinical trials. In contrast to the large HF clinical trials and other HF registries (34), UNITE-DNA includes patients with preserved LVEF, a large proportion of women and AA, and wide age range. The landmark HF randomized clinical trials have mostly been conducted in patients that are younger, with systolic dysfunction, Caucasian, and male compared to the general HF patient population (21). The heterogeneity of UNITE-DNA is an advantage for the generalizability of results, but heterogeneity could also be a disadvantage and limit power for detecting genetic associations. UNITE-DNA enrollment took place exclusively in outpatient HF specialty clinics, which may lead to selection bias and limit generalization of results to other HF patient care settings. Another limitation of the UNITE-DNA registry design is the mode of death is not known, which may limit the power to detect associations of cardiovascular variables (e.g.. systolic BP, HR, and etiology) with mortality if the patient died from something other than HF. On the other hand, all-cause mortality is a gold standard endpoint in clinical practice.

Interestingly, the HF phenotype in the UNITE-DNA AA patients was very different compared to the Non-AA. The AA UNITE-DNA patients had a higher percentage of women, were younger, had a lower percentage of ischemic etiology, higher percentage with a history of hypertension, increased diastolic BP, increased GFR, and increased BB dose compared to the Non-AA patients. The younger age, increased rate of hypertension, and decreased rate of ischemic etiology are consistent with findings in the

literature (35). Recent data is encouraging and demonstrates that AA HF patients have similar or better in-hospital mortality rates, and HF care is equitable in racial/ethnic groups (36). Although beyond the scope of this dissertation, future research should focus on HF in AA because 1) the disease burden of HF in the U.S. is expected to grow (37), 2) the AA population is expected to double by 2050 (38), and 3) AA have a disproportionate greater burden of HF (39).

The HF phenotype was also drastically different in ischemic versus non-ischemic UNITE-DNA patients. UNITE-DNA patients with an ischemic etiology had a higher percentage of males, Non-AA, diabetes, hypertension, increased age, decreased HR, GFR, and anti-RAAS use, and more deaths compared to the patients with non-ischemic disease. The increased age, percentage of males, diabetes, and deaths are consistent with previous findings in the literature (40). Although not confirmed in the analysis herein, data in the literature suggests that prognosis and drug response differs by HF etiology (41). These drastic differences in the HF phenotype, prognosis, and drug response by etiology may reflect underlying genetic differences between the ischemic and non-ischemic HF etiologies. Risk stratification and the tailoring of pharmacotherapeutic regimens according to HF etiology may provide future opportunities for personalization of HF medicine.

Objective #2: Determine the independent clinical factors associated with survival in UNITE-DNA. The significant, independent clinical risk factors for all-cause mortality found in UNITE-DNA were increased age, the presence of diabetes, increased NYHA class, decreased systolic blood pressure, decreased LVEF, and

decreased GFR. The direction and magnitude of association for these clinical risk factors are similar to other HF patient populations published in the literature. Increased age is not surprising, as studies in the very elderly with HF have demonstrated they have extremely high risk for mortality (6). The micro- and macro-vascular complications accompanied with diabetes possibly explain the increased risk of mortality in the HF patients with diabetes, although evidence also demonstrates that diabetes independently contributes to myocardial damage, hypertrophy, and fibrosis (42). Symptomatic HF is probably one of the most important predictors of mortality, as even a crude measurement such as NYHA class consistently predicts mortality (6). The relationship of systolic blood pressure with mortality in HF may seem paradoxical, as decreased systolic blood pressure would be protective in hypertensive patient populations. However low systolic blood pressure is consistently a poor prognostic sign in HF (7). Low systolic blood pressure may be a sign of poor systolic capacity of the heart coupled with poor vascular responses and declining renal function, but low blood pressure also prevents the use and titration of life-saving HF medications such as ACE inhibitors. Decreased LVEF is also consistently associated with poor prognosis, and in UNITE-DNA it may reflect non-response to ventricular remodeling drugs such as ACE inhibitors, aldosterone antagonists, and BB. Poor renal function may contribute to increased mortality risk in HF due to the resulting electrolyte abnormalities and dose-limiting consequences for life-saving HF medications such as aldosterone antagonists.

Several established mortality risk factors in the literature (e.g. gender, race, heart rate, etc.) were not confirmed in UNITE-DNA. However this was probably due to lack of power as opposed to underlying differences in the UNITE-DNA patient population

because the estimates for the risk factors were similar to those in the literature. This is also evident because more clinical risk factors were statistically significant in the overall UNITE-HF registry (n = 1304).

Objective #3: Determine the independent association of BB treatment with survival in UNITE-DNA. The major limitation to evaluating BB treatment response in UNITE-DNA is non-randomization to treatment groups. Randomization (in large sample sizes) ensures balancing of known and, perhaps more importantly, unknown characteristics between the treatment groups and therefore true BB effects can be concluded. However, it is reasonable to suspect that certain biases are present in an observational study such as UNITE-DNA. Specifically, the UNITE-HF providers may have been hesitant to prescribe BB in the more severe HF patients because of the negative inotropic effects. Indeed, BB were once contraindicated in HF. This type of bias would falsely increase the BB response estimate. However there could also be competing bias, in that over the course of UNITE-DNA follow-up, BB were becoming adopted as standard of care in HF. There may have been crossover of the untreated patients at baseline to the BB treated group, which would falsely decrease the BB response estimate. Adjustment with covariates in regression modeling helps controlling some of the bias for known confounding factors, but it still does not control enough of the bias to equal the balance achieved with a randomized, controlled trial. A further step toward balancing treatment groups is propensity matching. Indeed the UNITE-DNA propensity-matched sub-group was well-balanced, but statistical power is sacrificed when decreasing the UNITE-DNA sample size for propensity-matching (which is why the propensity score

was also used as a covariate). Also, propensity matching only balances the treatment groups based on *known* factors, leaving unknown factors that could possibly bias the results. This is where the unmeasured confounders analysis is valuable to help determine how large of an effect from an unmeasured confounder would have to be in order to change the result. Despite all of these analysis methods, BB was still associated with decreased mortality, and therefore conclusions made on pharmacogenetic interactions will still be meaningful.

Objective #4: Determine clinical factors associated with BB response in UNITE-DNA. Previous data in the literature demonstrates that certain clinical factors are possibly associated with BB response in patients with HF, such as gender (21), race (21), and etiology (23). However this was not confirmed in UNITE-DNA, even in unadjusted analyses (data not shown). This does not seem to be an issue of low power because even when the entire UNITE-DNA sample was included in the analyses (with simple imputation for missing covariates), the results were still negative. There is biologic plausibility for difference in BB response among these groups. For example, patients with ischemic cardiomyopathy may have irreversible damage to the myocardium, prohibiting the beneficial ventricular remodeling conferred by BB. Moreover, ischemic etiology is more common in males, which could possibly explain the decreased BB responsiveness by gender. In the overall UNITE-HF registry, the statistical interaction between race and BB was significant, indicating decreased BB responsiveness in AA. A possible explanation for decreased responsiveness in AA is the difference in HF phenotype between AA and Non-AA. Racial differences in drug response are also often attributed to

differences in care, socioeconomic status (43), and genetics. As mentioned above, data is encouraging that care is equivalent in racial groups (36), and certainly in UNITE-DNA the BB utilization and doses were similar between races. Although UNITE-DNA did not collect data on socioeconomic status, the next chapter begins to elucidate the association between genetic factors and BB response.

Table 11. UNITE-HF study sites and investigators that contributed subjects for this research.

Site	Investigator(s)
University of North Carolina (Coordinating center)	Kirkwood Adams, Carla Sueta, Amanda Garrand, J. Herbert Patterson
Duke University	Christopher O'Connor, Wendy Gattis
University of Iowa	Ron Oren
Cardiac Center LA	Jalal Ghali
University of Illinois-Chicago	Stephanie Dunlap
Case Western Reserve	Ileana Pina
Medical University of South Carolina	Adrian Van Bakel, Grady Hendrix
University of Florida	Alan Miller, Jun Chung
Stern Cardiovascular	Frank McGrew
University of Texas Medical Branch	Daniel Lenihan
Tulane University	Hector Ventura
University of Cincinnati	Lynne Wagoner, Ginger Conway

Table 12. Baseline characteristics in pooled UNITE-DNA and by race.

Baseline characteristics	Pooled n = 722	Non-AA n = 450 (62.5%)	AA n = 270 (37.5%)	p-value
male gender	455 (63%)	317 (70%)	136 (51%)	<.0001
age (years)	57 (13)	60 (13)	53 (13)	<.0001
NYHA class	2.50 (0.64)	2.51 (0.64)	2.50 (0.63)	0.6535
ischemic etiology	314 (44%)	222 (50%)	90 (34%)	<.0001
Hx of hypertension	488 (68%)	272 (61%)	215 (80%)	<.0001
Hx of diabetes	233 (33%)	140 (31%)	91 (34%)	0.4024
SBP (mmHg)	119 (22)	118 (20)	121 (24)	0.1192
DBP (mmHg)	70 (13)	69 (12)	72 (14)	0.0002
HR (beats/min)	75 (14)	75 (14)	76 (14)	0.1046
LVEF (%)	32 (15)	32 (15)	32 (17)	0.7141
Estimated GFR (mL/min)	66 (27)	63 (23)	71 (31)	<.0001
BB	484 (67%)	304 (68%)	179 (67%)	0.6851
BB dose (mg metoprolol CR equivalents)	112 (85)	105 (82)	124 (88)	0.0170
ACE inhibitor	577 (81%)	365 (82%)	211 (79%)	0.3248
ACE inhibitor or ARB	639 (90%)	402 (90%)	235 (88%)	0.3285
Loop diuretic	603 (84%)	367 (82%)	234 (87%)	0.1219
Spironolactone	172 (24%)	107 (24%)	65 (24%)	0.9628
Digoxin	516 (72%)	330 (74%)	185 (69%)	0.1321
Follow-up characteristics				
Days of follow-up	2410 (1288)	2475 (1285)	2302 (1290)	0.0808
Deaths	336 (47%)	204 (45%)	131 (49%)	0.4068

Table 13. Baseline characteristics in pooled UNITE-DNA and by BB treatment status.

Baseline characteristics	Pooled n = 722	BB yes n = 484 (67%)	BB no n = 238 (33%)	p-value
male gender	455 (63%)	307 (64%)	144 (62%)	0.5991
race (AA)	270 (38%)	179 (37%)	143 (39%)	0.6851
age (years)	57 (13)	56 (13)	59 (13)	0.0133
NYHA class	2.50 (0.64)	2.43 (2.67)	2.65 (0.57)	<.0001
ischemic etiology	314 (44%)	211 (45%)	100 (44%)	0.8322
Hx of hypertension	488 (68%)	325 (67%)	160 (69%)	0.5966
Hx of diabetes	233 (33%)	152 (32%)	156 (67%)	0.7112
SBP (mmHg)	119 (22)	119 (22)	121 (23)	0.2826
DBP (mmHg)	70 (13)	70 (13)	71 (12)	0.1614
HR (beats/min)	75 (14)	73 (13)	80 (14)	<.0001
LVEF (%)	32 (15)	32 (15)	31 (16)	0.5475
Estimated GFR (mL/min)	66 (27)	68 (26)	64 (27)	0.0603
BB dose (mg metoprolol CR equivalents)	112 (85)	112 (85)	0 (0)	N/A
ACE inhibitor	577 (81%)	403 (84%)	173 (75%)	0.0034
ACE inhibitor or ARB	639 (90%)	441 (92%)	197 (85%)	0.0058
Loop diuretic	603 (84%)	412 (85%)	191 (82%)	0.2069
Spironolactone	172 (24%)	119 (25%)	52 (23%)	0.5238
Digoxin	516 (72%)	344 (71%)	172 (74%)	0.5234
Follow-up characteristics				
Days of follow-up	2410 (1288)	2475 (1244)	2272 (1369)	0.0479
Deaths	336 (47%)	194 (40%)	140 (60%)	<.0001

Table 14. Baseline characteristics in pooled UNITE-DNA and by etiology.

Baseline characteristics	Pooled n = 722	Ischemic n = 314 (44%)	Non-ischemic n = 392 (56%)	p-value
male gender	455 (63%)	227 (72%)	218 (56%)	<.0001
race (AA)	270 (38%)	90 (29%)	174 (44%)	<.0001
age (years)	57 (13)	62 (11)	53 (13)	<.0001
NYHA class	2.50 (0.64)	2.62 (0.58)	2.43 (0.66)	<.0001
Hx of hypertension	488 (68%)	231 (74%)	247 (63%)	0.0031
Hx of diabetes	233 (33%)	126 (40%)	101 (26%)	<.0001
SBP (mmHg)	119 (22)	120 (23)	119 (22)	0.3849
DBP (mmHg)	70 (13)	70 (12)	70 (13)	0.5483
HR (beats/min)	75 (14)	73 (13)	78 (15)	<.0001
LVEF (%)	32 (15)	31 (15)	33 (16)	0.0876
Estimated GFR (mL/min)	66 (27)	62 (23)	70 (29)	<.0001
BB	484 (67%)	211 (68%)	263 (67%)	0.8322
BB dose (mg metoprolol CR equivalents)	112 (85)	105 (86)	113 (84)	0.3175
ACE inhibitor	577 (81%)	242 (78%)	324 (83%)	0.0941
ACE inhibitor or ARB	639 (90%)	267 (86%)	360 (92%)	0.0079
Loop diuretic	603 (84%)	264 (85%)	328 (84%)	0.6611
Spironolactone	172 (24%)	66 (21%)	103 (26%)	0.1115
Digoxin	516 (72%)	214 (69%)	294 (75%)	0.0604
Follow-up characteristics				
Days of follow-up	2410 (1288)	2198 (1292)	2566 (1271)	0.0002
Deaths	336 (47%)	180 (57%)	149 (38%)	<.0001

Table 15. Baseline characteristics in pooled UNITE-DNA and by vital status.

Baseline characteristics	Pooled n = 722	Alive n = 386 (53%)	Deceased n = 336 (47%)	*p-value
male gender	455 (63%)	236 (61%)	219 (65%)	0.2400
race (AA)	270 (38%)	139 (36%)	131 (39%)	0.4068
age (years)	57 (13)	54 (13)	61 (13)	<.0001
NYHA class	2.50 (0.64)	2.37 (0.69)	2.67 (0.53)	<.0001
ischemic etiology	314 (44%)	134 (36%)	180 (55%)	<.0001
Hx of hypertension	488 (68%)	246 (64%)	242 (72%)	0.0218
Hx of diabetes	233 (33%)	94 (25%)	139 (41%)	<.0001
SBP (mmHg)	119 (22)	120 (22)	119 (22)	0.3549
DBP (mmHg)	70 (13)	71 (13)	69 (12)	0.0035
HR (beats/min)	75 (14)	75 (15)	75 (13)	0.7670
LVEF (%)	32 (15)	34 (16)	30 (15)	0.0006
Estimated GFR (mL/min)	66 (27)	72 (25)	59 (27)	<.0001
BB	484 (67%)	290 (76%)	194 (58%)	<.0001
BB dose (mg metoprolol CR equivalents)	112 (85)	117 (88)	105 (80)	0.1388
ACE inhibitor	577 (81%)	320 (84%)	257 (78%)	0.0456
ACE inhibitor or ARB	639 (90%)	351 (92%)	288 (87%)	0.0439
Loop diuretic	603 (84%)	309 (80%)	294 (88%)	0.0059
Spironolactone	172 (24%)	98 (26%)	74 (22%)	0.3140
Digoxin	516 (72%)	261 (68%)	255 (77%)	0.0105
Follow-up characteristics				
Days of follow-up	2410 (1288)	3260 (807)	1438 (1021)	<.0001

Table 16. Baseline characteristics of propensity-matched dataset

Baseline characteristics	Pooled n = 406	BB yes n = 203 (50%)	BB no n = 203 (50%)	p-value
male gender	243 (60%)	129 (59%)	129 (61%)	0.7613
race (AA)	135 (36%)	68 (34%)	77 (38%)	0.3321
age (years)	58 (13)	59 (13)	58 (12)	0.5530
NYHA class	2.63 (0.56)	2.63 (0.53)	2.63 (0.59)	1.0000
ischemic etiology	173 (43%)	86 (43%)	87 (44%)	0.8507
Hx of hypertension	284 (70%)	147 (72%)	137 (69%)	0.3892
Hx of diabetes	126 (31%)	60 (30%)	66 (33%)	0.5411
SBP (mmHg)	120 (23)	119 (23)	120 (23)	0.6464
DBP (mmHg)	71 (13)	71 (14)	71 (12)	0.9816
HR (beats/min)	79 (14)	78 (14)	79 (14)	0.5504
LVEF (%)	31 (16)	31 (16)	31 (16)	0.7945
Estimated GFR (mL/min)	64 (26)	64 (24)	64 (28)	0.9970
BB dose (mg metoprolol CR equiv)	100 (82)	100 (82)	0 (0)	<.0001
ACE inhibitor	315 (78%)	158 (78%)	157 (77%)	0.9053
ACE inhibitor or ARB	357 (88%)	177 (87%)	180 (89%)	0.6476
Loop diuretic	345 (85%)	178 (88%)	167 (82%)	0.1265
Spironolactone	100 (25%)	51 (25%)	49 (24%)	0.8399
Digoxin	293 (72%)	145 (71%)	148 (73%)	0.7397
Follow-up characteristics				
Days of follow-up	2369 (1332)	2420 (1287)	2317 (1376)	0.4390
Deaths	210 (52%)	90 (44%)	120 (59%)	0.0029

Table 17. Baseline characteristics of propensity-matched patients and unmatched.

Baseline characteristics	Pooled n = 722	Propensity-matched n = 406 (56%)	Unmatched n = 316(44%)	p-value
male gender	455 (63%)	243 (60%)	212 (67%)	0.0398
race (AA)	270 (38%)	145 (36%)	125 (40%)	0.2860
age (years)	57 (13)	58 (13)	55 (14)	0.0023
NYHA class	2.50 (0.64)	2.63 (0.56)	2.34 (0.69)	<.0001
ischemic etiology	314 (44%)	173 (43%)	141 (46%)	0.4536
Hx of hypertension	488 (68%)	284 (70%)	204 (65%)	0.1037
Hx of diabetes	233 (33%)	126 (31%)	107 (34%)	0.4253
SBP (mmHg)	119 (22)	120 (23)	119 (21)	0.3974
DBP (mmHg)	70 (13)	71 (13)	69 (12)	0.1965
HR (beats/min)	75 (14)	79 (13)	71 (13)	<.0001
LVEF (%)	32 (15)	31 (16)	33 (15)	0.1805
Estimated GFR (mL/min)	66 (27)	64 (26)	69 (27)	0.0104
BB	484 (67%)	203 (50%)	281 (90%)	<.0001
BB dose (mg meto CR equiv)	112 (85)	100 (82)	118 (87)	0.0243
ACE inhibitor	577 (81%)	315 (77%)	262 (85%)	0.0120
ACE inhibitor or ARB	639 (90%)	357 (88%)	282 (92%)	0.1174
Loop diuretic	603 (84%)	345 (85%)	258 (83%)	0.4083
Spironolactone	172 (24%)	100 (25%)	72 (23%)	0.6669
Digoxin	516 (72%)	293 (72%)	223 (72%)	0.8911
Follow-up characteristics				
Days of follow-up	2410 (1288)	2369 (1332)	2465 (1230)	0.3204
Deaths	336 (47%)	210 (52%)	126 (40%)	0.0015

Table 18. Univariate proportional hazards analysis of 11 candidate clinical variables.

Variable	HR	95% CI	p-value
Gender (Male)	1.23	(0.98-1.54)	0.0762
Race (AA)	1.14	(0.92-1.42)	0.2321*
Age (10 years)	1.42	(1.30-1.55)	<.0001
Etiology (ischemic)	1.74	(1.40-2.17)	<.0001
Hx HTN (yes)	1.27	(1.00-1.62)	0.0473
Hx DM (yes)	1.75	(1.40-2.17)	<.0001
NYHA class (1 class)	1.77	(1.46-2.13)	<.0001
SBP (5 mmHg)	0.97	(0.95-0.99)	0.0350
HR (5 bpm)	0.98	(0.95-1.02)	0.3476*
LVEF (5%)	0.93	(0.89-0.97)	0.0002
GFR (10 mL/min)	0.85	(0.81-0.89)	<.0001

*Did not meet < 0.1 level of significance and was not entered into multivariable model

Table 19. Final multivariable clinical model.

Variable	HR	95% CI	p-value
Age (10 years)	1.32	(1.20-1.46)	<.0001
Hx DM	1.62	(1.28-2.03)	<.0001
NYHA class	1.60	(1.30-1.95)	<.0001
SBP (5 mmHg)	0.96	(0.94-0.99)	0.0072
LVEF (5%)	0.96	(0.92-0.99)	0.0363
GFR (10 mL/min	0.91	(0.87-0.96)	0.0008

Table 20. Adjusted interaction between 11 candidate clinical variables and BB response.

Variable	*p-value
Gender (Male)	0.1005
Race (AA)	0.1471
Age (10 years)	0.6225
Etiology (ischemic)	0.1357
Hx HTN	0.2166
Hx DM	0.1058
NYHA class	0.3073
SBP (5 mmHg)	0.9385
HR (5 bpm)	0.1706
LVEF (5%)	0.3647
GFR (10 mL/min	0.8958

*Bonferroni-adjusted level of statistical significance = 0.0091

Figure legends

Figure 4. The Kaplan-Meier estimated survival distribution function for all UNITE-DNA patients is shown over 10 years of follow-up. Censored observations are represented by small circles.

Figure 5. Survival curves for UNITE-DNA derived from the Cox proportional hazards model adjusted for age, diabetes, systolic blood pressure, NYHA class, LVEF, and GFR and stratified by patients treated with beta-blockers (n = 483) and patients not treated with beta-blockers (n = 232) at baseline.

Figure 6. The linear association between BB dose and reduction in mortality derived from the Cox proportional hazards model adjusted for age, diabetes, SBP, NYHA class, LVEF, and GFR is shown by 25, 50, 100, 150, and 200mg of metoprolol dose equivalents.

Figure 4. Kaplan-Meier survival curve for UNITE-DNA

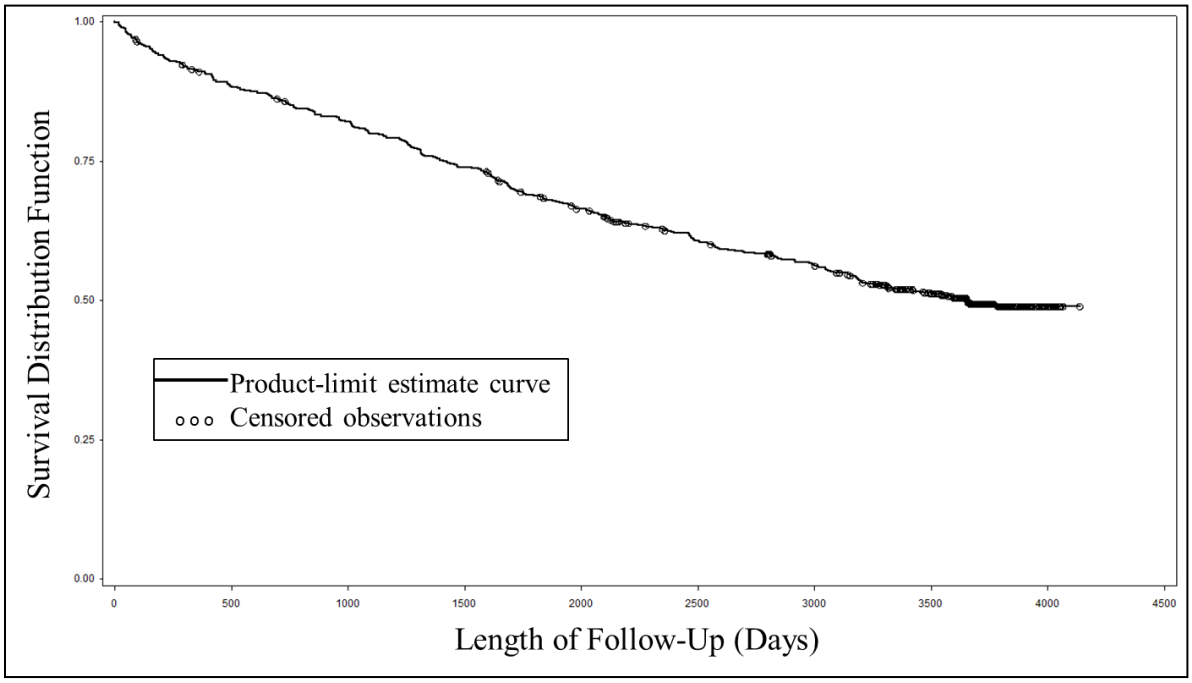


Figure 5. Adjusted survival curves by BB treatment in UNITE-DNA

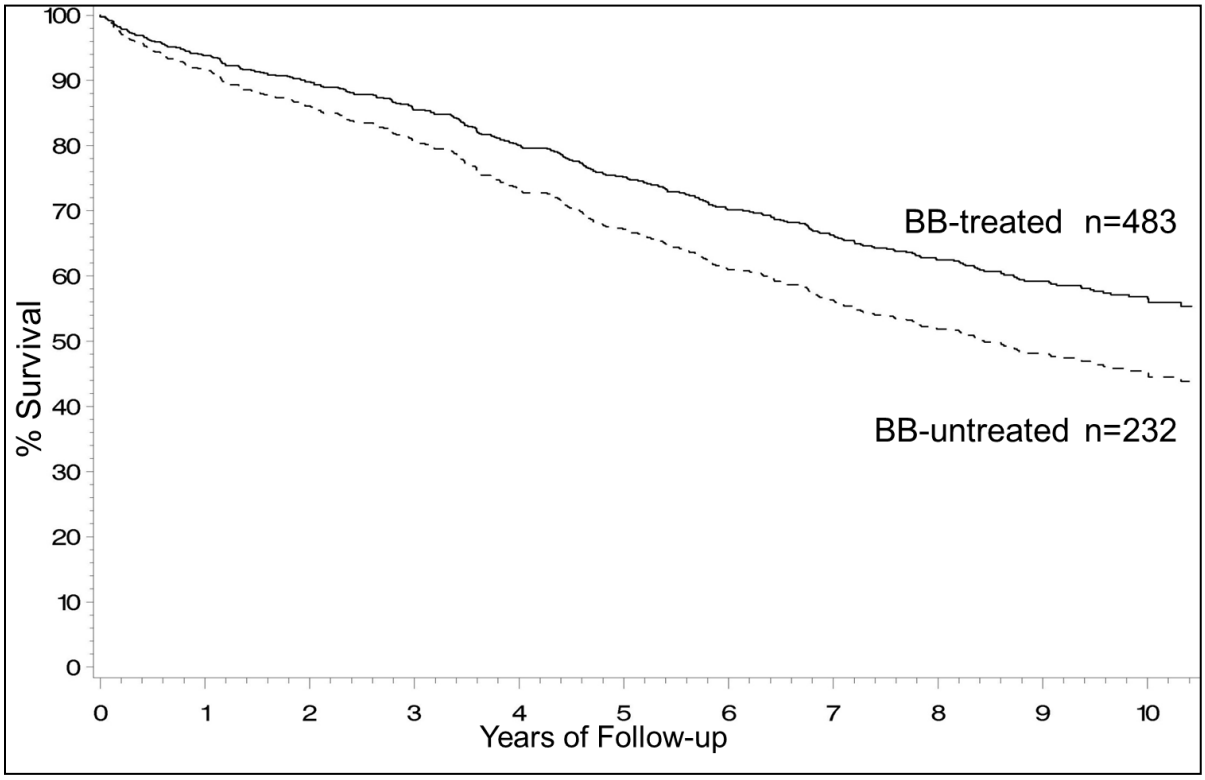
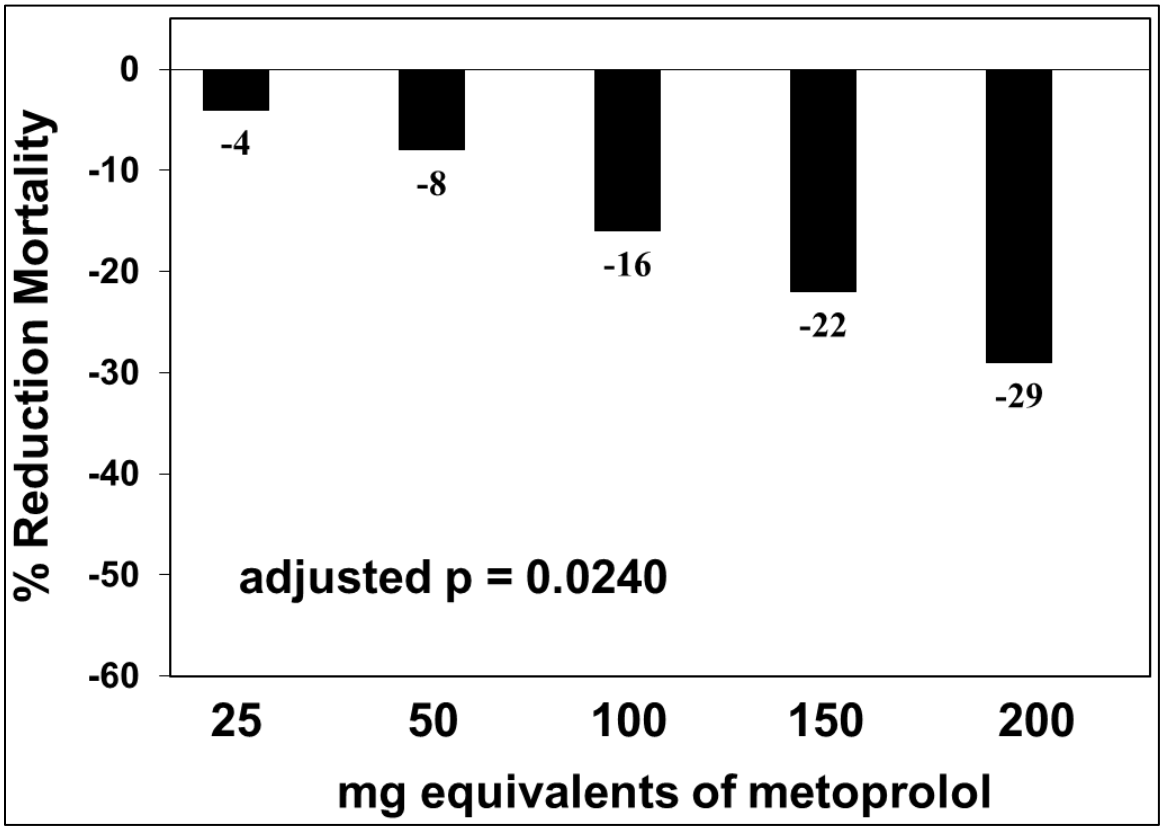


Figure 6. Adjusted BB dose associated survival benefit in all UNITE-DNA patients.



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CHAPTER V:
INDIVIDUAL GENETIC VARIANTS ASSOCIATED WITH HF PATIENT SURVIVAL AND BETA-BLOCKER RESPONSE

Summary

The previous chapter described the UNITE-DNA HF patient population and the clinical characteristics that are associated with survival and BB survival benefit. This chapter covers the individual, candidate genetic variants that are independently associated with HF patient survival and BB survival benefit. Although none of the 11 variants or diplotypes was significantly associated with survival, Ser49Gly in *ADRB1* was significantly associated with BB response after rigorous control for clinical covariates and multiple comparisons. BB treatment was associated with a statistically significant 46% reduction in mortality in Ser49-homozygotes but a non-significant 38% increase in Gly49-carriers.

Introduction

The clinical characteristics described in the previous chapter do not entirely explain the variability in HF survival and BB response, but data is emerging for genetic factors that may play a role (see Chapter II: Identification of Candidate Genetic Variants). When studying and interpreting genetic associations in common, complex diseases such as HF, the small effect sizes of common variants in common diseases and the potential for false positives must be considered. The common disease common variant hypothesis

(1) predicts that multiple common genetic variants contribute to common, complex diseases, and each variant has a small effect (e.g. odds ratios typically in the range of 1.2 to 1.5). The small effect sizes expected for common genetic variants in common diseases requires large sample sizes for the power to detect genetic associations, especially in comparison to and when controlling for clinical risk factors with large effects. However genetic variants have the potential for effect sizes similar to and independent of their clinical risk factor counterparts. For example, a single insertion/deletion variant in *ACE* accounts for half of the variance in plasma ACE levels (2). HF patients who possess the deletion allele have significantly higher plasma ACE levels and significantly higher risk of mortality (3). The odds ratio for the *ACE* genetic variant in the multivariable model was 1.69, which was higher than any other significant clinical variable in a study of 194 patients with idiopathic HF. For comparison, the other significant predictors of mortality in the multivariable analysis were New York Heart Association functional class (odds ratio = 1.42) and left ventricular end-systolic diameter (odds ratio = 1.07).

Because the genetic contribution to variability in HF outcomes is most likely polygenic, genetic association studies often test multiple genetic variants in multiple genetic models resulting in multiple statistical tests. Multiple statistical testing inflates Type I error (rejecting the null hypothesis when it is actually true). An extreme example would be GWAS, in which one million genetic variants could be tested for an association. If a GWAS investigator used alpha = 0.05, then 50,000 of the genetic variants could be reported as positive by chance alone. It has been speculated that 19 out of every 20 genetic marker-disease associations in the literature are false (4), which is supported by the pervasive lack of replication for many genetic markers (5) (Appendix

IV). Several reasons could possibly explain the lack of replication, such as true variation of the underlying association in different populations, misclassification of outcome, population stratification, or lack of power, but the failure to exclude chance is purported to be the number one cause of false discoveries (6). False discoveries can lead to wasted time and resources on leads that are eventually proven worthless, and the repeated lack of replication of genetic associations also leads to a loss of confidence in this type of research by the scientific community. Therefore, several methods have been developed to control for false discoveries when making multiple comparisons in genetic association studies.

Multiple comparison control methods can be broadly defined into two categories (4,6): those that control the family wise error rate (FWER) and those that control the false discovery rate (FDR). The family wise error rate is the rate of Type I error for a family of statistical tests, and the false discovery rate is the proportion of rejected null hypotheses that are false. Methods that control the FWER are more conservative than FDR methods, and FWER methods become increasing conservative with the number of statistical tests. For example, one of the most commonly used FWER controlling methods, presumably because of its simplicity, is the Bonferroni correction in which alpha is divided by the number of comparisons. Therefore, for a GWAS of 500,000 variants, the alpha = 10^{-7}. The Bonferroni correction for multiple comparisons can be overly conservative because achieving such low levels of Type I error increases Type II error (failing to reject the null hypothesis when in fact it is false), and hence extremely large sample sizes are required for the power to meet statistical significance. The incurred loss of power, even in medium-sized studies, has led many investigators to neglect

multiplicity control all together. FWER controlling methods assume independence of the statistical tests, which is unlikely in genetic association studies of many variants. Also, the FWER correction is dependent on the number of tests, which in genetic association studies can be arbitrary and based on factors such as budget, publication strategy, and genotyping capacity. The context of the genetic association study is also important. For example, exploratory studies may prefer less conservative methods and therefore permit a number of false discoveries in order to ensure the power for detecting true discoveries. On the other hand, validation studies may use more conservative methods since the genetic association has been previously reported, and the implications for the genetic association may be great (e.g. the conduct of a large, expensive, prospective clinical trial or change in clinical practice).

Both FWER and FDR methods were used for this chapter. The more conservative FWER controlling Bonferroni method was used as control for the primary hypotheses of this chapter: an independent association of the individual genetic variants or diplotypes with 1) survival and 2) BB survival benefit in an autosomal dominant inheritance model. The more conservative method was chosen for the two primary hypotheses because these hypotheses have been tested before, and thus UNITE-DNA is serving as a validation population. Also, the implications for the results are large because they concern HF patient survival and pharmacotherapy that is firmly entrenched in the HF treatment guidelines. For exploratory analyses of other inheritance models and sub-groups (i.e. racial and etiologic), a FDR of 5% modified for dependency was used. This is because the analyses are exploratory, and hence a small amount of false discoveries are permitted to allow for the power to detect other important leads.

Methods

Statistical analysis. The autosomal dominant inheritance model (major allele [most frequent] homozygotes versus heterozygotes plus minor allele homozygotes) was used to test the two primary hypotheses. The purpose of using the dominant inheritance model was two-fold. First, it maximizes the sample sizes of the comparison groups. Second, it improves potential future generalizability in clinical practice (e.g. if BB response differed only in a minor allele homozygotes sub-group, then it would only apply to a small percentage of patients). To assess the independent association of the 11 candidate variants and two diplotypes (diplotypes in *ADRB1* and *ADRB2*; see Chapter III: Characterization of Candidate Genetic Variants) with survival, each was tested in the Cox proportional hazards reduced model developed in Chapter IV: Characterization of Patient Population. The model was developed for the risk alleles determined in Chapter II, so that the expected HR would be >1 based on previous literature. Given n = 722, alpha = 0.1, and 8.9 years of follow-up, there was 80% power to detect a HR = 1.19 for a dichotomous variable in the univariate analysis. To assess the independent interaction between genotype and BB survival benefit, each of the 11 candidate variants and two diplotypes was tested for a multiplicative statistical interaction (variant*BB) within the reduced clinical model. If the interaction term was statistically significant, then the adjusted BB survival benefit within the genotype sub-groups was determined using stratified and contrast analysis. BB dose-associated survival benefit was assessed by including BB dose (in 25mg metoprolol equivalents) in the model as a continuous variable. Possible non-linearity of BB dose-associated survival benefit was assessed by

including polynomial terms in the model. The Bonferroni-adjusted alpha for the independent association of variants with survival was 0.05/13 = 0.0038 and for the genotype*BB interaction term was 0.1/13 = 0.0077 (multiplicative statistical interactions are underpowered). For any test that was statistically significant in the reduced model, it was also tested with the full and UNITE-HF clinical models described in Chapter IV for robustness. Also, for any variant that was statistically significant, baseline characteristics were compared between genotype sub-groups to determine possible differences in baseline characteristics that were not included as covariates in any of the above clinical adjustment models. Specific BBs (metoprolol and carvedilol) were assessed for significant genotype*BB interactions. Because UNITE-DNA is not a BB randomized clinical trial, any significant results for BB survival benefit were subjected to propensity matching (7). Patients treated and un-treated with BB were matched 1:1 using a greedy 8→1 matching algorithm (8). Because sample size is decreased with propensity matching, the propensity score was also used as a covariate in modeling the entire UNITE-DNA cohort. A sensitivity analysis was performed to evaluate the effect of a possible unknown or unmeasured confounder (9,10) on any significant results. HRs for BB versus no BB adjusted for an unknown binary confounder was derived assuming a HR = 2 for the unknown confounder. There were very few patients with missing values for any covariate (~10%). But to ensure that including patients with missing data for the multivariable analysis did not significantly affect the conclusions, analyses were repeated with simple imputation for missing data and hence all patients were included in the analysis.

Several exploratory analyses were performed in addition to the two primary hypotheses described above. To determine if the candidate genetic variants or haplotypes would *replace* any of the candidate clinical variables in the final multivariable model, the candidate genetic variants were subject to model building similar to Chapter IV. Specifically, a series of univariate proportional hazards regression models were fit, one model for each of 11 candidate variants, two haplotypes, and pre-defined clinical factors. A significance level of $p < 0.10$ was prospectively defined for inclusion in the multivariable analysis. Final inclusion in the multivariable model was determined using stepwise selection with a significance level of $p < 0.05$. Additional exploratory analysis tested the other inheritance models, recessive and additive (except for *ADRA2C* which can only be tested in the dominant inheritance model [see Chapter III]), for an independent association with survival or BB survival benefit. These analyses were also performed stratified by race, BB treatment, and etiology. A 5% false discovery rate (with modification for dependency) (11) was used to adjust the p-values for the many exploratory tests performed.

Results

Survival. For the primary hypothesis (independent association of candidate genetic variants or diplotypes with survival in a dominant inheritance model), none of the candidate genetic variants or diplotypes met the criteria for statistical significance as shown in Table 21. The results were similar when simple imputation was used for missing data. In the exploratory analyses, none of the candidate genetic variants or diplotypes replaced the clinical variables in the multivariable model because none of the

genetic variants had a univariate association with $p < 0.1$. When the other inheritance models (recessive and additive) and strata (by race, etiology, or BB treatment) were explored, none of the candidate genetic variants or diplotypes were independently associated with survival after correction with a 5% false discovery rate modified for dependency (Table 22).

Beta-blocker response. For the primary hypothesis (independent association of candidate genetic variants or diplotypes with BB response in a dominant inheritance model), one candidate genetic variant met the criteria for a statistical significance, *ADRB1* Ser49Gly, as shown in Table 23 (Appendix III) (12). The results were similar when simple imputation was used for missing data. The Ser49Gly*BB interaction term remained statistically significant in the full model ($p = 0.0030$) and UNITE-HF model ($p = 0.0028$). Race and history of hypertension were significantly different between genotype groups (Table 24), and these were included as covariates in the full clinical model which remained statistically significant. In the propensity matched dataset ($n = 406$; see Chapter IV), the Ser49Gly*BB interaction term p-value was $p = 0.0206$, and it was $p = 0.0032$ when the propensity score was used as a covariate for the entire UNITE-DNA cohort. The propensity-matched and propensity-adjusted stratified results are shown in Table 25. The adjusted stratified and contrast results for BB and BB dose response within the dominant inheritance genotype groups are shown in Table 26. BB dose response was only present in the Ser49-homozygotes and was approximately linear on the ln(HR) scale because polynomial terms in the model (e.g. BB*BB) were not statistically significant. The adjusted Ser49Gly*BB interaction was $p = 0.0161$ for metoprolol and $p = 0.0046$ for carvedilol specifically. Figure 7 shows the adjusted

survival curves stratified by BB and Ser49Gly genotype, and Figure 8 shows the adjusted BB dose-associated survival benefit by Ser49Gly genotype. An unknown binary confounder with a HR = 2 would have to be present in 70% more of the BB untreated patients than the BB treated patients to render the BB association in Ser49-homozygotes non-significant. In the exploratory analyses, the other inheritance models (recessive and additive) and strata (by race and etiology) did not have any statistically significant associations after adjustment for a 5% false discovery rate modified for dependency (Table 22).

Discussion

Survival. In this chapter, none of the 11 individual genetic variants or 2 diplotypes was independently and significantly associated with survival in UNITE-DNA. The negative results for survival are surprising, as each of the variants has established molecular effects which have been translated to HF relevant clinical outcomes (see Chapter II: Identification of Candidate Genetic Variants). The two most probable explanations for the negative survival results are selection bias and lack of power. The UNITE-DNA patient population comes from outpatient HF specialty clinics. Therefore it is possible that there is selection occurring before the patients even make it to the clinic. For example, if certain genetic variants were extreme risk factors for poor clinical outcome, patients may die or receive a heart transplant before they are even referred to the HF clinic. Also, UNITE-DNA was a heavily treated population. Therefore the patients may have been treated beyond any potential genetic effects. For example, the patients could have such extreme pharmacologic inhibition of the SNS and RAAS with a

combination of ACE inhibitor or ARB, BB, and aldosterone antagonist that any effects of genetic polymorphisms in the SNS and RAAS are neutralized. Indeed, this has been seen previously in the literature. The *ACE* indel was associated with clinical outcome in BB-untreated HF patients, but there was no influence of the *ACE* indel in patients treated with BB and ACE inhibitor (13). However, the exploratory analysis of UNITE-DNA also did not reveal an association of any of the genetic variants in BB-untreated patients after correction for multiple comparisons. There may also be true underlying differences in the genetic association for the genetic variants in the patient populations initially studied and UNITE-DNA. Power may also be an explanation for the negative survival results. According to the common disease common variant hypothesis (1), common genetic variants such as those studied herein are expected to have a small effect on common diseases such as HF. The expected odds ratios are typically in the range of 1.2 to 1.5, and there was only 80% power to detect a HR = 1.19 in univariate analysis of UNITE-DNA. Publication bias may have falsely elevated the expected odds ratios for common variants studied herein (14). Negative results, especially in smaller studies, may not be submitted for publication let alone accepted. Therefore any systematic review of published results would be misleading. Although the individual effects of the genetic variants may be small, the genetic variants come from the same physiologic systems (SNS and RAAS), and therefore may have additive effects when combined. This concept was explored in the next chapter.

***ADRB1* Ser49Gly and BB response.** There is strong biologic plausibility and prior literature to support the pharmacogenetic interaction between BB and Ser49Gly in

ADRB1. The beta-1 adrenergic receptor is the principal adrenergic receptor subtype expressed on human cardiomyocytes. It is the primary mediator of catecholamine-mediated increases in cardiac inotropy and chronotropy, as well as the cardiomyopathic effects leading to HF (15). Stimulation of myocardial beta-1 adrenergic receptors with catecholamines results in the activation of the heterotrimeric Gs protein, which, in turn, activates adenylyl cyclase and promotes the production of cAMP. The gene encoding the beta-1 adrenergic receptor (*ADRB1*) is localized to chromosome 10q24-q26 (16). *ADRB1* is intronless, consists of 1,714 base pairs, and codes for a 51.3 kDa protein consisting of 477 amino acid residues (17). The non-synonymous single nucleotide polymorphism, 145 A->G or rs1801252, causes the substitution of glycine for serine at amino acid 49 (Ser49Gly) in the N-terminal extracellular domain of the receptor (18,19). The Ser49 allele is common in humans, having an allele frequency of approximately 75-87% in Caucasians, Chinese, and AAs (20). However the Gly49 allele is conserved across other mammalian species (21), indicating that Ser49Gly may have functional relevance. Indeed, Ser49Gly has been widely studied *in vitro*, *ex vivo*, and *in vivo*.

In transfected cell experiments, the influence of Ser49Gly on agonist binding and basal and agonist-stimulated adenylyl cyclase activity is unclear. Levin *et al* found that Gly49-expressing cells have a higher affinity for beta-1 agonists (22), but Rathz *et al* found identical agonist binding affinities (23). Levin *et al* found that Gly49-expressing cells have 4-fold and 2-fold higher basal and maximally-stimulated adenylyl cyclase activity compared to Ser49, respectively (22), but Rathz *et al* found identical basal and agonist-stimulated adenylyl cyclase activities (23). However where the literature *is* consistent is with respect to antagonist binding and down-regulation. Ser49 and Gly49-

cells bind metoprolol equally, and Gly49 receptors are down-regulated after sustained agonist stimulation whereas Ser49 receptors are not. In patients with chronic HF and persistent sympathetic stimulation that is toxic to the cardiomyocyte, down-regulation of beta-1 receptors is thought to be a protective adaptation.

In non-failing, human right atrial tissue experiments, Molenaar *et al* found that basal and norepinephrine-stimulated contractions were similar among Ser49- and Gly49-containing tissues (24). This was also found by Sarsero *et al* using (-)-CGP 12177, a partial agonist BB which increases heart rate and force at high concentrations and is resistant to blockade with propranolol (25). Sarsero *et al* tested the potency (-)-CGP 12177 in failing hearts, but did not test for the influence of Ser49Gly. The results were consistent among patients treated and not treated with BBs, supporting the *in vitro* findings of Rathz *et al*. It is important to note that the transfected cell experiments demonstrated changes in down-regulation between Ser49 and Gly49 only after sustained stimulation with agonist. Therefore the experiments by Molenaar *et al* and Sarsero *et al* may not have allowed sufficient time for beta-1 receptor down-regulation, and the results may have been different in tissues from patients with failing hearts or sustained experimental exposure to agonist.

Ser49Gly has been studied for an association with many *in vivo* cardiovascular parameters such as blood pressure, heart rate, and left ventricular remodeling and the changes in these parameters in response to beta-blockade. As would be expected by the lack of down-regulation of Ser49-containing receptors, patients with Ser49 tended to have a better blood pressure response to beta-blockade (26-29). Although there are several negative studies as well (30-35). Ser49 is associated with higher heart rate at rest

(36,37) and in response to exercise (30). Although no association (31,33,38,39) and the reverse association with heart rate have also been reported (40). In response to BB, there is a similar negative chronotropic response among genotypes (34,37). A single study showed improved left ventricular end diastolic diameter, but not LVEF, in response to metoprolol in Ser49 homozygous HF patients compared to Gly49 carriers (41). However many studies failed to find an association with left ventricular remodeling response (34,42-46). An important consideration in studies of cardiovascular parameters is the time dependency of beta-1 receptor down-regulation demonstrated in the *in vitro* experiments. Specifically, short-term measurements of cardiovascular parameters may fail to detect differences among Ser49Gly genotypes. Another consideration is that receptor down-regulation is in response to persistent agonist stimulation, like that seen in HF. Therefore studies in healthy volunteers or cardiovascular disease with non-failing hearts may also not translate the *in vitro* findings. Moreover, left ventricular remodeling response to beta-blockade is time dependent, in which it may take up to one year to see a complete response (47).

Ser49Gly has been studied for its association with the development of several cardiovascular diseases, albeit with inconsistent results. The seminal paper by Podlowski *et al* describes the striking association of Ser49Gly with the development of idiopathic dilated cardiomyopathy (IDCM), but the study was very small (21). Podlowski *et al* performed a case-control study of 37 German IDCM patients and 40 healthy volunteers, the Gly49 allele was only detected in the IDCM patients resulting in an estimated odds ratio for the development of IDCM of 14.7 with Gly49 (21). A similar association was found in larger case-control studies of Italian (48) and Chinese patients (49). However, a

recent meta-analysis of 2642 cases and 3136 controls revealed an association with IDCM only among Asian patients and not European (50). There are also mixed results for the association of Ser49Gly with the development of hypertension, with one large meta-analysis showing no association (51) and another demonstrating an association (52).

Ser49Gly is not clearly involved with the development of cardiovascular disease, but it may alter the course of established cardiovascular disease. Indeed, this is supported by a retrospective study by Borjesson *et al* (19). In 184 patients with IDCM, the survival curve for Ser49-homozygous patients treated with BB was almost identical to Gly49-carrying patients not receiving BB. This was confirmed in a study by Magnusson *et al* (53), in which they added a prospective IDCM cohort (n = 190) to the retrospective cohort studied by Borjesson *et al* (53), and a study of 171 Italian IDCM patients found similar results in univariate analysis (54). Magnusson *et al* found that patients carrying Gly49 had a similar survival rate regardless of high-dose (>50% of target dose) or low-dose (≤ 50% of target dose) BB. However, in the group of patients treated with low-dose BB, patients carrying Gly49 had lower five-year mortality compared to patients homozygous for Ser49 (risk ratio = 0.24; p = .020). Like all genetic association literature, there are many things to consider when interpreting the association between Ser49Gly and survival such as study power, publication bias, phenotypic definition, and racial/ethnic stratification (55). Acknowledging these differences, the Magnusson *et al*, Borjesson *et al*, and the results described herein suggest that it is more critical that HF patients homozygous for Ser49 are treated with high dose BBs than Gly49-carriers.

***ADRB1* Arg389Gly and BB response.** Sympathetic stimulation of *ADRB1* results in activation of the Gs protein, which in turn activates adenylyl cyclase and the

production of cAMP (56). Arg389 of *ADRB1* displays increased coupling to Gs compared to Gly389 (57); hence Arg389 has greater basal and agonist-stimulated activity (57). Therefore it has been hypothesized that HF patients possessing Arg389 would have a greater response to BB. With respect to ventricular remodeling responses, this has been studied in a series of small HF cohorts.

Mialet-Perez et al retrospectively studied 224 patients with systolic dysfunction receiving carvedilol (58). They were the first to report that patients who were homozygous for Arg389 had a significantly greater improvement in LVEF after treatment with BB than patients who were homozygous for Gly389 (+8.7% ± 1.1% versus +0.93% ± 1.7%, respectively; $p < 0.02$). Patients who were heterozygous at position 389 had a similar improvement in LVEF compared to Arg389 homozygotes (7.02% ± 1.5%). This association was confirmed in three prospective studies totaling 345 patients among a variety of etiologies (ischemic and non-ischemic), BB (metoprolol and bisoprolol), and ethnic groups (Caucasian, AA, and Chinese) (41,44,45). However there are also three studies totaling 416 patients that failed to find a significant association (34,59,60). Given that the series of studies investigating LVEF response were small, it is difficult to conclude if Arg389Gly is a good predictor of LVEF response to BB. Notably, the previous, positive LVEF studies are consistent for the beneficial variant (Arg389). If the previous positive findings were purely spurious, spurious findings for the Gly389 variant would be expected as well. The data on UNITE-DNA presented herein cannot weigh into this issue because UNITE-DNA only had baseline LVEF data available. However, LVEF was similar among Arg389Gly genotype groups in UNITE-DNA at baseline (data not shown).

Liggett et al conducted a pharmacogenetic sub-study with a survival endpoint utilizing patients from the Beta-Blocker Evaluation of Survival Trial (BEST) (61). BEST was a randomized, placebo-controlled trial of the investigational beta antagonist bucindolol, which found that bucindolol did not significantly decrease mortality in HF patients overall (HR = 0.90; adjusted p = 0.13). However in the pharmacogenetic sub-study of 1040 patients (62), these investigators found survival benefit from bucindolol varied by genotype. Patients homozygous for Arg389 had a statistically significant improvement in survival compared to placebo (HR = 0.62; p = 0.03), whereas Gly389 carriers did not (HR = 0.90; p = 0.57). Importantly, these results do not seem to apply to BB currently used to treat HF.

White et al performed a pharmacogenetic sub-study (63) consisting of 600 patients from MERIT-HF (64), a randomized, controlled trial for the effectiveness of metoprolol CR/XL in chronic HF. They did not find an association of Arg389Gly with the primary outcome of all-cause mortality or hospitalization in either the metoprolol CR/XL or placebo treated groups. Cresci et al also found no association of Arg389Gly genotype with all-cause mortality in a prospective registry of two independently recruited U.S. HF populations where baseline BB therapy, if utilized, was predominantly metoprolol or carvediolol (65). These findings are also consistent with the lack of association of Arg389Gly genotype with mortality described by Sehnert et al in a prospective registry study of 637 patients that were all treated with BB (66). Consistent with these other studies, the UNITE-DNA data presented herein also does not support a pharmacogenetic interaction between Arg389Gly and currently used BB. Only a small study of 201 HF patients with a limited number of events reported by Biolo et al found

results consistent with Liggett et al, where metoprolol and carvedilol appeared to be more effective at high doses in decreasing HF-related mortality in patients carrying the Arg389 allele (67).

The discrepant pharmacogenetic results for bucindolol and the other currently used BB are most likely related to the unique pharmacological properties of bucindolol. Unlike currently used BB for HF, bucindolol has the additional pharmacologic property of marked sympatholysis, which contributed to the lack of mortality benefit in BEST (68). The marked sympatholysis with bucindolol is due to presynaptic beta-2 adrenergic receptor blockade unopposed by potent alpha adrenergic receptor blockade, which does not occur with carvedilol, metoprolol, or bisoprolol. Further supporting a bucindolol-specific Arg389Gly interaction, Liggett et al demonstrate in an *ex vivo* experimental model that the effects of the Arg389Gly variant differs by BB (62), and the *ex vivo* model is consistent with clinical findings. Specifically, bucindolol produced a significant, negative inotropic effect in Arg389-homozygous hearts, but not in Gly389-carrying hearts, which is consistent with the clinical finding that only Arg389-homozygous patients, and not Gly389-carriers, derive survival benefit from bucindolol. Accordingly, the *ex vivo* model demonstrated a similar negative inotropic response to carvedilol in Arg389-homozygous and Gly389-carrier hearts, which is consistent with the previous clinical findings and UNITE-DNA herein that Arg389-homozygotes and Gly389-carriers derive similar survival benefit from carvedilol. According to the MERIT-HF pharmacogenetic sub-study and UNITE-DNA herein, this may also be applicable to metoprolol (*ex vivo* experimental data for metoprolol not available).

***ADRB2* Gly16Arg and BB response**. In HF, chronic adrenergic stimulation causes down-regulation of the beta-1 adrenergic receptor but not the beta-2 adrenergic receptor. This causes a change in the ratio of beta-1:beta-2 from approximately 80:20 in healthy heart tissue to approximately 60:40 in the failing heart (69). Therefore the use of beta-1 selective versus non-selective BBs in HF remains a clinical issue. Although the density of the beta-2 adrenergic receptor in HF is unchanged compared to beta-1, the beta-2 receptor is subject to desensitization via functional uncoupling from the intracellular G protein, Gs (56). A glycine (Gly) at amino acid position 16 results in increased agonist-promoted desensitization compared to arginine (Arg) (70). The pharmacogenetic interaction between this variant and BB has not been studied *in vitro.* However it has been hypothesized that because Gly16 allows for greater desensitization of the beta-2 receptor, HF patients possessing Gly16 have "genetic beta-blockade." "Genetic beta-blockade," or the lack thereof, may interact with exogenously administered BB.

Six clinical studies tested the Gly16Arg variant in 738 HF patients (34,43,44,60,71,72), and none found a significant association between Gly16Arg and BB response with respect to BB tolerability, LVEF, or LVFS. Importantly, in three of these studies, the patients received beta-1 selective BB, which could have limited the power to detect a pharmacogenetic interaction with Gly16Arg. Acknowledging this limitation, and the negative results in UNITE-DNA, it seems unlikely that this variant could have a clinically meaningful pharmacogenetic interaction with BB.

***ADRB2* Gln27Glu and BB response**. A glutamine (Glu) at amino acid position 27 in the beta-2 adrenergic receptor is resistant to agonist-promoted desensitization (70),

and in contrast to Gly16Arg, there is ventricular remodeling data to support a pharmacogenetic interaction with BB. Although the pharmacogenetic interaction has not been studied *in vitro*, it has been hypothesized that patients with Glu27 will be more responsive to BB because they have more sensitive beta-2 receptors. Indeed, this has been confirmed in three clinical studies evaluating LVEF changes. Kaye et al were the first to report this pharmacogenetic interaction in a retrospective study of 80 HF patients on at least 4 months of carvedilol (72). They defined good responders as having an increase in LVEF of at least 10%, or an increase in LVFS of at least 5%. Patients homozygous for Gln27 had a significantly lower proportion of good responders than patients who were carrying Glu27 (26% versus 63%, $p = 0.003$). These findings were confirmed in two prospective studies by Troncoso et al (73) and Metra et al (60), which totaled 216 patients with systolic dysfunction and receiving carvedilol. Troncoso et al and Metra et al also found that Glu27 was associated with a favorable BB response in other parameters such as heart rate (73), malondialdehyde levels (a marker of oxidative stress) (73), and pulmonary wedge pressure both at rest and peak exercise (60). However, there are four studies that did not find a significant association between Gln27Glu and ventricular remodeling response to BB (34,41,43,44), and there is no previous data on this variant and BB survival benefit. Ventricular remodeling is not a perfect surrogate for survival, and therefore according to the UNITE-DNA data herein, the Gln27Glu pharmacogenetic interaction may be limited to ventricular remodeling differences that do not translate into survival differences.

***ADRA2C* indel and BB response.** The function of the alpha-2C adrenergic receptor is pre-synaptic auto-inhibition of norepinephrine release. An indel variant in

ADRA2C results in a four amino acid loss at positions 322-325. The deletion results in the loss of normal auto-inhibitory receptor function and hence increased presynaptic release of norepinephrine (74). Although not studied *in vitro*, it is possible that the deletion is associated with BB response, especially when it is inherited with other genetic variants affecting sympathetic activity. For example, HF patients with *ADRB1* Arg389 (with increased agonist-promoted activity) and the *ADRA2C* deletion (with increased presynaptic release of norepinephrine) could have enhanced beta-adrenergic receptor activity and hence greater response to beta-blockade.

Lobmeyer et al investigated the possible interaction between *ADRB1* Arg389, the *ADRA2C* indel, and BB response in 54 HF patients with systolic dysfunction (75). The deletion carriers had an increased improvement in LVEF compared to insertion homozygotes (+6% versus +1%; $p = 0.045$). Synergy between the *ADRB1* and *ADRA2C* variants was supported by the magnitude of results, in that patients both homozygous for Arg389 and a deletion carrier exhibited the greatest LVEF response compared to all other genotypes (+12% versus +2% as the greatest change in all other genotypes; $p < 0.05$ for all comparisons). Nonen et al also investigated ventricular changes (LVFS) in response to BB and *ADRA2C* status in 80 IDC patients, but did not find a significant association (43). Therefore like the UNITE-DNA results, the Nonen et al data may indicate that the *ADRA2C* indel is not meaningful for BB response alone, but according to the Lobmeyer et al data, it may be important in combination with *ADRB1* Arg389Gly.

The complexity of adrenergic regulation through *ADRA2C* was highlighted in a pharmacogenetic sub-study consisting of 1040 patients from BEST (61) by Bristow et al (76). Although Lobmeyer et al found that deletion carriers experienced greater LVEF

improvement with BB, Bristow et al found that deletion carriers did not experience survival benefit from beta-blockade (HR = 1.09; p = 0.80) (76). However Bristow et al found that the insertion homozygous patients experienced survival benefit (HR = 0.70; p = 0.025). Importantly, the BB investigated by Bristow et al was bucindolol. Bristow et al previously showed that the marked sympatholysis caused by bucindolol results in increased mortality and HF hospitalizations compared to patients with little or no sympatholytic response (68). Indeed, this was the case in deletion-carriers. In bucindolol-treated patients, a comparison of homozygous *ADRA2C* insertion and deletion carriers revealed that deletion carriers had a 3.1-fold greater reduction in norepinephrine (p = 0.001). As described above, marked sympatholysis is unique to bucindolol; therefore the pharmacogenetic interaction between the *ADRA2C* indel and bucindolol survival benefit may not be applicable to other BB, and this idea is supported by the negative results for a pharmacogenetic interaction in UNITE-DNA.

***GRK5* Gln41Leu and BB response**. The function of the G-protein receptor kinases is to desensitize ligand-occupied G-protein coupled receptors such as beta-adrenergic receptors (77). The Leu41 allele more effectively desensitizes agonist-stimulated responses (78). Because HF patients with Gln41 may have more sensitive beta-adrenergic receptors, it has been hypothesized that HF patients with Gln41 would have a greater response to beta-blockade. Although not studied *in vitro*, this potential pharmacogenetic interaction has been examined both retrospectively and prospectively in HF patients (78).

In a case-control study, Liggett et al found a significant pharmacogenetic interaction, but only in the AA sub-group (n = 242) and not in European-Americans (n =

568). They then confirmed these findings in a prospective, observational study of a second cohort of 375 AAs with HF, where they found that only individuals who were homozygous for Gln41 had significantly improved transplant-free survival with BB treatment (HR = 0.22; $p < 0.001$). There was no difference in this outcome in patients carrying Leu41 with or without BB (HR = 0.78; p = 0.53). Cresci et al found similar results in a cohort of AA HF patients (65). In the overall cohort, there was a trend for a BB treatment effect (HR = 0.698; p = 0.1). However in a sub-group of *ADRB1* Gly389 homozygous/*GRK5* Gln41 homozygous AAs, BB did provide mortality benefit (HR: 0.385; p = 0.012). When these investigators matched AAs and Caucasians by *ADRB1* Gly389 homozygous/*GRK5* Gln41 homozygous genotype and BB treatment, survival was similar in the two races. These findings must be considered with some caution due to the limited number of events in the first prospective cohort and the overlapping composition of the study populations in the Liggett et al and Cresci et al cohorts.

The previous literature indicates that the *GRK5* Gln41Leu pharmacogenetic interaction with BB is limited to AAs. The primary hypothesis in this chapter covered the entire UNITE-DNA cohort, but the exploratory analyses, which included race stratification, still did not replicate the previous findings in AAs. The Liggett et al, Cresci et al, and the UNITE-DNA data herein all come from well-executed pharmacogenetic studies; therefore it is difficult to determine the true pharmacogenetic interaction of *GRK5* Gln41Leu with BB. There are some important differences between the previous *GRK5* literature and UNITE-DNA that may explain the discrepancy in results. There may be regional differences in the pharmacogenetic interaction. The Liggett et al and Cresci et al patients were predominantly from Cincinnati, whereas the UNITE-DNA patients are

predominantly from the southeastern U.S. Indeed global regional differences in BB response have been reported (79). The data supporting the *GRK5* Gln41Leu and BB pharmacogenetic interaction is only significant in AAs. The genetic admixture of the AAs in Cincinnati may be different than in the southeastern U.S., but ancestry-informative markers are not available in UNITE-DNA. The Liggett et al and Cresci et al studies only included HF patients with systolic dysfunction. If the pharmacogenetic interaction is only applicable to patients with systolic dysfunction, then the inclusion of HF patients with preserved ejection fraction in UNITE-DNA may limit the ability to detect the association.

RAAS variants and BB response. Although BB are deemed adrenergic antagonists, biologic plausibility supports a pharmacogenetic interaction between BB response and RAAS variants as well as the SNS variants. The rate-limiting step in the cascade of enzymatic events leading to the formation of angiotensin II is the release of renin. The release of renin is increased with increased SNS activity (Figure 2), and treatment of HF patients with BB results in marked suppression of renin levels (80). The inhibition of the RAAS by BB is one of the potential mechanisms of BB survival benefit. Because BB inhibit the rate limiting step of the RAAS cascade and the RAAS plays a major role in the pathophysiology of HF, functional RAAS variants were also tested for a pharmacogenetic interaction with BB response in addition to SNS variants. However the largely negative results for the RAAS variants do not support this hypothesis. It is possible that BB inhibition of the RAAS does not play a large enough role in the mechanism of BB survival benefit in HF patients for a pharmacogenetic interaction to be detected or meaningful. Except for the *ACE* indel, previous studies testing a

pharmacogenetic interaction between RAAS variants and BB response have not been published. Therefore, the negative results for the other four RAAS variants (i.e. *AGT* G-6A, *AGTR1* A1166C, *CYP11B2* T-344C, and the *BDKRB2* indel) in UNITE-DNA are novel and cannot be placed in the context of any available literature. Although Chapter II summarizes the functional effects of these RAAS variants and their association with HF patient physiologic and clinical outcomes, which led to the general hypothesis for a BB pharmacogenetic interaction.

The role of *ACE* in the RAAS is to convert angiotensin I to angiotensin II, resulting in downstream effects including sodium and water retention and vasoconstriction. Since its discovery, the 287 base pair indel in intron 16 of *ACE* has been the most studied cardiovascular-relevant variant. The *ACE* indel accounts for half of the variance in serum ACE levels (2), with the deletion allele conferring significantly higher levels. Because the *ACE* deletion results in higher RAAS activity and BBs decrease RAAS activity, this led to the specific hypothesis that HF patients with the *ACE* deletion would have a greater response to BB. The literature testing this hypothesis is mixed, in that two studies support this hypothesis (13,81), while a single study (82) and the UNITE-DNA data presented herein do not.

In 2001, McNamara et al were the first to publish the pharmacogenetic interaction between BB and the *ACE* indel in a cohort of 328 HF patients followed for a median 21 months (13). In the overall cohort, there was a trend for increased transplant-free survival in patients receiving BB ($p = 0.065$). However when *ACE* indel sub-groups were analyzed individually, only patients homozygous for the deletion had a significant improvement in transplant-free survival from beta-blockade (deletion homozygous: p =

.007; insertion homozygous: p = 0.74; heterozygous: p = 0.59). These results were validated in another study published by McNamara et al in 2004 (81), when the size of the same cohort increased from 328 to 479. de Groote et al also tested this pharmacogenetic interaction in 199 HF patients, but the study did not yield a significant result with respect to LVEF, peak VO_2, or cardiac survival (82).

In UNITE-DNA, there was no association between the *ACE* indel and BB survival benefit. The negative results in UNITE-DNA are surprising because this research is very similar to the studies by McNamara et al. For example, the study designs by McNamara et al are also prospective, observational cohorts recruited from a HF specialty clinic, and the enrollment period was similar to UNITE-DNA (1996-2001 in McNamara et al vs. 2000-2002 in UNITE-DNA). The patients in UNITE-DNA and the McNamara et al studies had similar anti-RAAS treatment rates (~90%) and minor allele frequency of the *ACE* indel (~44% for the insertion allele). The BB treatment rate was much lower in the McNamara et al studies (~37% in McNamara et al vs. 67% in UNITE-DNA), but the distribution of specific BB was similar and also BB doses. The association between BB and mortality reduction overall was also similar between UNITE-DNA and the McNamara et al studies (~30% reduction).

However, there are several important differences between the UNITE-DNA study herein and the studies by McNamara et al that could potentially explain the discrepant results. The studies by McNamara et al were from a single center, the University of Pittsburgh, and therefore their results may only be applicable to that particular patient population. Indeed, the *ACE* indel pharmacogenetic interaction was not replicated in the multicenter UNITE-DNA (predominantly from the southeastern U.S.) or by de Groote et

al (82) (a single center study in France). Notably, the de Groote et al study was smaller than both the UNITE-DNA and McNamara et al cohorts, and de Groote et al did not report a power calculation. McNamara et al only enrolled patients with systolic dysfunction, but the type of cardiac dysfunction is not available for UNITE-DNA. Therefore, including patients without systolic dysfunction in UNITE-DNA may limit the ability to detect the pharmacogenetic interaction if the interaction is driven by patients with systolic dysfunction only. This is plausible since the large BB clinical trials did not demonstrate survival benefit in HF patients with preserved ejection fraction. The McNamara et al cohorts also had a higher percentage of men (75% vs. 63%), less AAs (9% vs. 37%), and lower baseline LVEF (24% vs. 32%). Interestingly, the analyses in the McNamara et al studies were not adjusted for clinical covariates (only a log-rank test was used). Baseline differences existed between the BB treatment groups (e.g. LVEF and the number of women) in the McNamara et al studies, and therefore these differences should be adjusted for in the survival analysis. Whether the *ACE* indel and BB pharmacogenetic interaction found by McNamara et al would remain significant despite covariate adjustments is unknown. Notably, the univariate UNITE-DNA analysis did not reveal a signal for this pharmacogenetic interaction. The UNITE-DNA data presented herein is more definitive than the McNamara et al studies because it is multicenter, and there is substantially more power due to longer follow-up and sample size. Therefore according to the UNITE-DNA and de Groote et al data, it is unlikely that the *ACE* indel and BB pharmacogenetic interaction would be applicable to other HF patient populations than those studied by McNamara et al.

Table 21. Independent association of 11 candidate genetic variants and 2 haplotypes with survival.

Risk allele	HR	95% CI lower	95% CI upper	*p-value
ADRB1 Ser49	0.79	0.62	0.99	0.0461
ADRB1 Arg389	0.88	0.71	1.11	0.2769
ADRB2 Arg16	1.00	0.79	1.27	0.9797
ADRB2 Glu27	1.08	0.86	1.36	0.4976
GRK5 Gln41	0.92	0.67	1.26	0.6070
ADRA2C Del	1.24	0.98	1.58	0.0782
ACE Del	0.98	0.77	1.24	0.8542
AGT -6A	1.31	1.03	1.65	0.0252
AGTR1 1166C	0.89	0.71	1.12	0.3237
CYP11B2 -344C	0.84	0.67	1.05	0.1224
BDKRB2 Ins	1.16	0.91	1.48	0.2356
ADRB1 diplotype Ser49/Arg389	0.69	0.53	0.90	0.0065
ADRB2 diplotype Arg16/Glu27	0.76	0.45	1.29	0.3085

*Bonferroni-adjusted alpha = 0.0038

Table 22. Ten lowest p-values in the exploratory analyses for an association with survival and beta-blocker survival benefit prior to and after correction for 5% FDR modified for dependency.

Survival			BB survival benefit		
Test	*Raw p	FDR corrected p	Test	*Raw p	FDR corrected p
Variant: *ADRB1* Ser49Gly Model: Dominant Stratum: BB-treated	0.0004	0.4989	Variant: *ACE* indel Model: Recessive Stratum: Ischemic etiology	0.0002	0.193
Variant: *ADRB1* Ser49Gly Model: Additive Stratum: BB-treated	0.0009	0.4989	Variant: *ADRB1* Ser49Gly Model: Dominant Stratum: Non-ischemic	0.0028	1
Variant: *ADRB1* Ser49-Arg389 Model: 2 copies vs. <2 copies Stratum: BB-treated	0.0010	0.4989	Variant: *ADRB1* Ser49Gly Model: Additive Stratum: All patients	0.0044	1
Variant: *ADRB1* Ser49-Arg389 Model: 2 vs. 1 vs. 0 Stratum: BB-treated	0.0014	0.5089	Variant: *ADRB1* Ser49Gly Model: Additive Stratum: Non-ischemic	0.0053	1
Variant: *AGT* G-6A Model: Additive Stratum: Ischemic etiology	0.0017	0.5089	Variant: *ACE* indel Model: Additive Stratum: Ischemic etiology	0.0066	1
Variant: *AGT* G-6A Model: Dominant Stratum: Ischemic etiology	0.0021	0.5132	Variant: *ADRB1* Ser49-Arg389 Model: 2 vs. 1 vs. 0 copies Stratum: Ischemic	0.0168	1
Variant: *GRK5* Gln41Leu Model: Recessive Stratum: Not BB-treated	0.0024	0.5132	Variant: *BDKRB2* indel Model: Recessive Stratum: Non-ischemic	0.0195	1

Variant: *GRK5* Gln41Leu Model: Recessive Stratum: Non-AA	0.0076	1	Variant: *ADRB1* Ser49Gly Model: Dominant Stratum: AA	0.0223	1
Variant: *AGTR1* A1166C Model: Recessive Stratum: Not BB-treated	0.0099	1	Variant: *AGTR1* A1166C Model: Additive Stratum: Non-AA	0.0279	1
Variant: *CYP11B2* T-344C Model: Dominant Stratum: BB-treated	0.0128	1	Variant: *ADRB1* Ser49-Arg389 Model: 2 vs. 1 vs. 0 copies Stratum: All patients	0.0312	1

*Adjusted for reduced model covariates but not for multiple comparisons

Table 23. Adjusted interaction between 11 candidate genetic variants and 2 haplotypes and BB survival benefit.

Risk allele	*p-value
ADRB1 Ser49	**0.0018**
ADRB1 Arg389	0.8542
ADRB2 Arg16	0.5318
ADRB2 Glu27	0.1427
GRK5 Gln41	0.2754
ADRA2C Del	0.6272
ACE Del	0.6959
AGT -6A	0.6823
AGTR1 1166C	0.6236
CYP11B2 -344C	0.0962
BDKRB2 Ins	0.9871
ADRB1 diplotype Ser49/Arg389	0.0309
ADRB2 diplotype Arg16/Glu27	0.8525

*Bonferroni-adjusted alpha = 0.0077

Table 24. Baseline characteristics by Ser49Gly genotype

	Pooled n = 722	Ser49Ser n = 491	Ser49Gly + Gly49Gly n = 228	p-value
Male gender n(%)	455 (63.1%)	312 (63.5%)	140 (61.7%)	0.6295
Age mean(sd)	57.0 (13.2)	56.8 (13.5)	57.5 (12.6)	0.5073
Race (AA)	270 (38%)	159 (33%)	111 (49%)	**<.0001**
NYHA functional class mean(sd)	2.50 (0.64)	2.50 (0.65)	2.51 (0.60)	0.8587
Ischemic etiology n(%)	314 (44.5%)	212 (44.1%)	101 (45.5%)	0.7246
Hx of hypertension n(%)	488 (68.0%)	317 (64.8%)	169 (74.8%)	**0.0080**
Hx of diabetes n(%)	233 (32.5%)	162 (33.3%)	70 (30.8%)	0.5189
SBP mean(sd)	119 (22)	119 (22)	121 (23)	0.3321
DBP mean(sd)	70 (13)	70 (12)	71 (14)	0.4802
HR mean(sd)	75 (14)	75 (14)	76 (14)	0.3386
LVEF mean(sd)	32 (15)	32 (15)	32 (16)	0.7837
Serum creatinine mean(sd)	1.47 (1.37)	1.43 (1.35)	1.58 (1.44)	0.1856
Estimated GFR (ml/min)	66.3 (26.5)	67.1 (26.7)	64.7 (26.3)	0.2739
BB n(%)	484 (67.4%)	331 (67.8%)	152 (67.0%)	0.8176
ACE inhibitor n(%)	577 (80.8%)	401 (82.5%)	174 (77.3%)	0.1026
ACE inhibitor and ARB n(%)	13 (1.9%)	9 (1.9%)	4 (1.8%)	1.0000
ACE inhibitor or ARB n(%)	639 (89.5%)	440 (90.5%)	196 (87.1%)	0.1669
Loop diuretic n (%)	603 (84.0%)	408 (83.6%)	193 (84.6%)	0.6303
Any diuretic n (%)	635 (88.4%)	434 (88.9%)	199 (87.7%)	0.6200
Digoxin n(%)	516 (72.0%)	353 (72.5%)	161 (70.9%)	0.6656
Spironolactone n (%)	172 (24.1%)	118 (24.4%)	54 (23.8%)	0.8636
Days of follow-up mean(sd)	2410 (1288)	2453 (1263)	2330 (1332)	0.2356
Number of deaths (%)	336 (46.5%)	223 (45.4%)	111 (49.7%)	0.4138

Table 25. Propensity-matched and propensity-adjusted Ser49Gly-stratified BB response.

Propensity-matched (n = 406)				
Genotype	HR	95% CI lower	95% CI upper	p-value
Ser49-homozygotes (n = 279)	0.59	0.42	0.83	0.0025
Gly49-carriers (n = 125)	1.45	0.85	2.48	0.1767
Propensity-adjusted (n = 719)				
Genotype	HR	95% CI lower	95% CI upper	p-value
Ser49-homozygotes (n = 491)	0.58	0.43	0.78	0.0003
Gly49-carriers (n = 228)	1.20	0.75	1.93	0.4472

Table 26. Stratified and contrast-derived HR for BB in Ser49Gly genotypes adjusted with reduced clinical model.

BB as dichotomous variable (on BB vs. off BB)				
Stratified				
Genotype	HR	95% CI lower	95% CI upper	p-value
Ser49-homozygotes	0.54	0.41	0.72	<.0001
Gly49-carriers	1.38	0.89	2.12	0.1462
Contrast				
Genotype	HR	95% CI lower	95% CI upper	p-value
Ser49-homozygotes	0.57	0.43	0.75	<.0001
Gly49-carriers	1.26	0.83	1.91	0.2859
BB dose as continuous variable (25mg metoprolol equivalent increase)				
Stratified				
Genotype	HR	95% CI lower	95% CI upper	p-value
Ser49-homozygotes	0.92	0.87	0.97	0.0012
Gly49-carriers	1.01	0.96	1.07	0.6990
Contrast				
Genotype	HR	95% CI lower	95% CI upper	p-value
Ser49-homozygotes	0.92	0.88	0.97	0.0010
Gly49-carriers	1.01	0.96	1.06	0.6798

Figure legends

Figure 7. Survival curves for UNITE-DNA derived from the Cox proportional hazards model adjusted for age, diabetes, systolic blood pressure, NYHA class, LVEF, and GFR are shown and were stratified by Ser49Gly genotype (Ser49-homozygous and Gly49-carriers) and BB treatment (treated or not treated with BB at baseline).

Figure 8. The linear association between BB dose and reduction in mortality derived from the Cox proportional hazards model adjusted for age, diabetes, SBP, NYHA class, LVEF, and GFR is shown by 25, 50, 100, 150, and 200mg of metoprolol dose equivalents and stratified by Ser49Gly genotype.

Figure 7. Adjusted survival curves stratified by Ser49Gly genotype and BB treatment.

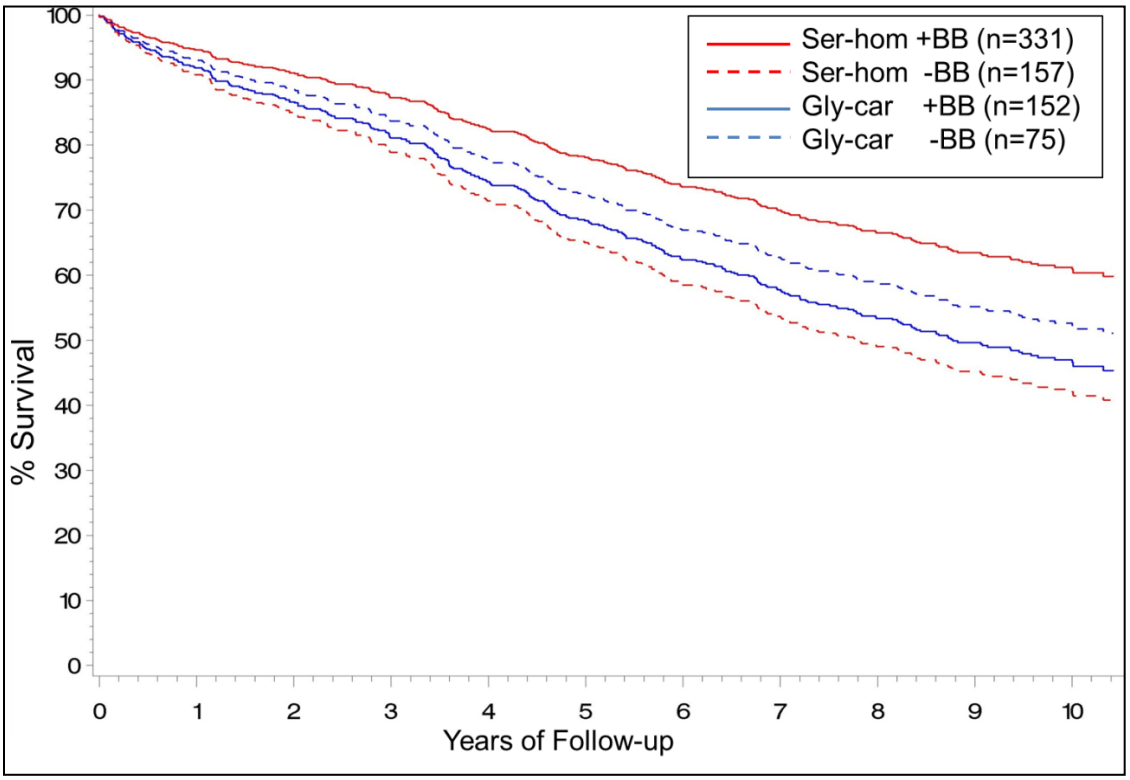
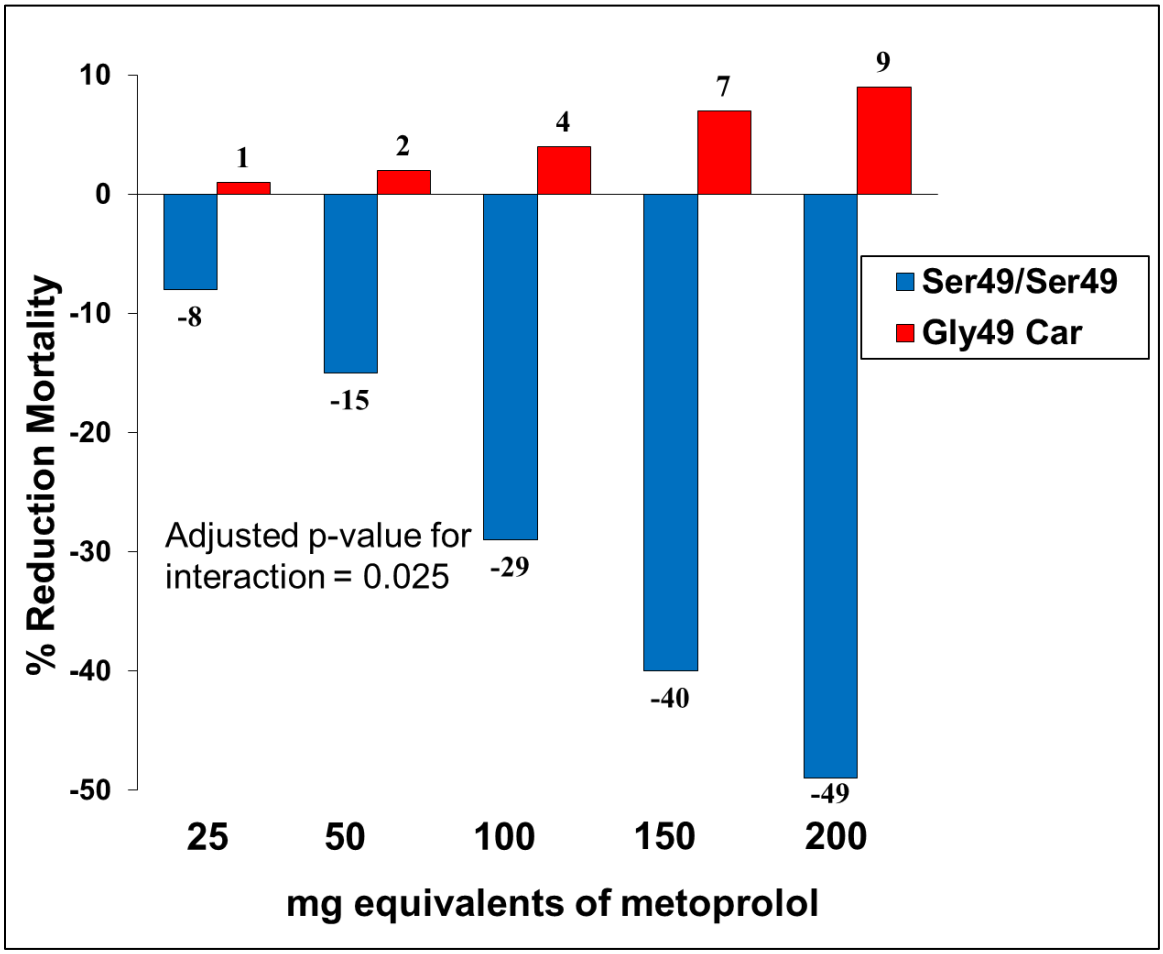


Figure 8. Adjusted BB dose-associated survival benefit by Ser49Gly genotype.



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CHAPTER VI:
GENETIC RISK SCORES ASSOCIATED WITH HF PATIENT SURVIVAL AND BETA-BLOCKER RESPONSE

Summary

The previous chapter covered the association of the individual genetic variants with HF survival and BB survival benefit. The goal of this chapter is to assess the additive association of genetic variant combinations, via genetic risk scores (GRS), with HF patient survival and BB survival benefit. Neither the simple nor internally-weighted genetic risk scores were associated with HF patient survival or BB survival benefit. The genetic risk scores also did not add to the predictability of clinical risk factors for mortality or reclassify HF patients to new mortality risk categories.

Introduction

Common, complex diseases such as HF are influenced by multiple genetic and environmental factors. The importance of genetic factors in HF is exemplified by the heritability of HF risk factors (e.g. hypertension [1], increased left ventricular mass [2], and coronary heart disease[3]), the increased risk of HF in children of parents with HF (4), and the individual associations of genetic variants with HF patient survival (5-9) and drug response (Appendices I & II) (10,11). However, as the number and prevalence of genetic risk factors increases, the contribution of each individual genetic variant decreases. For example, in another multifactorial disease such as diabetes, eight

statistically significant genetic variants in a GWAS only explained 0.04% to 0.5% of the total variance in diabetes risk (12). Moreover, common genetic variants (i.e. minor allele frequency greater than 5%) have modest associations with clinical outcomes in common, complex diseases; the odds ratios typically range from 1 to 2 (13). An example specific to HF would be the 287-bp intron 16 deletion in *ACE*; in a study of 193 HF patients, homozygotes for the deletion had an adjusted odds ratio of 1.69 for death compared to all other patients (5). This example of the modest risk associated with individual common genetic variants limits their clinical application. However, common genetic variants can be aggregated into a genetic risk score (GRS), with the expectation that a high GRS would be sufficient to influence clinical decision-making.

GRS models are evolving, but currently there are four general GRS models in the literature (14): simple (15,16), internal weighted (17), external weighted (18), and polygenic (19). The simple GRS model is the sum of all risk alleles (0, 1, or 2) for all variants, and only involves two parameters in the model:

$$simple\ GRS = \sum_{i=1}^{I} SNP_i$$

$$y = \beta_0 + \beta \sum_{i=1}^{I} SNP_i$$

The simple GRS has the advantage of easier application in clinical practice because it does not involve complicated calculations. A disadvantage of the simple GRS is that it gives equal weight to each variant, which is unlikely to be biologically plausible and may not accurately capture the true genetic risk contribution from each variant. This can be overcome by weighting the variants, which is generally calculated by multiplying the number of risk alleles for each variant by the weight for that variant, and then taking the

sum across all variants. The weight for each variant can be derived from internal or external data. The internal weighted GRS uses the beta coefficients directly from the original dataset model:

$$internal\ weighted\ GRS = \sum_{i=1}^{I} \beta_i SNP_i$$

$$y = \beta_0 + \sum_{i=1}^{I} \beta_i SNP_i$$

A disadvantage of this method is the number of parameters in the model is equal to the number of variants +1. The major disadvantage of using an internal weighted GRS is the bias inherent in using the same dataset to both create and test a GRS model. This bias can be overcome by using an external weighted GRS:

$$external\ weighted\ GRS = \sum_{i=1}^{I} w_i SNP_i$$

$$y = \beta_0 + \beta \sum_{i=1}^{I} w_i SNP_i$$

The weight for each variant is usually the log odds ratio calculated from an independent GWAS or meta-analysis of the SNP. This model, like the simple GRS, only has two parameters. The disadvantage of the external weighted GRS is that GWAS or meta-analysis data is not always available for the genetic variants, and the patient population from which the weight is derived may differ from the patient population in which it is tested. A disadvantage that is common to all three GRS methods discussed thus far (simple, internal weighted, and external weighted) is that they assume an additive association, in which heterozygotes have intermediate risk between low-risk

homozygotes and high-risk homozygotes. This disadvantage can be overcome using a polygenic GRS model:

$$polygenic\ GRS = \sum_{i=1}^{I} \beta_{i1} x_{i1} + \sum_{i=1}^{I} \beta_{i2} x_{i2}$$

$$y = \beta_0 + \sum_{i=1}^{I} \beta_{i1} x_{i1} + \sum_{i=1}^{I} \beta_{i2} x_{i2}$$

Briefly, two dummy variables are considered for each variant, so ultimately the three possible genotypes are coded as a three-level class variable (14). The polygenic GRS does not assume an additive risk model, and therefore it is more flexible if the underlying genetic model is unknown. The major disadvantage of the polygenic GRS is that the number of parameters is dramatically increased in the model (2 x number of variants + 1), which can lead to over-fitting and loss of power. Currently, the best GRS model is unknown, but the appropriate GRS model selection may depend on a number of factors: the number of variants in the panel, sample size, number of covariates, underlying inheritance models, variant effect sizes, etc (14).

These GRS models have been applied in a variety of common, complex diseases such as prostate cancer (20), coronary heart disease (21,22), type 2 diabetes (23), and primary cardiovascular events (15). These are successful examples in which the patients with a high GRS had significantly higher risk for clinical outcome compared to patients with a low GRS, and the association was stronger than with the individual variants alone. Notably, the study by Kathiresan *et al* (15) used genetic variants associated with an intermediate phenotype (i.e. LDL), which when aggregated were significantly associated with a clinical phenotype (i.e. primary cardiovascular events). This logic may also translate to HF, in which the aggregate of multiple genetic variants affecting intermediate

phenotype (i.e. SNS and RAAS activity), could be associated with clinical phenotype (i.e. mortality) (24). Mechanistically this is plausible, as data from separate HF studies demonstrate that genetic variants individually can affect SNS and RAAS activity *in vitro* and are associated with clinical outcome. For example, an Arg389Gly substitution in the beta-1 adrenergic receptor (*ADRB1*) results in decreased coupling to the intracellular stimulatory protein Gs (25). This Arg389Gly *ADRB1* variant was associated with survival and beta-blocker response in HF patients (9). In another study, a Gln41Leu substitution in the G-protein coupled receptor kinase 5 (*GRK5*), which enhances beta-adrenergic receptor intracellular uncoupling, was also associated with HF patient survival and beta-blocker response (8). Because both of these variants affect beta-adrenergic intracellular coupling, which is necessary for receptor function, they could have an additive association with clinical outcome.

One may argue how a GRS could be useful in HF when the intermediate phenotype, SNS and RAAS activity, can be measured directly such as with plasma norepinephrine and angiotensin II levels. However there are several reasons for measuring a GRS instead of, or in addition to, plasma norepinephrine and angiotensin II levels. In Katherisan *et al* (15), a GRS composed of SNPs associated with LDL was associated with primary cardiovascular events *independent of* actual LDL levels. A possible explanation is that genotype represents life-long exposure to LDL, or in the case of HF it would be life-long exposure to SNS and RAAS activity. Genotype also represents activity at the intracellular and receptor levels, which are not represented by plasma levels. For example, although a HF patient's plasma norepinephrine levels could be elevated, it may not manifest adverse effects if the patient also possesses genetic

polymorphisms that decrease intracellular coupling to stimulatory G proteins (e.g. *ADRB1* Arg389Gly and *GRK5* Gln41Leu described above). In addition, plasma levels are dynamic, whereas genotype is stable from birth. Therefore the GRS can be measured early in life at stages where plasma norepinephrine and angiotensin II are normal. The GRS also has the advantage of only requiring a single measurement, and plasma levels of SNS and RAAS activity may require serial measurements, for example, before and after new therapies. Genotyping assays are easier and more accurate than bioanalytical assays because there are only a few possible discrete results, and genotyping also has the advantage of multiplexing. Most importantly, plasma levels are not an accurate reflection of SNS or RAAS activity because plasma levels are determined by both the rate of release and rate of clearance. The reduced blood flow to the kidneys that occurs in HF may falsely elevate plasma levels (26).

In addition to *ADRB1* Arg389Gly and *GRK5* Gln41Leu described above, several other common SNS and RAAS variants in the literature have functional effects and are associated with HF physiologic or clinical outcomes (see Chapter II: Identification of Candidate Genetic Variants). Because these variants are common, a given HF patient would be expected to possess multiple functional SNS and RAAS genetic variants. However, to date, no genetic association study in HF patients has integrated multiple genetic variants that affect SNS and RAAS activity into a clinical outcomes model. A HF GRS could have profound clinical implications. For example, a HF GRS could be used to identify patients who have increased genetic risk of mortality independent of clinical risk factors. It is also possible that HF patients with a high GRS may need additional pharmacotherapy compared to patients with low GRS.

Because of the potential advantages and clinical implications of the GRS in HF, the objective of this chapter was to test the independent association, additive predictability, and discriminative capability of a simple GRS for HF patient survival and BB response. The simple GRS model was chosen because this is the first application of the GRS method to HF; the easy calculation would facilitate clinical application; and the small number of parameters required in the final model. The external weighted GRS model could not be performed because there is no GWAS or meta-analysis data available for genetic variants in HF. The internal weighted GRS model was not chosen because of the increased number of parameters in the model and the bias inherent in creating and testing the model in the same patient population. The polygenic GRS model was not chosen because of the dramatically increased number of parameters in the model, and currently there is not a replication patient population to rule out over-fitting of the polygenic GRS model.

Methods

Genetic risk score calculation. The panel of 11 variants identified in Chapter II: Identification of Candidate Genetic Variants was used. Based on the molecular, physiologic, and clinical phenotypes in the previous literature, the assigned risk alleles have been summarized in Table 27. Only patients with complete genotypes for all 11 variants had a GRS calculated and were included in the analysis (n = 701). The GRS for each UNITE-DNA patient was calculated by summing the number of risk alleles (0, 1, or 2) for each variant, with the exception of the *ADRA2C* indel. The *ADRA2C* indel genotyping assay was unable to discriminate deletion homozygotes from heterozygotes

(see Chapter III: Characterization of Candidate Genetic Variants), and therefore insertion homozygotes had 0 added to their GRS and deletion-carriers had 1 added to their GRS. Therefore the range of possible GRS was 0 to 21.

Statistical analysis. Descriptive statistics for the GRS included mean, standard deviation, median, minimum, maximum, and quintiles. Baseline characteristics and cumulative incidence of death among GRS quintiles were tested using the Cochran-Armitage test for trend. The distributions of GRS in UNITE-DNA patients that died and did not die during the 10-year follow-up were plotted and mean GRS compared using Student's t-test. The GRS was analyzed as a continuous variable in a Cox proportional hazards model unadjusted and adjusted with the reduced model of clinical covariates (see Chapter IV: Characterization of Patient Population) for association with time to all-cause mortality. Cumulative incidence curves and the hazard ratio with 95% confidence intervals for GRS quintiles (with the lowest GRS quintile as the reference) were also derived from the Cox model. Receiver-operating-characteristic (ROC) curves were plotted for the clinical covariates with and without the GRS, with incidence of all-cause mortality at 10 year follow-up as the outcome. The C statistic, a measure of the area under the ROC curve, was calculated with and without the GRS, and was compared using a nonparametric approach (27). Net reclassification index and integrated discrimination improvement were also calculated with incidence of all-cause mortality at 10 year follow-up as the outcome (28). Secondary subgroup analyses included stratification by age (using median), race (AA and non-AA), etiology (ischemic and non-ischemic), and beta-blocker treatment (on BB at baseline and not).

To assess the association between GRS and BB response, multiplicative interaction terms (GRS*BB and GRS*BB dose) were incorporated into the Cox proportional hazards model unadjusted and adjusted with the reduced model of clinical covariates (see Chapter IV: Characterization of Patient Population). BB response in the highest GRS quintile was compared to the lowest GRS quintile. The alpha level of statistical significance for all tests was defined as 0.05, except where multiple comparisons were made and alpha was adjusted using a Bonferroni correction. All statistical analyses were performed using SAS v.9.2 (Cary, NC, USA).

Results

The number of patients with complete genotypes for all 11 variants was 701 out of 722. The mean ± sd, median, minimum, and maximum GRS were 11.2 ± 1.72, 11, 6, and 17, respectively, which were similar among all sub-groups (age [above and below median = 58 years], race [self-reported AA and non-AA], etiology [ischemic and non-ischemic HF], and beta-blocker treatment) (Table 28). Clinical characteristics, drug utilization, and vital status were similar among GRS quintiles (Table 29), except the percentage of AAs decreased as the GRS quintile increased. The distribution of GRS in alive (mean ± sd = 11.2 ± 1.70) and deceased patients (mean ± sd = 11.1 ± 1.75) were similar in the entire cohort (p = 0.5393; Figure 9) and all sub-groups (data not shown). The GRS was not associated with time to all-cause mortality in univariate models (data not shown) or multivariable models as a continuous variable (Figure 10) or in quintiles (Table 30), and this was similar among all clinical sub-groups. The area under the ROC

curves were similar for the clinical risk factors alone (area = 0.7489) versus the clinical risk factors plus GRS (area = 0.7487; p = 0.6550; Figure 11). The net reclassification index and integrated discrimination improvement were both equal to zero because none of the patients were reclassified by the addition of GRS to the clinical model.

An exploratory post-hoc analysis of an internal-weighted GRS using all 11 variants and a J48 decision tree-pruned (29) variant panel was performed. The HR for the internal-weighted GRS's was tested for external generalizability using 10-fold cross-validation and the 95% CI estimated using a bootstrap technique with 10,000 samples (30,31). The 10-fold cross-validated HR and bootstrapped 95% CI for an internal-weighted GRS using all 11 variants was HR = 0.74 and 95% CI = 0.28-1.35. The J48 decision tree did not prune any of the 11 variants, and hence a reduced internal-weighted GRS could not be carried out.

The BB*GRS adjusted interaction using GRS as a continuous variable was not statistically significant in all of the UNITE-DNA patients or any of the sub-groups (Table 31). However, when comparing BB response in the highest GRS quintile to the lowest, patients in the highest GRS quintile seemed to have a greater response to BB (n = 62; adjusted HR = 0.29; 95% CI = 0.12-0.71; p = 0.0070) compared to the patients in the lowest GRS quintile (n = 242; adjusted HR = 0.85; 95% CI = 0.56-1.30; p = 0.4618). There was not a statistically significant interaction between BB dose and GRS in all patients, but there was a statistically significant interaction between BB dose and GRS in the non-ischemic sub-group of patients (p = 0.0011) (Table 31). In the non-ischemic etiology patients, BB dose response improved as the GRS increased. In the highest GRS quintile non-ischemic patients (n = 30), every 25mg metoprolol equivalent increase in

dose yielded an adjusted HR = 0.64, 95% CI = 0.38-1.07, and p = 0.0885. However in the lowest GRS quintile non-ischemic patients (n = 130), every 25mg metoprolol equivalent increase in dose yielded an adjusted HR = 1.13, 95% CI = 1.03-1.24, and p = 0.0098.

Discussion

This is the first report to incorporate multiple genetic variants known to affect the SNS or RAAS into a clinical outcomes model for HF. The simple GRS was not independently associated with time-to-mortality. The simple GRS did not add to the predictability of clinical risk factors for mortality, nor did it reclassify HF patients into new mortality risk categories. Even when the internal-weighted GRS method was explored it was non-significant, and 10-fold cross-validation revealed that the internal-weighted GRS would not be generalizable to other patient populations. There are several possible explanations for these negative GRS results. Only a few variants within the complex physiologic systems such as the SNS and RAAS were included in the panel. There are many variants in the genes included in this panel that were not genotyped, and there are entire genes that were not represented in the panel (e.g. *ADRB3, SLC6A2*, *REN*, *NOS*). Many more variants may be required to adequately capture the genetic effects on the activity of these systems. Simulated studies of GRS demonstrate that the addition of much more common variants with small effect sizes or a few rare variants with large effect sizes may improve discrimination (32,33). Indeed, as many as 500 genetic loci may underlie the risk for another common complex disease, type 2 diabetes mellitus (34). It is also possible that system-level counter-regulatory mechanisms may neutralize adverse

genetic effects in the SNS and RAAS, such as arginine vasopressin, endothelin, natriuretic peptides, which were not included in this study.

A major limitation of this study is the lack of the direct measurement of the intermediate phenotype, e.g. plasma norepinephrine and angiotensin II levels. Therefore the individual variant associations with SNS and RAAS activity were not validated. Also, the individual associations between the variants and survival (see Chapter V: Assessment of Candidate Genetic Variants and Survival) were not validated. However the rationale for the GRS is that common variants will only have a modest (and perhaps undetectable) association with outcomes in common diseases, but in aggregate the association may be detectable. Indeed this is the case for examples in the literature: studies did not validate the individual association of the variants in the GRS, but when combined there was a significant association (24,35,36). Another possible limitation is that the risk allele designation was incorrect. The risk allele designations were based solely on previous literature and not associations determined in UNITE-DNA to prevent bias. However it is possible that the previous literature was false positives or exaggerated due to publication bias, but even the internal-weighted GRS was negative. Using the simple GRS model also has limitations. Specifically, each variant was given equal weight, which is not biologically plausible. However this limitation would be more important when using variants with highly variable effect sizes. For example, we only tested common variants, in which the expected associations in common diseases are usually odds ratios of 1 to 2 (13). This is exemplified in other common diseases where weighting the score did not substantially improve predictive value (36). The simple GRS model also assumes additive risk per allele. Notably, the additive genetic model performs well even when the

true genetic model is unknown or wrongly specified (37). There are also the limitations inherent to the registry study design (see Chapter IV: Characterization of Patient Population), and we were not able to discriminate *ADRA2C* deletion homozygotes from heterozygotes. Hence, only 1 was added for deletion-carriers. However due to the largely negative results, this is unlikely to have a large effect.

Given the sacrifice of power, the lack of association within all of the sub-groups was somewhat surprising. The sub-groups were chosen based on an increased theoretical likelihood for an association and on previous literature. Because the GRS represents possible long-term exposure to high SNS and RAAS activity, adverse effects of a high GRS, if present, would be expected to manifest in younger patients (35). Racial sub-groups were also considered because the frequency of the risk alleles varies by race. Because patients with an ischemic HF etiology may have irreversible damage to their myocardium, it is possible that the GRS would more likely have an association in patients with a non-ischemic etiology. Beta-blocker treatment sub-groups were analyzed because pharmacogenetic studies demonstrate that, in general, individual SNS and RAAS variants conferring higher activity are associated with increased beta-blocker response (Appendix I) (10).

Interestingly, BB response differed between the highest and lowest GRS, but the sample sizes are very small and thus must be considered exploratory. Although it is biologically plausible because the patients with the highest GRS would be expected to have the highest SNS and RAAS activation, and hence would derive the most benefit from SNS and RAAS inhibition with BB. This was statistically significant when looking at BB dose in non-ischemic patients. Given the very small sample size again, this is

biologically plausible as patients with ischemic etiology may have irreversible damage to the myocardium, and the beneficial ventricular remodeling response may not be as profound in patients with ischemic etiology compared to patients with non-ischemic. Of course this would need to be replicated in a prospective cohort.

Table 27. Designation of risk alleles for 11 variant panel used in GRS calculation

Gene	Variant	Literature-defined risk allele	References
ADRB1	Ser49Gly	Ser	(6,38-40)
	Arg389Gly	Arg	(9,25)
ADRB2	Gly16Arg	Arg	(41,42)
	Gln27Glu	Glu	(41,42)
ACE	Ins/Del	Del	(43,44)
ADRA2C	Ins/Del	Del	(45,46)
GRK5	Gln41Leu	Gln	(8)
AGT	G-6A	A	(47,48)
AGTR1	A1166C	C	(49-51)
CYP11B2	T-344C	C	(52,53)
BDKRB2	Ins/Del	Ins	(54-56)

Table 28. Mean ± sd, median, minimum, and maximum GRS in all patients and sub-groups

Group	Mean ± sd	Median	Minimum	Maximum
All patients	11.2 ± 1.7	11	6	17
Age < 58	11.3 ± 1.6	11	7	17
Age > 58	11.1 ± 1.8	11	6	17
Non-AA	11.4 ± 1.8	11	6	17
AA	10.8 ± 1.6	11	6	16
Ischemic etiology	11.3 ± 1.8	11	6	17
Non-ischemic etiology	11.1 ± 1.6	11	6	16
BB treated	11.2 ± 1.7	11	7	16
No BB	11.2 ± 1.7	11	6	15

Table 29. Baseline clinical characteristics, drug utilization, and vital status by GRS quintiles

Variable	1st quintile GRS = 0-10 (n=242;35%)	2nd quintile GRS = 11 (n=157;22%)	3rd quintile GRS = 12 (n=156;22%)	4th quintile GRS = 13 (n=84;12%)	5th quintile GRS = 14-17 (n=62;9%)	p-value
Male	168 (64%)	97 (62%)	96 (62%)	58 (69%)	36 (58%)	0.8188
Age	58 (13)	56 (13)	55 (14)	60 (12)	55 (13)	0.2225
African-Am	111 (42%)	66 (42%)	57 (37%)	27 (32%)	9 (15%)	0.0001
NYHA class	2.5 (0.6)	2.5 (0.7)	2.5 (0.6)	2.5 (0.6)	2.6 (0.7)	0.3397
Ischemic	115 (45%)	66 (43%)	59 (39%)	42 (51%)	32 (52%)	0.4137
Hx of HTN	180 (69%)	110 (71%)	104 (68%)	56 (67%)	38 (61%)	0.2925
Hx of DM	87 (34%)	41 (26%)	49 (31%)	34 (40%)	22 (35%)	0.3883
SBP (mmHg)	120 (22)	119 (23)	119 (21)	119 (19)	117 (24)	0.4019
DBP (mmHg)	70 (13)	71 (14)	71 (11)	69 (13)	68 (11)	0.1285
Heart rate (bpm)	76 (15)	74 (12)	77 (14)	74 (14)	72 (14)	0.0922
LVEF (%)	33 (14)	31 (16)	32 (17)	32 (16)	31 (15)	0.6481
GFR (ml/min)	65 (26)	68 (27)	68 (26)	65 (31)	65 (22)	0.9463
ACE inhibitor	210 (81%)	130 (83%)	122 (79%)	63 (77%)	52 (85%)	0.8623
ACE inhibitor *or* ARB	233 (90%)	140 (89%)	138 (89%)	72 (88%)	56 (92%)	0.9645
BB	177 (68%)	109 (69%)	102 (66%)	54 (64%)	42 (69%)	0.7690
BB dose (mg metop equiv)	105 (82)	119 (84)	106 (89)	115 (77)	105 (103)	0.7869
Loop diuretic	221 (84%)	133 (85%)	127 (82%)	71 (85%)	51 (85%)	0.9127
Digoxin	183 (70%)	114 (73%)	111 (73%)	62 (74%)	46 (75%)	0.3123
Spironolactone	61 (24%)	41 (26%)	35 (23%)	20 (24%)	15 (25%)	0.9881
Length of follow-up (days)	2393 (1307)	2345 (1339)	2477 (1262)	2486 (1299)	2471 (1090)	0.3257
Deaths n(%)	125 (48%)	70 (45%)	73 (47%)	42 (50%)	26 (42%)	0.7772

Table 30. Adjusted survival by GRS quintiles.

GRS quintile	HR	95% CI	p-value
1 (GRS = 0-10)	reference		
2 (GRS = 11)	1.20	0.87-1.65	0.2660
3 (GRS = 12)	1.20	0.87-1.64	0.2658
4 (GRS = 13)	1.01	0.69-1.47	0.9697
5 (GRS = 14-17)	0.82	0.51-1.32	0.4257

Table 31. Adjusted interaction between the GRS and BB in all patients and sub-groups.

BB yes or no	
Group	*p-value
All patients	0.2433
Age < 58	0.0239
Age > 58	0.7198
Non-AAs	0.7580
African Americans	0.0831
Ischemic etiology	0.7687
Non-ischemic etiology	0.0249
BB dose	
Group	*p-value
All patients	0.1334
Age < 58	0.2808
Age > 58	0.5321
Non-AAs	0.9833
African Americans	0.0170
Ischemic etiology	0.3083
Non-ischemic etiology	0.0011

*Bonferroni-adjusted alpha = 0.1/7 = 0.0143

Figure legends

Figure 9. The normal approximation for the distribution of GRS in UNITE-DNA patients that were still alive at 10-year follow-up and those that have died during follow-up is shown.

Figure 10. The HR for GRS in the Cox proportional hazards model adjusted for age, diabetes, systolic blood pressure, NYHA class, LVEF, and GFR where necessary and stratified by age (at median), race (AA and Non-AA), etiology (ischemic and non-ischemic), and BB treatment (treated or not treated with BB at baseline).

Figure 11. Receiver-operation characteristic (ROC) curves for clinical risk factors alone and clinical risk factors plus the GRS are shown. The area under the ROC curve for the clinical risk factors alone was 0.7489 and with the GRS was 0.7487 ($p = 0.6550$).

Figure 9. Distribution of GRS in alive and deceased patients.

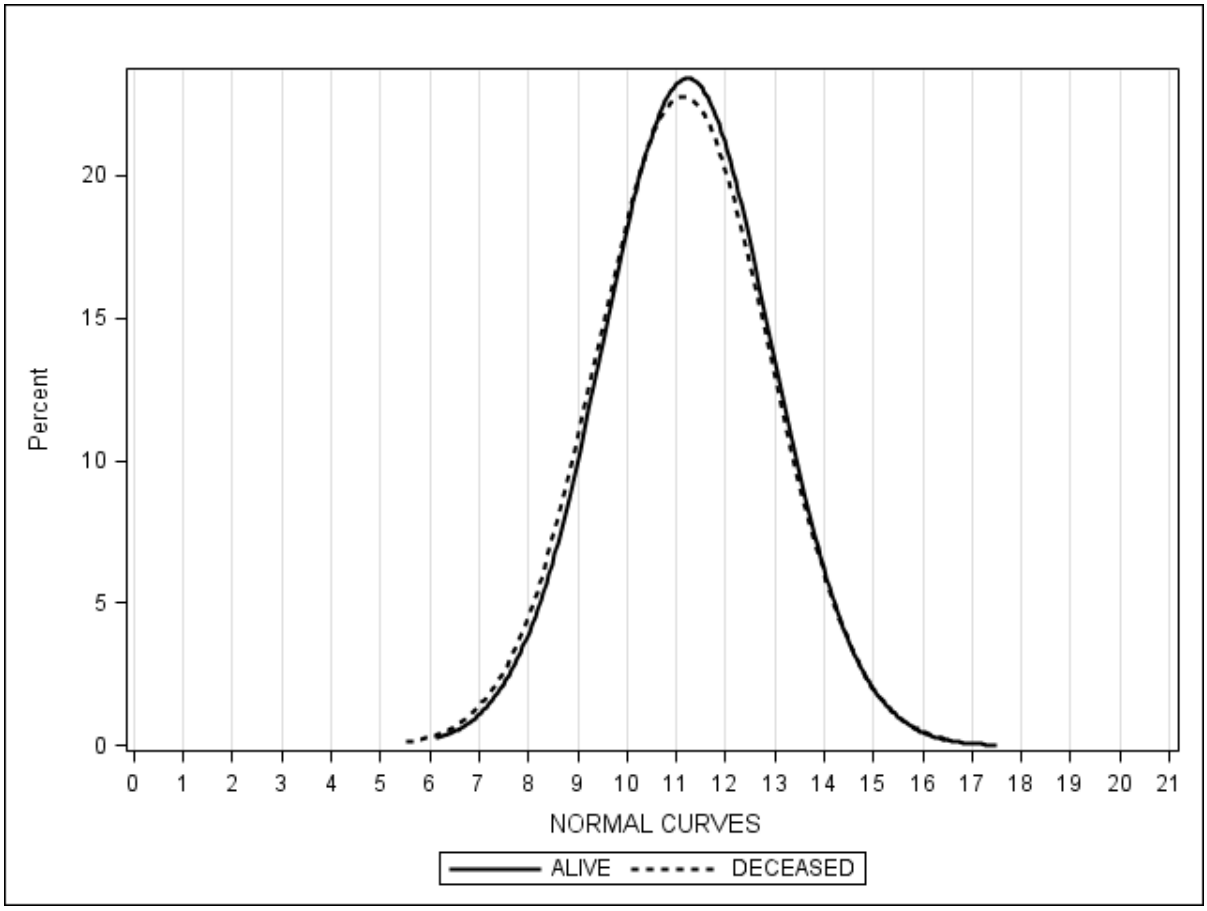


Figure 10. Adjusted HR for GRS in all UNITE-DNA patients and sub-groups.

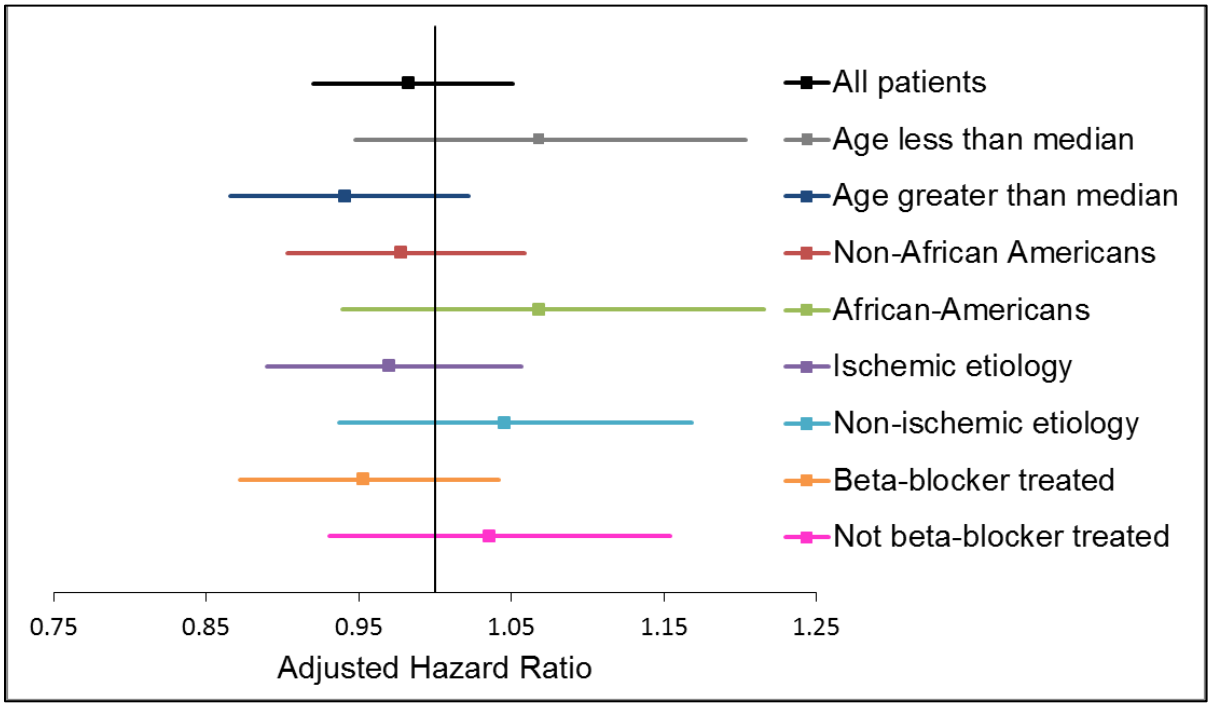
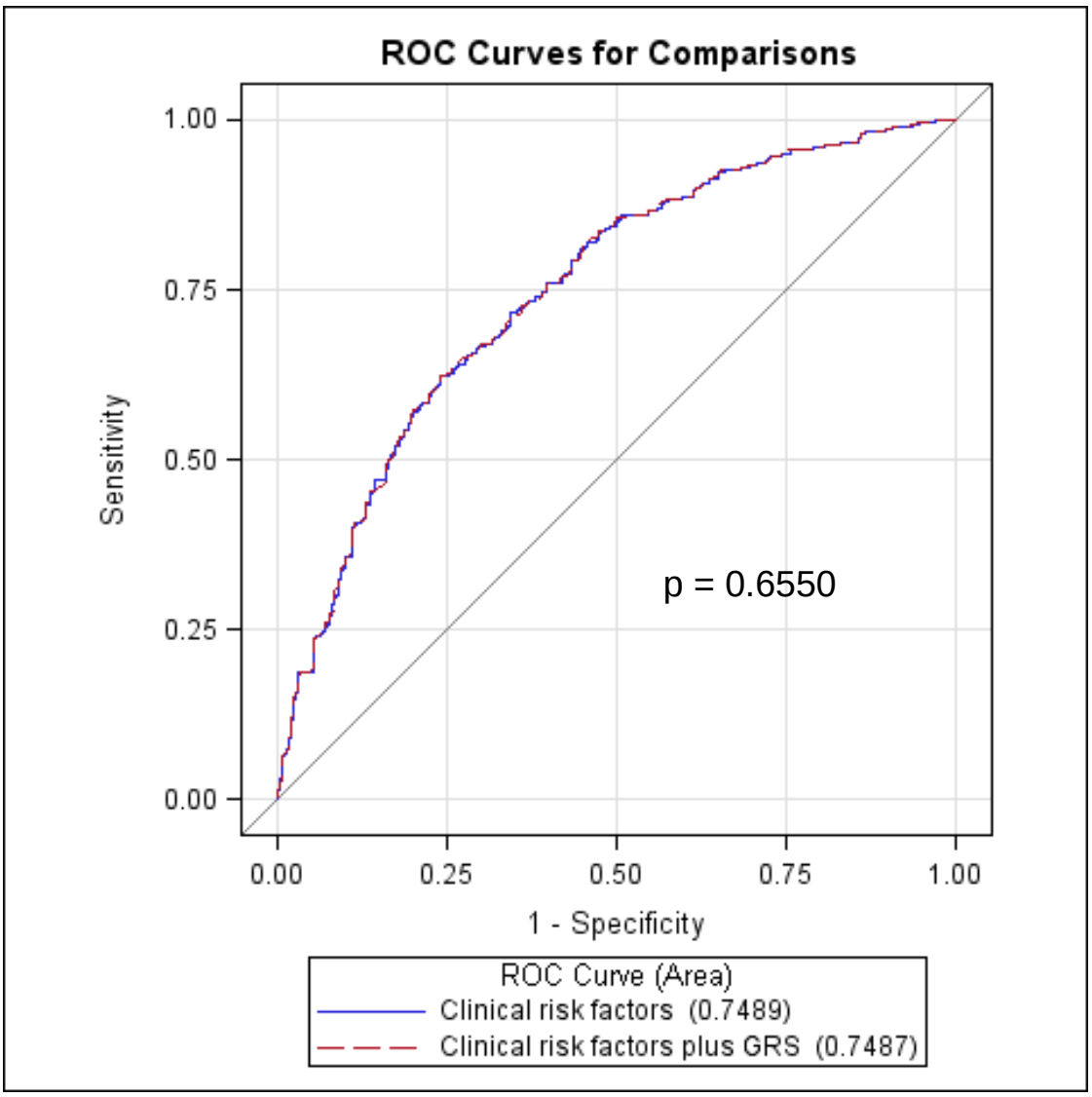


Figure 11. ROC curves for clinical risk factors ± GRS.



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CHAPTER VII:
GENE-GENE INTERACTIONS ASSOCIATED WITH HF PATIENT SURVIVAL AND BETA-BLOCKER RESPONSE

Summary

The previous chapter covered the additive association of the candidate genetic variants with survival and BB survival benefit, and this chapter covers the association of gene-gene interactions (epistasis) with HF patient survival and BB survival benefit. The classification and regression tree (CART) algorithm, a recursive partitioning data mining method, was used with 15 clinical and 13 genetic input variables to detect gene-gene interactions associated with the binary endpoint of 10-year all-cause mortality in UNITE-DNA. There were no epistatic interactions associated with HF patient survival or BB survival benefit in UNITE-DNA overall or in the AA patients, but in the non-AA patients there was a pharmacogenetic gene-gene interaction between *ADRB1* Ser49-Arg389 diplotype and *AGTR1* A1166C. In the non-AA patients aged less than 60 and treated with BBs, the mortality rate was approximately 3-fold higher (24% vs. 73%) if patients had the *ADRB1* Ser49-Arg389/Gly49-Arg389 diplotype and carried *AGTR1* 1166C. Although the CART algorithm was 10-fold cross-validated, these results need to be validated in an independent cohort.

Introduction

Common genetic variants, individually, explain very little variability in polygenic traits (such as survival and BB response) in complex diseases (such as HF), leading to the failure of many genetic association studies (1-6). One of the potential explanations for this phenomenon is epistasis, or gene-gene interactions. The association of an individual genetic variant with a complex phenotype will be missed if it is tested individually but involved in epistasis. Epistasis has long been recognized to be fundamentally important in complex, non-Mendelian traits such as HF (7), but detecting epistasis is statistically challenging. Traditional, parametric statistical methods can be used to detect epistasis by incorporating multiplicative interaction terms (e.g. SNP1*SNP2) into the model (e.g. multiple linear, logistic, or Cox proportional hazards regression). However there are many limitations in using traditional statistical methods for detecting epistasis; the following parametric regression assumptions must be met: data are normally distributed, equal variances, independent observations, independent variables are not correlated, linear association between variables and outcome, and the sample is random. Traditional regression models also cannot handle the highly dimensional nature of genetic data because the power of the model is dramatically decreased with an increasing number of parameters. Traditional regression models are typically only used to test pairwise interactions because testing higher order interactions (i.e. 3-way, 4-way, etc.) dramatically increases the number of models, and hence, multiple testing becomes a major issue. Testing more than one genetic inheritance model (e.g. dominant, recessive, or additive) also contributes to the multiple comparisons issue. Statistically significant marginal effects are necessary to detect interactions; therefore purely epistatic

interactions or those variants with slight marginal effects will be missed when using traditional statistics. Observations with missing values are typically excluded or the missing values need to be imputed. Although traditional statistical methods are widely accepted, the clinical application of a gene-gene interaction traditional statistical model to an individual patient is difficult and requires calculations.

Because of the many limitations of traditional statistical methods for detecting epistasis, novel data-mining techniques have been developed (8) and are preferred (9). Data mining is a burgeoning new technology which takes advantage of recent technological advances in computational power, using computer algorithms that automatically sift through databases seeking regularities or patterns. Data mining and traditional statistical analysis are not mutually exclusive, but rather they are closely related. Data mining utilizes traditional statistics, for example, in algorithm definitions or correction for over-fitting. The major difference between data mining and traditional statistical analysis is that statistics are typically used to test formal hypotheses, but data mining recognizes patterns in data that *leads* to formal hypotheses. Data mining has several advantages over traditional statistical methods for detecting epistasis; data mining is non-parametric and essentially hypothesis, assumption, and model free. Data mining also inherently handles observations with missing values. There are no multiple comparisons issues because data mining methods inherently test for higher-order interactions and all genetic inheritance models. Data mining methods also have output models that are easily interpreted and clinically applicable without calculation.

Data mining methods for epistasis can be defined into three categories: recursive partitioning, combinatorial, or neural network (9). The recursive partitioning methods, or

tree-based methods, split the data using logical if-then conditions to best classify the observations according to a binary outcome. The recursive partitioning methods have several advantages: 1) handle a large number of input variables, 2) fast computation times (even for very large datasets), 3) suited to deal with different types of genetic heterogeneity, 4) the results are presented in an easily interpretable final model, and 5) they can detect interactions without *strong* marginal effects. The major limitation of the recursive partitioning methods is dependence on *slight* marginal effects because the splits are based on a single variable. The combinatorial approaches are not dependent on marginal effects, and therefore they are ideal for detecting purely epistatic interactions (9). Combinatorial methods exhaustively search over all possible variable combinations to find the combinations that best predict the outcome (9). Due to this exhaustive search, the major disadvantage of the combinatorial approaches is extremely long computational time. Long computational time is also a major disadvantage of neural networks. Neural networks "learn" to make predictions for new datasets from a training dataset based on the hypothesized processes of the brain (9). Neural networks consist of inter-connected nodes arranged in layers through which the input signal is processed. The major disadvantage of the combinatorial and neural network approaches is output that is complex and thus difficult to interpret and apply clinically.

For this dissertation research, a data mining method was chosen to test the general hypothesis that gene-gene interactions between and within functional genetic variants in the SNS and RAAS will be associated with HF patient survival and BB response. Because data mining was chosen, a specific hypothesis on which genetic variants and how many genetic variants would interact and the nature of the interaction is not

necessary. The disadvantage when using a data mining method to test a general hypothesis is that any positive gene-gene interactions would be considered hypothesis-generating, and therefore it would need to be prospectively validated in an adequately powered study. A data mining method was chosen over traditional statistical methods because the data mining methods do not rely on parametric assumptions or statistically significant marginal effects, they can handle highly dimensional data, can test for higher order interactions, handles observations with missing values, and avoid multiple comparisons issues. Of the three data mining methods described above (i.e. recursive partitioning, combinatorial, and neural network), recursive partitioning was chosen because the output is easily interpreted and the epistatic interactions are placed in a clinical context. Recursive partitioning also has the advantage fast computation time compared to the combinatorial and neural network methods. There are several different types of recursive partitioning algorithms, such as CART, J48, or C4.5 (8), but the CART algorithm was specifically chosen because it has previously been applied to HF for clinical input variables (10), allowing a comparison of this CART model accuracy to an independent CART model in HF patients. Also, other recursive partitioning algorithms (e.g. J48 and C4.5) are prone to over-fitting, limiting the generalizability of the results to HF patient populations other than UNITE-DNA.

Methods

Recursive partitioning. The open source Waikato Environment for Knowledge Analysis (WEKA) version 3.6.7 (8) implementation of the classification and regression tree (CART) algorithm (11) was used to detect epistasis in UNITE-DNA. The binary

outcome variable was 10-year all-cause mortality. Fifteen clinical and 13 genetic input variables were included in the analysis (Table 32). The *ADRB1* and *ADRB2* diplotypes were estimated using PHASE is version 2.1.1. The CART algorithm segregates different values of the input variables through a decision tree composed of progressive binary splits (11). Each parent node in the decision tree produces two child nodes, which in turn can become parent nodes producing additional child nodes. Every value of each input variable is considered as a potential split, and the split is made to maximize the purity of the resultant leaf nodes (e.g. 100% deceased and 0% alive or 0% deceased and 100% alive). If variables have missing values, a surrogate value is imputed based on all of the other variables with non-missing values in the patient. This procedure is analogous to replacing a missing value in a linear model by regressing on the non-missing value most highly correlated with it, but it is more robust (11). The partitioning process continues until all of the patients in UNITE-DNA are classified, and then the tree is pruned using minimal cost-complexity pruning (11) to optimally balance tree complexity with tree generalizability. Because there is not a validation cohort, internal validation using 10-fold cross validation was used during the tree building process. Tree accuracy was assessed using area under the receiver operating characteristic curve (AUC ROC) and misclassification rate. Because allele frequencies and the heart failure phenotype are dramatically different between the AA and non-AA patients, decision trees were also derived in racial strata.

Results

When recursive partitioning all of the UNITE-DNA patients, the variables that stayed in the model for predicting 10-year all-cause mortality (in order of importance) were age (less than or greater than 62 years), GFR (less than or greater than 43 mL/min), heart failure etiology (ischemic or non-ischemic), and systolic blood pressure (less than or greater than 107 mmHg) (Figure 12). There were no gene-gene interactions, or even single genetic variants, that were predictive of mortality in all of the UNITE-DNA patients. The ROC AUC was 0.663 and the misclassification rate was 35%. Notably, the accuracy of the UNITE-DNA model is similar to a much larger study of hospitalized HF patients in the ADHERE registry (n = 33,046), in which the ROC AUC was 0.687 (10). Specifically in the non-AA patients, the tree is much larger (Figure 13). Ten variables were important for predicting 10-year all-cause mortality, including a pharmacogenetic gene-gene interaction between *ADRB1* and *AGTR1* in the non-AA patients aged less than 60 treated with BB (Figure 14). If the non-AA patients did *not* have the *ADRB1* Ser49-Arg389/Gly49-Arg389 diplotype, were treated with BB, and aged less than 60, then their 10-year all-cause mortality rate was only 19% (n = 147). However, if the non-AA patients did have the *ADRB1* Ser49-Arg389/Gly49-Arg389 diplotype, carried *AGTR1* 1166C, were treated with BB, and aged less than 60, then their mortality rate was 73% (n = 12). If the non-AA patients did have the *ADRB1* Ser49-Arg389/Gly49-Arg389 diplotype, were homozygous for *AGTR1* 1166A, treated with BB, and aged less than 60, then their mortality rate was only 24% (n = 9). The ROC AUC was 0.645 and the misclassification rate was 36% for the non-AA model. In the AA patients, there were no pharmacogenetic gene-gene interactions, but a single genetic variant was important for

predicting mortality: *ADRB2* Arg16Gly (Figure 15). If the AA patients had a GFR greater than 54 mL/min, age less than 73, LVEF less than 23, diastolic blood pressure greater than 67, and were homozygous for *ADRB2* Gly16, then their mortality rate was 82% (n = 10), but if they carried *ADRB2* Arg16 then their mortality rate was only 29% (n = 29). The ROC AUC was 0.616 and the misclassification rate was 37% for the AA model.

Discussion

This is the first report using data mining to detect gene-gene interactions associated with HF clinical outcome. When considering all of the UNITE-DNA patients, there were no gene-gene interactions. Because the allele frequencies and HF phenotypes are dramatically different between non-AA and AA HF patients, decision trees were also derived separately in racial strata, and a pharmacogenetic gene-gene interaction between *ADRB1* and *AGTR1* was identified in the non-AA. Only one previously published study has evaluated the association between gene-gene interactions and clinical outcome in HF patients (12). This study by Kardia *et al* tested a novel hypothesis, but the methodology is fraught with limitations. In Kardia *et al*, 655 Caucasian patients were genotyped for 16 variants in *ADRA2C* and 17 variants in *ADRB1*. The patients were followed for a mean 3.16 years to the endpoint of death or heart transplant. Kardia *et al* used traditional statistical methods, despite the numerous limitations of this approach as described in the introduction, to detect intra- and inter-genic epistasis. Kardia *et al* only tested for pairwise interactions, and they used a 30% FDR to control for multiple comparisons and leave-one-out cross validation due to the lack of a validation cohort. Covariates used to

adjust the Cox proportional hazards model were age at initial diagnosis, BB usage, hypertension status, and sex. Kardia *et al* found two genetic variants with statistically significant main effects, but these same two genetic variants were also involved in gene-gene interactions, making the interpretation of the main effects impossible. Three variants in *ADRA2C* and five variants in *ADRB1* were involved in eight cross-validated epistatic interactions, resulting in two-locus genotype classes with significant relative risks ranging from 3.02 to 9.23. The clinical interpretation of this data is difficult because Kardia *et al* only tested pairwise interactions, and it is unknown whether higher-order interactions are associated with outcome. Higher-order interactions are highly likely, given that the eight cross-validated epistatic interactions stem from within and between two highly related genes. It is impossible to determine a given HF patient's mortality risk from this data because there may be synergy between the risk-increasing epistatic pairs. Alternatively, a HF patient's risk cannot be estimated if they possessed a combination of protective and harmful epistatic pairs. In addition, because multiple tests were performed, the probability of these associations being falsely positive is very high. The FDR method they used to control for false discoveries allowed 30% to be false, meaning at least two of the eight epistatic interactions they discovered are probably false.

The recursive partitioning data mining method used herein addresses many of the limitations encountered by Kardia *et al* for detecting gene-gene interactions associated with HF clinical outcome. Because all of the input variables are added to the model simultaneously, there are no multiple comparisons issues. Also, the Kardia *et al* study did not study AAs, whereas UNITE-DNA includes both AA and non-AA patients analyzed together and separately. The output for the CART algorithm is easily interpreted and in a

clinical context. Known functional variants were studied herein, whereas Kardia *et al* studied variants with unknown functional consequences. In addition, nine genes were tested in UNITE-DNA whereas Kardia *et al* only tested two genes. The recursive partitioning data mining method also does not depend on the assumptions necessary for the parametric regression approach used by Kardia *et al* i.e. data is normally distributed, equal variances, independent observations, independent variables are not correlated, linearity, or a random sample. As evident in the Kardia *et al* study, statistically significant marginal effects are necessary to detect epistatic interactions. Whereas recursive partitioning only depends on *slight* marginal effects of each single variant. Kardia *et al* also only tested a dominant genetic inheritance model. Recursive partitioning can handle a large number of input variables (no loss of degrees of freedom with increasing number of parameters like regression models). Importantly, recursive partitioning has its own limitations. Although it is not dependent on *strong* marginal effects, recursive partitioning is dependent on *slight* marginal effects since the splits are made on a single variable. To overcome this limitation, data mining methods that could potentially be used in future work are the combinatorial and neural network methods (9), which are capable of detecting purely epistatic interactions. Another limitation of recursive partitioning is the very small sample sizes in the leaf nodes, which limits the generalizability of the results to large patient populations and makes recruitment of subjects for adequately powered prospective studies difficult.

It should be emphasized that data mining methods are not hypothesis-testing, but rather hypothesis-generating. For instance, the general hypothesis for this study was that gene-gene interactions between functional variants within the SNS and RAAS will be

associated with survival in patients with HF. A specific hypothesis on which and how many of the genetic variants will interact was not made. Because of the small sample sizes in the leaf nodes, the pharmacogenetic gene-gene interaction between *ADRB1* and *AGTR1* can now *lead to* a prospective, adequately-powered study tested with traditional statistical methods. The gene-gene interaction needs to be validated both statistically and biologically, but the statistical interaction needs to be validated prior to the biological interaction. Although this finding was 10-fold cross-validated, it was only found in a single cohort. Figure 16 shows a proposed research pathway for implementation of the pharmacogenetic gene-gene interaction identified in the non-AA into clinical practice. The next step for this gene-gene interaction would be retrospective validation in another HF patient population. One such cohort could be from the study by Cresci *et al* (13). Cresci *et al* studied 2,460 HF patients, of which 1,392 were Caucasian and treated with BBs. Also estimating the number of those patients under the age of 60 and considering the frequencies of the *AGTR1* 1166C allele and the *ADRB1* Ser49-Arg389/Gly49-Arg389 diplotype, about 20 of the patients from the Cresci *et al* study would fall into the high risk category (i.e. *ADRB1* Ser49-Arg389/Gly49-Arg389 diplotype and *AGTR1* 1166AA) and 425 patients into the low risk category (i.e. not *ADRB1* Ser49-Arg389/Gly49-Arg389 diplotype or with the diplotype but carrying *AGTR1* 1166C) for the gene-gene interaction. Assuming the survival rates are 3-fold different between the high and low risk group, the Cresci *et al* cohort would have 77% power to detect this difference. Although this may be underpowered considering the "winner's curse (Appendix IV) (14)," where the initial report of a genetic association is exaggerated due to sampling bias. If retrospectively validated in an independent cohort (or cohort backwards;

"trohoc") such as Cresci *et al*, the next step would be to initiate a new prospective cohort. Assuming the difference in mortality rates between the high-risk and low-risk groups is 2-fold at 5 years of follow-up, 312 patients (156 per high risk and low risk genotype group) would be necessary for 80% power at alpha = 0.05. Alternatively, because the high risk group would be only be approximately one or two out of every 100 patients in an outpatient HF specialty clinic (making recruitment difficult), 80% power at alpha = 0.05 for 5 years of follow-up and 2-fold difference in mortality rate could also be achieved by enrolling 96 high risk patients and 1150 low risk patients. The advantage being lower enrollment of the high risk patients, but the disadvantage is that total enrollment would be much greater (n = 1246). However if there is still a significant difference in mortality due to the pharmacogenetic gene-gene interaction after retrospective and prospective cohort validation, then the next step would be to determine effective interventions for the high-risk patients. Although effective interventions could not be tested until the biological interaction is characterized.

As described in detail in Chapter II, there is clear functional, physiologic, and clinical plausibility for an association of each the 11 candidate genetic variants with survival and BB response in patients with HF. However the biologic plausibility specifically for this pharmacogenetic gene-gene interaction found in the following select group of patients is difficult to describe: non-AA, aged less than 60, treated with BBs, with the *ADRB1* Ser49-Arg389/Gly49-Arg389 diplotype, and carrying *AGTR1* 1166C. It is possible that a gene-gene interaction involving the SNS and RAAS was able to be detected in non-AA and not the AA because the SNS and RAAS play a smaller role in AA patients compared to non-AA (15). For example, despite more severe LV

dysfunction, AA patients have either similar or lower norepinephrine levels and they do not respond to pharmacologic neurohormonal inhibition as well as Caucasians (16). It is also possible that a genetic association would be detected in younger patients (aged less than 60 years) compare to older (aged greater than 60 years) because genetic variation represents long-term exposure to high SNS and RAAS activity. Therefore adverse effects of a genetic variant, if present, would be expected to manifest in younger patients (17). If the genetic variant has weak or no association with survival, then as patients age they would be more likely to succumb to comorbidities. However, a limitation of the UNITE-DNA registry design is that we cannot determine the cause of death.

As described in detail in Chapter II, the *ADRB1* Ser49 allele is relatively resistant to down-regulation compared to the Gly49 allele (18,19), and Arg389 couples to the stimulatory intracellular G-protein more than the Gly389 allele (20). Hence, the Ser49-Arg389 diplotype would be considered the most active form of the beta-1 adrenergic receptor, and it would also be the most responsive to BB inhibition. Therefore it would be expected that with an increasing number of Ser49-Arg389 haplotypes (0, 1, or 2) there would be increasing BB responsiveness. Because the Ser49-Arg489/Gly49-Arg389 diplotype only has one copy of the most active Ser49-Arg389 haplotype, patients with this diplotype would be expected to have an intermediate BB response compared to other diplotypes. Therefore it is not clear why patients with the least responsive diplotype (i.e. Gly49-Arg389/Gly49-Arg389) is low risk and grouped with the presumably most responsive diplotype (i.e. Ser49-Arg389/Ser49-Arg389). *AGTR1* 1166C has increased sensitivity to AII (21,22), is associated with left ventricular dysfunction in coronary artery disease patients (23), diastolic HF (24), and decreased survival in patients with HF

(25). It is not surprising that a variant from the RAAS is important in BB treated patients since there are beta-1 receptors in the kidney that mediate the release of renin (26). The CART results are the opposite of what would be expected based on the previous literature because UNITE-DNA patients carrying *AGTR1* 1166C had *lower* mortality than those homozygous for 1166A. Further research defining the potential biological interaction between *AGTR1* A1166C and the *ADRB1* Ser49-Arg389/Gly49-Arg389 diplotype is necessary.

The biological interaction between *ADRB1* and *AGTR1* has not been directly tested in experimental models, and it is difficult to determine from a statistical interaction at what level the interaction is occurring. For example, the biological interaction could be occurring at the transcriptional, translational, functional, or physiological level, each of which could be tested with a variety of experimental models. The best course of action would be studying the interaction at the most basic level, and then to gradually increase study complexity to the system level. For example, initial studies at the basic level could evaluate the protein-protein interaction in transcriptional regulation using the yeast two-hybrid system (27). A wide variety of animal models of HF exist to test more complex, system level gene-gene interactions (28). For example, rodent models are relatively inexpensive (compared with large-animal models), and manipulation of mouse genetics allows gain or loss of function of specific genes in specific cell types at specific times. Limitations of animal models of HF include: 1) difficulty in mimicking the variety of causes of HF (e.g. hypertension, ischemia, genetics, valvular disorders, etc.) and 2) animal models are often developed on a defined genetic background that does not reflect the genetic diversity of human HF populations (28). Fly and fish HF models are also

available, and these models are particularly well-suited to study specific genes due to the ease in which their genes can be modified. The limitations of fly and fish models are that they are far removed from the complexity of the adult mammalian heart (28).

The difficulty in deriving a biological explanation for this gene-gene interaction highlights the inherent difficulty in defining biological epistasis from statistical epistasis (29). Biological epistasis is the result of physical interactions among biomolecules within and between biochemical pathways or physiologic systems in an individual. In contrast, statistical epistasis is usually defined as deviation from linearity in a mathematical model or simply the relationship between multiple genotypes with phenotype in a population is not predictable from the individual genetic variants alone (29). The extent to which statistical evidence of epistasis from population studies, such as UNITE-DNA, is predictive of biological epistasis from experimental studies remains elusive (29). Currently, it is not possible to connect biological and statistical epistasis in humans, but with the promise of systems biology it may be possible in the future (30).

Table 32. Clinical and genetic variables input into the CART algorithm.

Clinical input variables	Genetic input variables
1. History of diabetes	1. *ADRB1* Ser49Gly
2. Age (years)	2. *ADRB1* Arg389Gly
3. Gender	3. *ADRB1* diplotype
4. HF etiology (ischemic vs. non-ischemic)	4. *ADRB2* Arg16Gly
5. History of hypertension	5. *ADRB2* Gln27Glu
6. Systolic blood pressure (mmHg)	6. *ADRB2* diplotype
7. Diastolic blood pressure (mmHg)	7. *ACE* 287bp deletion
8. Heart rate (beats per minute)	8. *ADRA2C* 12bp deletion
9. Left ventricular ejection fraction (%)	9. *GRK5* Gln41Leu
10. NYHA functional class	10. *AGT* G-6A
11. Race (self-identified Caucasian, AA, or other)	11. *AGTR1* A1166C
12. BB treatment status	12. *CYP11B2* T-344C
13. Specific BB (atenolol, metoprolol XL, metoprolol IR, carvedilol, bisoprolol, other)	13. *BDKRB2* 9bp deletion
14. BB dose (in mg metoprolol equivalents)	
15. Glomerular filtration rate (mL/min)	

Figure legends

Figure 12. Decision tree output from recursive partitioning all UNITE-DNA patients using the CART algorithm in WEKA version 3.6.7 is shown. Progressive binary splits of the UNITE-DNA data are made, considering all input variables and their values, to best classify UNITE-DNA patients according to the binary outcome variable of 10-year all-cause mortality. Red highlighted arrows are the split values associated with increased risk of 10-year all-cause mortality, and green highlighted arrows are the split values associated with decreased risk of 10-year all-cause mortality. N = number of patients that have died out of the total number of patients remaining after the preceding splits/total number of patients remaining after the preceding splits.

Figure 13. Complete decision tree output from recursive partitioning non-AA UNITE-DNA patients using the CART algorithm in WEKA version 3.6.7 is shown. Progressive binary splits of the UNITE-DNA data are made, considering all input variables and their values, to best classify UNITE-DNA patients according to the binary outcome variable of 10-year all-cause mortality. Red highlighted arrows are the split values associated with increased risk of 10-year all-cause mortality, and green highlighted arrows are the split values associated with decreased risk of 10-year all-cause mortality. N = number of patients that have died out of the total number of patients remaining after the preceding splits/total number of patients remaining after the preceding splits.

Figure 14. The branches of the decision tree derived from the non-AA UNITE-DNA patients using the CART algorithm leading to the gene-gene interaction is shown. N = number of patients that have died out of the total number of patients remaining after the preceding splits/total number of patients remaining after the preceding splits.

Figure 15. Complete decision tree output from recursive partitioning AA UNITE-DNA patients using the CART algorithm in WEKA version 3.6.7 is shown. Progressive binary splits of the UNITE-DNA data are made, considering all input variables and their values, to best classify UNITE-DNA patients according to the binary outcome variable of 10-year all-cause mortality. Red highlighted arrows are the split values associated with increased risk of 10-year all-cause mortality, and green highlighted arrows are the split values associated with decreased risk of 10-year all-cause mortality. N = number of patients that have died out of the total number of patients remaining after the preceding splits/total number of patients remaining after the preceding splits.

Figure 16. A research pathway to the clinical implementation of the pharmacogenetic gene-gene interaction identified in the non-AA UNITE-DNA patients is proposed.

Figure 12. Decision tree for all UNITE-DNA patients.

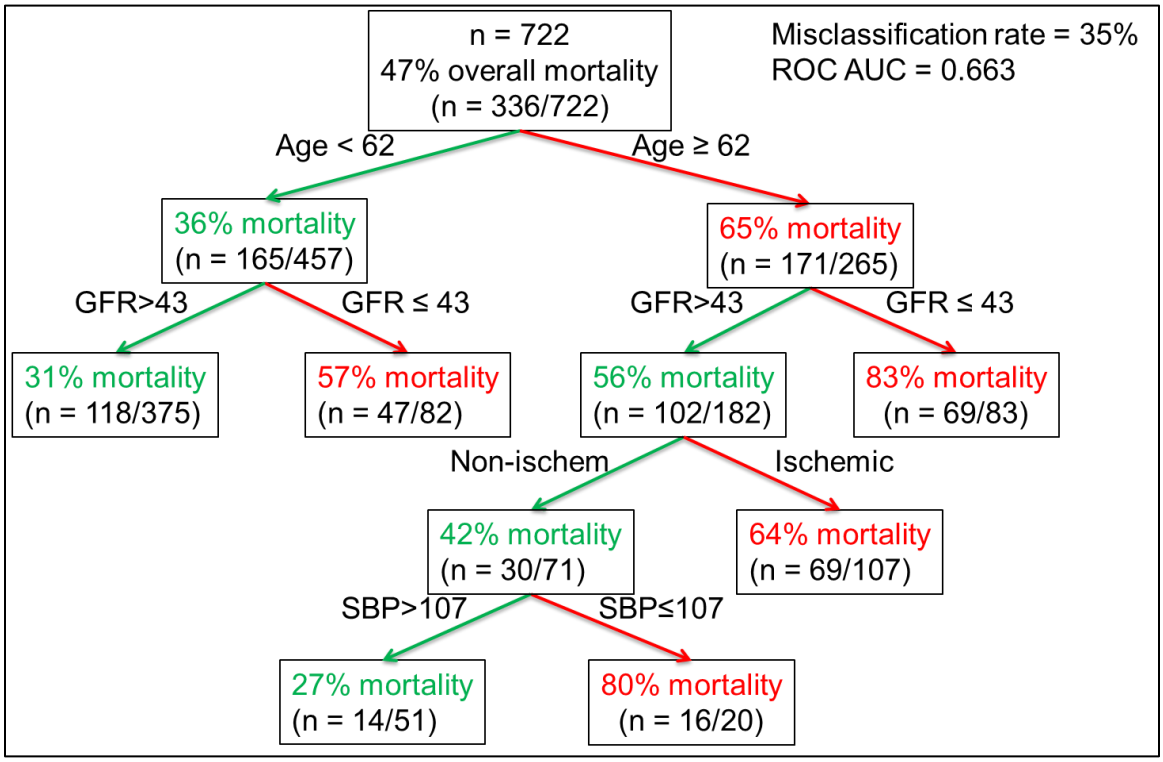


Figure 13. Complete decision tree for Non-AA UNITE-DNA patients.

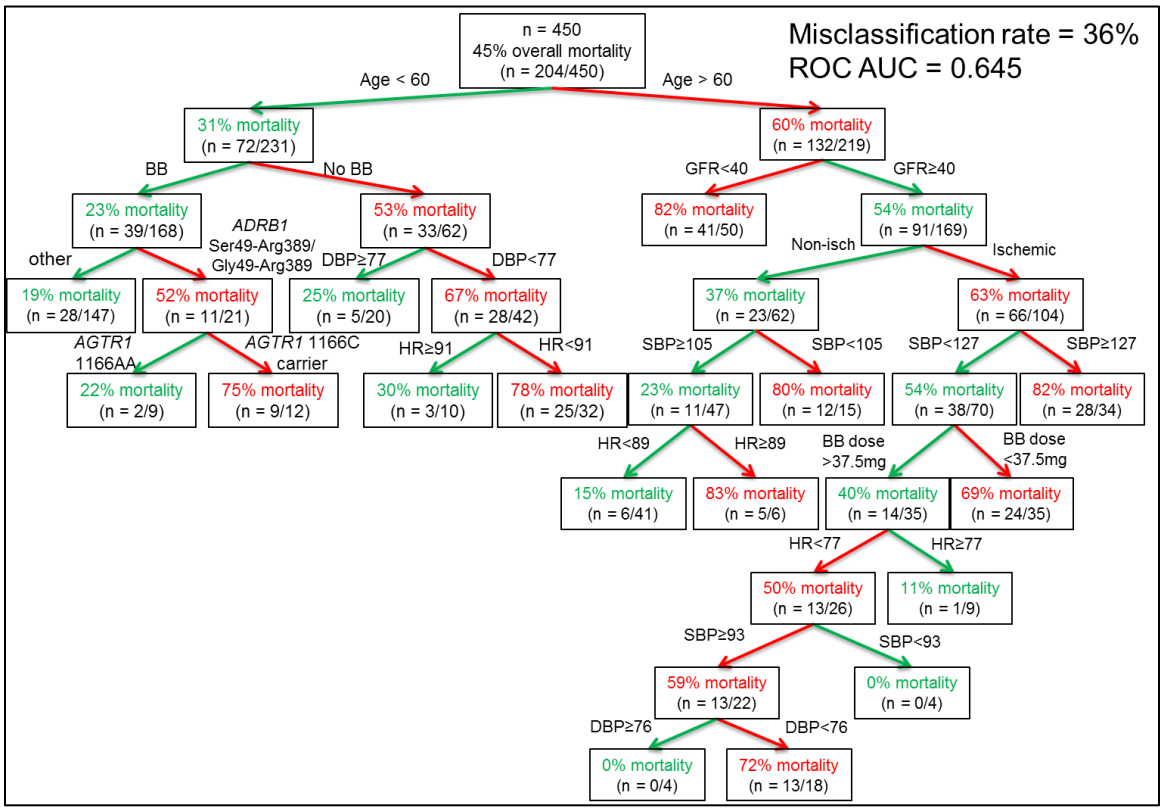


Figure 14. Branches of Non-AA decision tree leading to gene-gene interaction.

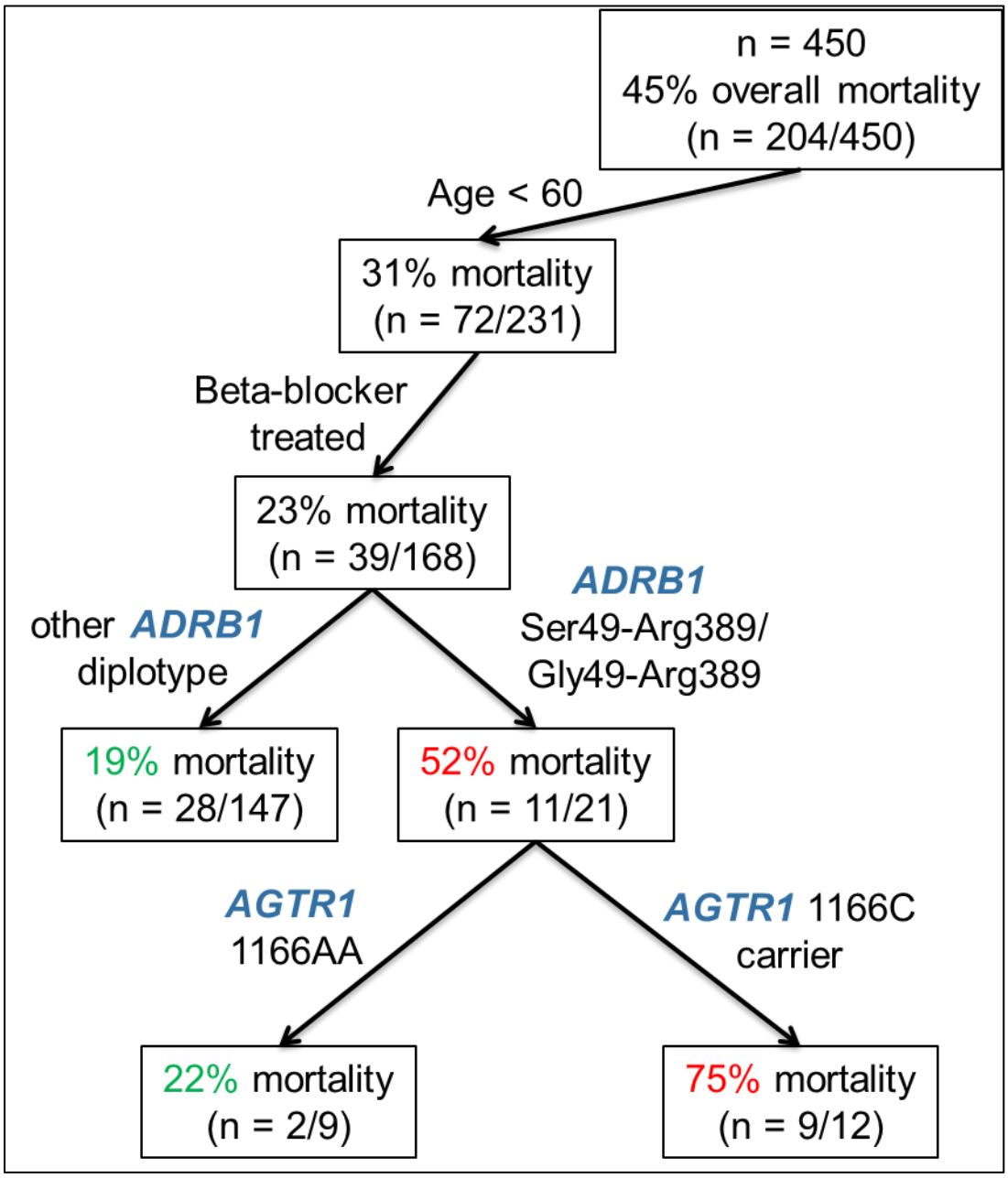


Figure 15. Complete decision tree for AA UNITE-DNA patients.

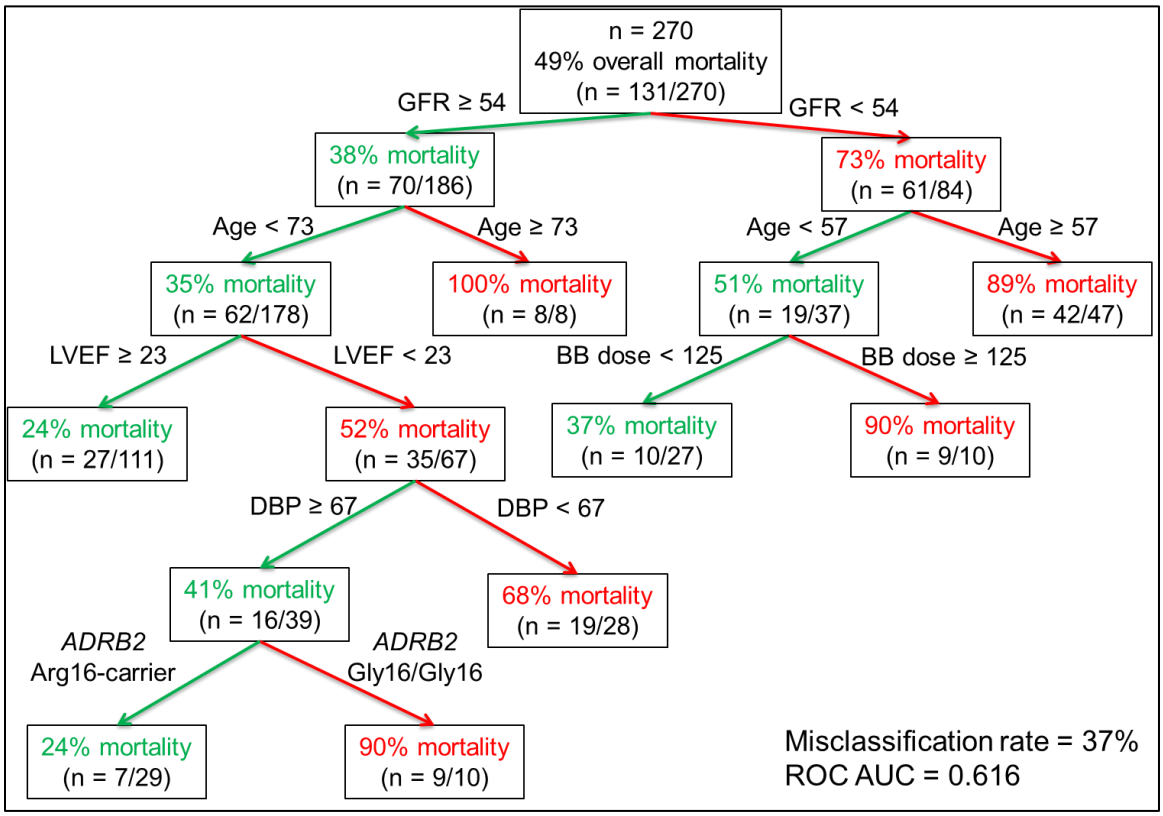
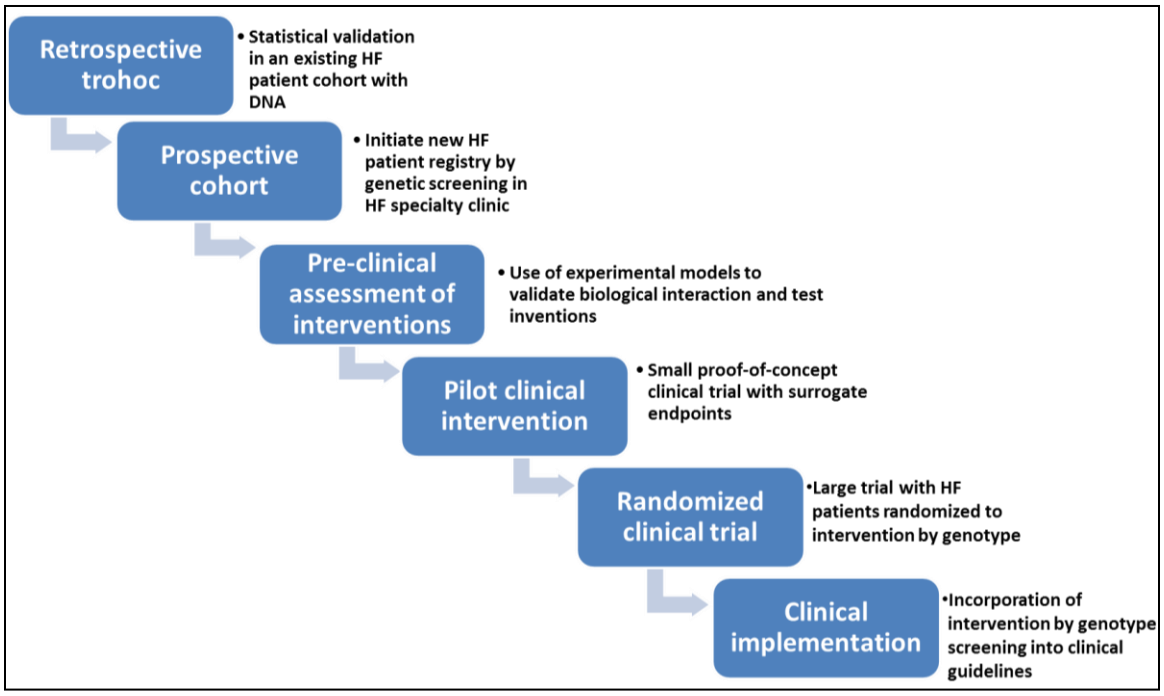


Figure 16. Research pathway to clinical implementation of the pharmacogenetic gene-gene interaction in non-AA.



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CHAPTER VIII:
DISCUSSION AND PERSPECTIVE

Summary

This chapter has several goals: 1) address the gaps in and limitations of the previous HF genetic association literature, 2) describe how this dissertation research fills the gaps in or overcomes the limitations of the previous HF genetic association literature, 3) summarize the findings of this dissertation research, 4) discuss these research findings within the context of the broader body of literature, 5) evaluate the potential clinical impact of these findings, and 6) propose areas for future investigation.

Discussion

HF is an enormous public health problem, with immense cost, incidence, prevalence, morbidity, and mortality. Hyperactivity of the SNS and RAAS is the primary contributor to HF pathophysiology, and, in patients with HF, it is associated with hemodynamic abnormalities (1), symptoms (2), and survival (3-6). The survival rates in patients with HF are highly variable, ranging from 93% (7) to 25% (8) per year, and individual patient responses to SNS and RAAS pharmacologic inhibition in HF are also highly variable. Pharmacologic inhibition of the SNS and RAAS with BB significantly decreases morbidity and mortality, on average, in large HF clinical trials (9,10), but long-term optimal dosing of BB fails to improve LVEF greater than 5% in as many as 43% of

HF patients (11). Unfortunately, clinical characteristics do not entirely explain the variability in HF patient survival rates and BB response. However genetic variation affects SNS and RAAS activity, and thus genetic variation may be associated with HF patient survival and BB response. Therefore the collective objective of this dissertation research was to determine the association of genetic variation in the SNS and RAAS with HF patient survival and BB response.

Several common, functional genetic variants in the SNS and RAAS are individually associated with HF physiologic (e.g. ventricular remodeling) or clinical outcomes (e.g. survival or BB response) in the literature. For example, an insertion/deletion variant in the gene that encodes for angiotensin-converting enzyme (*ACE*) accounts for half of the variance in plasma ACE levels (12). The *ACE* deletion allele is found in approximately one-half of HF patients, and it is associated with significantly higher plasma ACE levels (12), higher risk of mortality (13), and increased BB response (14). Like the *ACE* genetic variant, 11 total common variants supported by prior functional and clinical literature were chosen for this dissertation research: *ADRB1* Ser49Gly and Arg389Gly, *ADRB2* Gly16Arg and Gln27Glu, *ACE* 287 bp Ins/Del, *ADRA2C* 12 bp Ins/Del, *GRK5* Gln41Leu, *AGT* G-6A, *AGTR1* A1166C, *CYP11B2* T-344C, and *BDKRB2* 9bp Ins/Del.

The literature on these 11 genetic variants dates back 15 years. To date, no genetic test has been applied in HF clinical practice, and limitations of the previously published studies may have led to the lack of clinical application. Early genetic association studies with HF patient survival were conducted prior to the widespread use of BB (13,15). Therefore the confounding effect of BB on the genetic association with survival could not

be determined nor could BB pharmacogenetic associations. There has also been conflicting and non-replicated results in the HF genetic association literature. For example, two genetic variants in the beta-2 adrenergic receptor (*ADRB2* Gly16Arg and Gln27Glu) were associated with lower risk of worsening HF (16). However the same two variants have also been associated with higher risk of adverse outcome (17), or no association at all (15). Additionally, the majority of previous studies tested only one to three genetic variants, and therefore the independence of each genetic association, or the relative contribution of each variant, cannot be verified. For example, each of the following genes have been associated with HF patient survival in separate studies: *ACE* (13), *CYP11B2* (18), and *AGT* (19). All three of these genes are part of the RAAS, and therefore they may not have independent effects. And finally, many of the initial, positive genetic associations in HF have yet to be validated (20,21).

Validation of independent genetic associations needs to be performed in an adequately powered, well-characterized, and extensively genotyped patient cohort, which was accomplished in aim #1 (Chapter V). None of the eleven variants were significantly associated with HF patient survival, but a single genetic variant, *ADRB1* Ser49Gly, was significantly associated with BB response despite rigorous adjustment for clinical covariates and multiple comparisons. BB treatment was associated with a statistically significant 46% reduction in mortality in Ser49-homozygotes but a non-significant 38% *increase* in Gly49-carriers. This finding is consistent with two European studies of patients with IDCM (22,23) and also the hypertension literature, in that patients with Ser49 have a better blood pressure response to BB (24-27). Notably, there are other BB HF pharmacogenetic studies of Ser49Gly with a survival endpoint that were negative

(28,29), and the studies with intermediate endpoints (e.g. ventricular remodeling response to BB) are not supportive of this pharmacogenetic interaction (11,28,30-33). Therefore, acknowledging that these studies have many limitations, the weight of the evidence for this pharmacogenetic interaction between Ser49Gly and BB still needs to be increased prior to any prospective studies. Future research should focus on validating this pharmacogenetic association in another adequately-powered patient cohort with a large proportion of patients not treated with BB. The clinical implications for this finding, if validated, are profound because Ser49Gly could be used to identify genetic non-responders to BB therapy. HF patients could be screened for the Ser49Gly genetic variant in the clinic, and Gly49-carriers could be targeted for closer clinical monitoring and/or titration of other life-saving HF medications (e.g. RAAS inhibitors).

Another important limitation of the previous HF genetic association literature is that several different SNS and RAAS genetic variants had positive associations but in separate studies. Because these genetic variants are part of the same physiologic systems, it is reasonable to think that they could have additive effects. Indeed, these genetic variants are common, so a given HF patient would possess multiple, functional, SNS and RAAS genetic variants. In common, complex diseases such as HF, common genetic variants typically have weak associations with clinical outcomes (34), which could explain the largely negative results in Chapter V. The additive association of multiple SNS and RAAS genetic variants has not been previously studied, which was accomplished in aim #2 (Chapter VI) via simple and internally-weighted GRS's (35). Neither the simple nor internally-weighted genetic risk scores were associated with survival. The GRS's also did not add to the predictability of clinical risk factors for

mortality or reclassify HF patients to new mortality risk categories. There are several possible explanations for these negative results: only a few variants from the vastly complex SNS and RAAS physiologic systems were included, the literature reported associations upon which candidate selection was made were falsely positive, or there simply is not an additive association of the genetic variants. The second reason, that the literature reported associations upon which the candidate selection was made, is supported by the failed validation in aim #1. Unfortunately, these findings in aim #2 are consistent with several other negative GRS studies for cardiovascular disease in the literature (36-38), but this may still be a viable methodology in HF due to the successful GRS application in a variety of other common, complex diseases such as prostate cancer (39), coronary heart disease (40,41), type 2 diabetes (42), and primary cardiovascular events (43). As more genetic variants are discovered and validated in HF, future research could focus on using more advanced GRS methods such as the polygenic score (35) or the incorporation of rare genetic variants with larger effect sizes. A successful GRS in HF is clinically important because it could be used to accurately estimate the risk of mortality in HF, which enables informed decisions by providers, patients, and patients' families on HF medications, devices, heart transplantation, and end-of-life care.

Although additive associations of the genetic variants were not found, it is possible that there are synergistic associations or gene-gene interactions. For example, Liggett *et al* reported an Arg389Gly substitution in the beta-1 adrenergic receptor (*ADRB1*) which results in decreased coupling to the intracellular stimulatory protein Gs (44). The Arg389Gly *ADRB1* variant was associated with survival and beta-blocker response in HF patients (45). In another study by Liggett *et al*, a Gln41Leu substitution in

the G-protein coupled receptor kinase 5 (*GRK5*), which enhances beta-adrenergic receptor intracellular uncoupling, was also associated with HF patient survival and beta-blocker response (46). Because both of these variants affect beta-adrenergic intracellular coupling, which is necessary for receptor function, they could have a synergistic association with clinical outcome. Synergy, or other forms of gene-gene interactions, would not be detected in an additive model such as a GRS. Epistasis, or gene-gene interactions, is another possible explanation for the failure of other genetic association studies (28,47-51) and aims #1 and #2 because the association of an individual genetic variant with a complex phenotype will be missed if it is tested individually but involved in epistasis. A single gene-gene interaction study in patients with HF has been published (52), but they used traditional statistical methods fraught with limitations. Traditional statistical methods depend on parametric assumptions, a pre-specified genetic inheritance model, and cannot handle high dimensional data. Therefore the objective of aim #3 (Chapter VII) was to determine if gene-gene interactions within and between the SNS and RAAS were associated with survival in patients with HF.

A recursive partitioning data mining method, CART, was chosen to test the gene-gene interaction hypothesis in aim #3. Data mining methods have several advantages over traditional statistical methods for detecting epistasis because they are essentially assumption and model free and they can handle high dimensional data. There were no epistatic interactions associated with HF survival or BB response in UNITE-DNA overall or in the AA patients, but in the non-AA patients there was an interaction between the *ADRB1* Ser49-Arg389 diplotype and *AGTR1* A1166C. In the non-AA patients aged less than 60 and treated with BB, the mortality rate was approximately 3-fold higher (24% vs.

73%) if patients had the *ADRB1* Ser49-Arg389/Gly49-Arg389 diplotype and carried *AGTR1* 1166C. This is a novel association, and it is difficult to explain in the context of the available literature. The biological interaction between *ADRB1* and *AGTR1* has not been directly tested in experimental models, and it is difficult to determine from a statistical interaction at what level the interaction is occurring (e.g. transcriptional, translational, functional, or physiological level). Although the CART algorithm was 10-fold cross-validated, future research should focus on validating this association in an independent HF patient cohort. If validated, this finding has important clinical implications. Like the GRS, screening for this gene-gene interaction could potentially enable informed decisions by providers, patients, and patients' families on HF medications, devices, heart transplantation, and end-of-life care.

Perspective

This dissertation research has the potential to make an impact on both HF clinical practice and HF genetic association research. The *ADRB1* Ser49Gly pharmacogenetic interaction is the major finding in this dissertation research, and it is the closest to potential clinical application. If validated, the Ser49Gly variant could be used to tailor pharmacotherapy in HF patients. In a syndrome that is as fatal and prevalent as HF, and with the widespread use of beta-blockers, screening for this single genetic variant could have a profound public health impact. This research also makes an impact on the field of HF genetic association research, by using one of the largest community HF patient cohorts with the longest follow-up to date, testing multiple genetic variants, and the novel application of advanced analytical methods such as GRS's and data mining.

Besides the potential for an impact, this dissertation research also highlights the need for shifts in the current HF clinical practice and research paradigms. The current HF clinical practice paradigm more resembles generalized medicine instead of personalized medicine. (Personalized medicine as defined by the implementation of advanced "omic" technologies). For example, beta-blockers are recommended in all HF patients with an LVEF < 40%. However the results of this dissertation research challenge that recommendation because beta-blockers may not be effective in HF patients carrying the *ADRB1* Gly49 allele. Of course, the guideline recommendation for beta-blockers in HF is based on large clinical trials demonstrating an average benefit in large HF patient populations. Such large clinical trials are designed to evaluate average population benefit and not individual patient benefit. Therefore the current HF clinical research paradigm is bolstering the practice of generalized medicine and not personalized medicine.

In order to achieve personalized HF medicine, the HF clinical research paradigm must change. Given the size of the human genome, 1) a randomized clinical trial for every potential personalized intervention is not possible, 2) traditional statistical methods can no longer handle the amount of data available, and 3) current sample sizes do not meet the demands for statistical distinction. Randomized clinical trials yield gold-standard evidence, but, in the future, other levels of evidence must also be accepted if personalized HF medicine is to become a reality. For example, drug dosage is empirically adjusted according to the renal function of a patient if the drug is known to be renally cleared, and this practice was adopted without a randomized clinical trial for every renally cleared drug. Traditional statistical methods developed in the 1960's and 70's,

such as the Bonferroni correction and Cox regression, were developed for pencil and paper calculation out of necessity. Now that computers are widely available, more advanced statistical methods, such as data mining, should be used. And finally, the current methods for recruitment of HF patients for clinical research are not meeting the demands for the large sample sizes needed to carry out genetic association research. However this could change in the future with the aid of electronic medical records and opt-out instead of opt-in research participation. In conclusion, HF clinical practice and research has come a long way, but it still has a long way to go.

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APPENDIX I:

Genetic Tailoring of Pharmacotherapy in Heart Failure: Optimize the old, while we wait for something new

Talameh JA, McLeod HL, Adams KF Jr, Patterson JH. Genetic tailoring of pharmacotherapy in heart failure: optimize the old, while we wait for something new. J Card Fail. 2012 Apr;18(4):338-49.

Abstract

Background: The combination of angiotensin-converting enzyme (ACE) inhibitors and beta-adrenergic receptor blockers remains the essential component of heart failure (HF) pharmacotherapy. However the individual patient responses to these pharmacotherapies widely vary. The variability in response cannot be entirely explained by clinical characteristics, but genetic variation may play a role. Therefore the purpose of this review is to examine our current state of understanding for beta-blocker and ACE inhibitor pharmacogenetic literature in HF.

Methods: Beta-blocker and ACE inhibitor pharmacogenetic studies performed in patients with HF were identified from the PubMed database from 1966 to July 2011.

Results: Thirty beta-blocker and ten ACE inhibitor pharmacogenetic studies in patients with HF were identified. The ACE deletion variant was associated with greater survival benefit from ACE inhibitors and beta-blockers compared to the ACE insertion. Ser49 in the beta-1 adrenergic receptor, the insertion in the alpha-2C adrenergic receptor, and Gln41 in G-protein coupled receptor kinase 5 are associated with greater survival benefit

from beta-blockers, compared to Gly49, the deletion, and Leu41, respectively. However many of these associations have not been validated.

Conclusions: The HF pharmacogenetic literature is still in its very early stages, but there are promising candidate genetic variants that may identify which HF patients are most likely to benefit from beta-blockers and ACE inhibitors and patients that may require additional therapies.

Key Words

Heart failure; beta blocker; ACE inhibitor; pharmacogenetic; pharmacogenomic; polymorphism; variant

Introduction

Advances in pharmacotherapy over the past two decades have significantly improved heart failure (HF) morbidity and enhanced survival. The combination of angiotensin-converting enzyme (ACE) inhibitors and beta-adrenergic receptor blockers remains the essential component of HF pharmacotherapy.(1) Both drugs significantly improve survival; particularly beta-blockers, which result in a 35% reduction in mortality when added to ACE inhibitors and have been firmly entrenched in HF evidence-based guidelines since the late 1990's.(2,3) However, physiological actions, side effects, and efficacy vary substantially on a patient to patient basis. For example, ACE inhibition may fail to suppress angiotensin II in HF patients and aldosterone escape is common. (4) The prevalence and severity of ACE inhibitor-induced cough varies significantly and angioedema, a rare but potentially serious side effect, remains unpredictable. (5) Treatment with beta-blockers results in widely variable effects on left ventricular ejection fraction (LVEF). A distinct minority of patients (approximately 25%) experience a marked and sustained improvement in ventricular function, while others have no change or rarely may experience a decline.(6) HF patients may experience worsening of their symptoms during beta-blocker titration, requiring increased diuretic doses and rarely discontinuation of beta-blocker therapy.(7) Unfortunately, variability in ACE inhibitor and beta-blocker clinical response is typically not predictable based on clinical characteristics. Clearly, a better understanding of the basis of variable therapeutic response to ACE inhibitors and beta-blockers would be clinically useful.

Genetic variation is expected to account for a significant part of the individual patient response to cardiovascular medication. Differences in genetic coding have been

shown to influence pharmacokinetics and pharmacodynamics, which may translate into clinical outcomes such as therapeutic efficacy and adverse events. Whether genetic tailoring of ACE inhibitors and beta-blockers will improve the response to these agents in HF is unknown. Therefore it is important to understand the current state of pharmacogenetic literature for HF. The purpose of this review is to specifically examine genetic variants influencing the response to the mainstay of HF pharmacotherapy: ACE inhibitors and beta-blockers. For papers examining the association between genetic variants and HF predisposition or survival the reader is directed elsewhere.(8-11) We will discuss 1) the *in vitro* data supporting the mechanisms for pharmacogenetic interactions in HF, 2) the translation of mechanistic data to clinical pharmacogenetic studies in patients with HF, and 3) if the current state of the literature is sufficient for routine genetic tailoring of ACE inhibitor and beta-blocker pharmacotherapy in patients with HF.

Methods

Beta-blocker and ACE inhibitor pharmacogenetic studies were identified in the PubMed database from 1966 to July 2011 by combining the following search terms: heart failure, variant, polymorphism, pharmacogenetics, pharmacogenomics, beta blocker, ACE inhibitor, and each individual drug name. Studies were also identified from the reference lists of articles. Studies were limited to those performed in patients with heart failure and those published in English.

Results

Beta-blockers

Thirty beta-blocker pharmacogenetic studies in patients with HF have been published from 2000 to 2011, and the genetic variants studied for an association with (FDA-approved) beta-blocker response in HF patients are summarized in Table 1. These studies are heterogeneous in many aspects: design, sample size, endpoint, HF patient population, specific beta-blockers, and genetic variants tested. The study designs include retrospective, prospective non-randomized cohorts, pharmacogenetic sub-studies of randomized clinical trials, and a meta-analysis. The smallest study investigated 33 HF patients,(12) whereas the largest study included 2,460.(13) The endpoints ranged from intermediate phenotypes such as heart rate and LVEF to clinical outcomes such as survival. Some studies only included HF patients with idiopathic dilated cardiomyopathy (IDC), but the majority studied HF patients with systolic dysfunction from ischemic and non-ischemic etiologies. Most studies investigated FDA-approved beta-blockers for HF such as carvedilol and metoprolol succinate. Some report findings for the investigational agent bucindolol.(14-16) The majority of studies tested genetic variants related to the sympathetic adrenergic system, but there were some that investigated genetic variants related to the renin-angiotensin-aldosterone system. (17-19) The following sections will review the most commonly studied genetic variants in the HF beta-blocker pharmacogenetic literature.

Beta-1 adrenergic receptor

The largest amount of pharmacogenetic data for beta-blockers is for the primary drug target, the beta-1 adrenergic receptor (ADRB1). ADRB1 is the principal beta-

adrenergic subtype expressed on the cardiac myocyte, and it mediates cardiac contractility. The gene for ADRB1 is localized to chromosome 10q24-q26.(20) The gene is intronless, consists of 1,714 base pairs, and codes for a 51.3 kDa protein consisting of 477 amino acid residues. There are two variants in ADRB1 that have been studied: an amino acid substitution of glycine for serine at position 49 (Ser49Gly) and a glycine substituted for arginine at position 389 (Arg389Gly) in the receptor. These variants are common in the general population, and there are racial differences in their frequencies. The frequency of these variants and other variants that will be discussed in this review are presented in Table 2.

Beta-1 adrenergic receptor variant: Ser49Gly

Functionally, Gly49 results in greater agonist-promoted down-regulation of ADRB1 compared to Ser49.(21,22) Down-regulation of the beta-adrenergic receptors is thought to be a protective adaptation in HF, where chronic sympathetic activity is toxic to the cardiac myocyte.(23) This concept suggests that the Gly49 variant may be protective in patients with heart failure and patients with this variant may be less responsive to beta block. In contrast, *in vitro* experiments also demonstrate that cells expressing Gly49 are more sensitive to the inhibitory effects of metoprolol.(21) But whether HF patients possessing Gly49 treated with metoprolol would respond favorably despite down regulation of this receptor has not been determined. A number of clinical studies have examined the association of the Ser49Gly genotype with ventricular remodeling parameters such as LVEF, left ventricular end diastolic diameter (LVEDD), and left ventricular fractional shortening (LVFS) and outcomes during beta blockade, with complex and somewhat inconsistent results that will discussed in detail below.

Terra et al studied 54 patients with systolic dysfunction receiving at least three months of the target or highest tolerated dose of metoprolol CR/XL.(24) Patients carrying Gly49 had a significant decrease in LVEDD compared to Ser49 homozygous patients in response to metoprolol CR/XL (-2 mm vs. +2 mm; p = 0.003). However changes in LVEF were not significantly different between Ser49Gly sub-groups. de Groote et al studied 199 patients with systolic dysfunction and at least three months of the maximum tolerated dose of bisoprolol or carvedilol.(25) They found no difference in LVEF or RVEF among Ser49Gly sub-groups, but de Groote et al did not compare LVEDD responses. Nonen et al studied LVFS response in 80 patients with IDC on at least six months of a variety of beta-blockers, in which they did not find an influence of Ser49Gly.(26)

There are limitations to the Terra et al,(24) de Groote et al,(25) and Nonen et al(26) studies. It is possible that the duration of beta-blocker therapy was too short, and it may take at least one year to see a complete LVEF response.(27) HF etiology is also an important consideration when using ventricular remodeling endpoints. Patients with an ischemic etiology may have irreversible damage to the myocardium. These limitations were addressed by Chen et al in a study of 135 non-ischemic cardiomyopathy patients after 1.5 years of treatment with carvedilol, and there was still no significant impact of Ser49Gly on LVEF.(28)

Although there is little data supporting an interaction between Ser49Gly and ventricular remodeling response after beta-blocker treatment, Ser49Gly may have an impact on long-term response to beta-blockers. Because Gly49 is protective against chronic sympathetic stimulation, it has been hypothesized that long-term exogenous beta-

blockade is more critical for HF patients with Ser49. Indeed, this is supported by a retrospective study by Borjesson et al.(29) In 184 patients with IDC, the survival curve for Ser49 homozygous patients treated with beta-blockers was almost identical to Gly49 carrying patients not receiving beta-blockers. This was confirmed in a study by Magnusson et al,(30) in which they added a prospective IDC cohort (n = 190) to the retrospective cohort studied by Borjesson et al.(30) Magnusson et al found that patients carrying Gly49 had a similar survival rate regardless of high-dose (>50% of target dose) or low-dose (≤ 50% of target dose) beta-blocker. However, in the group of patients treated with low-dose beta-blocker, patients carrying Gly49 had lower five-year mortality compared to patients homozygous for Ser49 (risk ratio = 0.24; p = .020).

The Borjesson et al and Magnusson et al studies need to be interpreted cautiously. These studies consisted of entirely Swedish patients, and Biolo et al did not confirm these findings prospectively in a Brazilian population (n = 201) that included ischemic etiology.(31) Racial and ethnic stratification is especially a concern for genetic association studies due to differences in allele frequencies, haplotype structure, and the genetic admixture of populations.(32) Acknowledging these differences, the Magnusson et al and Borjesson et al studies suggest that it is more critical that patients homozygous for Ser49 are treated with high dose beta-blockers, and that Gly49 carrying patients receive equal benefit regardless of high- or low-dose beta-blocker.

Beta-1 adrenergic receptor variant: Arg389Gly

Sympathetic stimulation of ADRB1 results in activation of the Gs protein, which in turn activates adenylyl cyclase and the production of cAMP.(33) Arg389 of ADRB1 displays increased coupling to Gs compared to Gly389;(34) hence Arg389 has greater

basal and agonist-stimulated activity.(34) Therefore it has been hypothesized that HF patients possessing Arg389 would have a greater response to beta-blockers. With respect to ventricular remodeling responses, this has been studied in a series of small HF cohorts.

Mialet-Perez et al retrospectively studied 224 patients with systolic dysfunction receiving carvedilol.(35) They were the first to report that patients who were homozygous for Arg389 had a significantly greater improvement in LVEF after treatment with beta-blocker than patients who were homozygous for Gly389 (+8.7% ± 1.1% versus +0.93% ± 1.7%, respectively; $p < 0.02$). Patients who were heterozygous at position 389 had a similar improvement in LVEF compared to Arg389 homozygotes (7.02% ± 1.5%). This association was confirmed in three prospective studies totaling 345 patients among a variety of etiologies (ischemic and non-ischemic), beta-blockers (metoprolol and bisoprolol), and ethnic groups (Caucasian, African-American, and Chinese).(24,28,36) However there are also three studies totaling 416 patients that failed to find a significant association.(25,37,38) Given that the series of studies investigating LVEF response were small, it is difficult to conclude if Arg389Gly is a good predictor of LVEF response to beta-blocker. However the positive studies are promising because they are consistent for the beneficial variant (Arg389). If the positive findings were purely spurious, one would expect spurious findings for the Gly389 variant as well.

Liggett, Bristow and colleagues conducted a ground-breaking prospective pharmacogenetic sub-study utilizing patients from the Beta-Blocker Evaluation of Survival Trial (BEST) study.(39) BEST was a randomized, placebo-controlled trial of the investigational novel beta antagonist bucindolol, which found that bucindolol did not significantly decrease mortality in HF patients (HR = 0.90; adjusted $p = 0.13$). However

in the pharmacogenetic sub-study of 1040 patients,(14) these investigators found response to bucindolol varied by genotype. Patients homozygous for Arg389 had a statistically significant improvement in survival compared to placebo (HR = 0.62; p = 0.03), whereas Gly389 carriers did not (HR = 0.90; p = 0.57). In contrast, these results do not seem to apply to beta-blockers currently used to treat HF. White et al performed a pharmacogenetic sub-study (40) consisting of 600 patients from MERIT-HF,(41) a randomized, controlled trial for the effectiveness of metoprolol CR/XL in chronic HF. They did not find an association of Arg389Gly with the primary outcome of all-cause mortality or hospitalization in either the metoprolol CR/XL or placebo treated groups. Cresci et al also found no association of Arg389Gly genotype with all-cause mortality in a prospective registry of two independently recruited US heart failure populations where baseline beta blocker therapy, if utilized, was predominantly metoprolol or carvediolol. (13) These findings are also consistent with the lack of association of Arg389Gly genotype with mortality described by Sehnert et al in a prospective registry study of 637 patients that were all treated with beta-blockers.(42) Only a small study of 201 HF patients with a limited number of events reported by Biolo et al found results consistent with Liggett et al, where metoprolol and carvedilol appeared to be more effective at high doses in decreasing HF-related mortality in patients carrying the Arg389 allele.(31) Although additional study is needed, these discrepant results are most likely related to the unique pharmacological properties of bucindolol which include marked suppression of the beta-1 receptor activity in patients homozygous for Arg389.

Beta-2 adrenergic receptor

In HF, chronic adrenergic stimulation causes down-regulation of ADRB1, but not the beta-2 adrenergic receptor (ADRB2). This causes a change in the ratio of ADRB1:ADRB2 from approximately 80:20 in healthy heart tissue to approximately 60:40 in the failing heart.(43) Therefore the use of beta-1 selective versus non-selective beta-blockers in HF remains a clinical issue. The gene for ADRB2 is localized to chromosome 5q31-q32.(44) ADRB2 consists of a single exon of 2015 nucleotides which encodes a 413 amino acid protein. There are three variants in ADRB2 that have been studied: Gly16Arg, Gln27Glu, and Thr164Ile.

Beta-2 adrenergic receptor variant: Gly16Arg

Although the density of ADRB2 in HF is unchanged compared to ADRB1, ADRB2 is subject to desensitization via functional uncoupling from the intracellular G protein, Gs.(33) A glycine (Gly) at amino acid position 16 results in increased agonist-promoted desensitization compared to arginine (Arg).(45) The pharmacogenetic interaction between this variant and beta-blockers has not been studied *in vitro.* However it has been hypothesized that because Gly16 allows for greater desensitization of ADRB2, HF patients possessing Gly16 have "genetic beta-blockade." "Genetic beta-blockade," or the lack thereof, may interact with exogenously administered beta-blocker.

Six clinical studies tested the Gly16Arg variant in 738 HF patients,(25,26,28,38,46,47) and none found a significant association between Gly16Arg and beta-blocker response with respect to beta-blocker tolerability, LVEF, or LVFS. Importantly, in three of these studies the patients received beta-1 selective beta-blockers, which could have limited the power to detect a pharmacogenetic interaction with

ADRB2. Acknowledging this limitation, it seems unlikely that this variant could have a clinically meaningful pharmacogenetic interaction with beta-blockers.

Beta-2 adrenergic receptor variant: Gln27Glu

A glutamine (Glu) at amino acid position 27 in ADRB2 is resistant to agonist-promoted desensitization,(45) and in contrast to Gly16Arg, there is clinical literature to support a pharmacogenetic interaction with beta-blockers. Although the pharmacogenetic interaction has not been studied *in vitro*, it has been hypothesized that patients with Glu27 will be more responsive to beta-blockers because they have more sensitive ADRB2. Indeed, this has been confirmed in three clinical studies evaluating LVEF changes. Kaye et al were the first to report this pharmacogenetic interaction in a retrospective study of 80 HF patients on at least 4 months of carvedilol.(47) They defined good responders as having an increase in LVEF of at least 10%, or an increase in LVFS of at least 5%. Patients homozygous for Gln27 had a significantly lower proportion of good responders than patients who were carrying Glu27 (26% versus 63%, $p = 0.003$). These findings were confirmed in two prospective studies by Troncoso et al(12) and Metra et al,(38) which totaled 216 patients with systolic dysfunction and receiving carvedilol. Troncoso et al and Metra et al also found that Glu27 was associated with a favorable beta-blocker response in other parameters such as heart rate,(12) malondialdehyde levels (a marker of oxidative stress),(12) and pulmonary wedge pressure both at rest and peak exercise.(38) There are four studies that did not find a significant association between Gln27Glu and ventricular remodeling response to beta-blockers.(24-26,28) Although the majority of the data still support the pharmacogenetic

interaction because the negative studies are small (n < 200) and most included beta-1 selective beta-blockers.

Beta-2 adrenergic receptor variant: Thr164Ile

An isoleucine (Ile) substitution for threonine (Thr) at amino acid position 164 in ADRB2 has profound effects on receptor function *in vitro.* Ile164 demonstrates a substantial decrease in basal and agonist-stimulated activity due to defective coupling of the receptor to the stimulatory G protein, Gs.(48) Ile164 also has a lower affinity for beta-blockers.(48) This is a rare allele (Table 2) so definitive studies are lacking, but Liggett et al found suggestive evidence of a counterintuitive adverse association between the presence of the Ile164 genotype and poor outcome in HF.(9) This finding, coupled with the observation that Ile164 also has a lower affinity for beta-blockers, led to the hypothesis that HF patients with Ile164 would be less responsive to beta-blockade. However an exploratory clinical pharmacogenetic study by Littlejohn et al had an even more surprising finding.(49) These investigators retrospectively studied the association of survival with Ile164 genotype in 443 patients with HF. The Thr164 homozygotes demonstrated the expected mortality benefit from an average 3.09 years of beta-blockade (55.2% mortality rate without beta-blocker and 39.5% mortality rate with beta-blocker; p = 0.004). Only 14 patients were heterozygous for Ile164, and no homozygotes were found. Surprisingly, the beta-blocker effect was reversed in patients carrying Ile164. There was a 2-fold higher mortality rate in the seven Ile164 heterozygous patients treated with beta-blocker (57.1%), compared to the seven Ile164 heterozygous patients not treated with beta-blocker (28.6%). This could be due to the excessive impairment of cardiac function via the combination of dysfunctional ADRB2 and pharmacologic

blockade. The difference in mortality between beta-blocker treated and untreated Ile164 heterozygous patients was not statistically significant (p = 0.247), but the sample size was small. Three other studies failed to find a significant association.(25,26,28)

Alpha-2C adrenergic receptor

The function of the alpha-2C adrenergic receptor (ADRA2C) is pre-synaptic auto-inhibition of norepinephrine release. The gene is localized to chromosome 4p16.3 - p16.3. An insertion/deletion variant in ADRA2C results in a four amino acid loss at positions 322-325. The deletion results in the loss of normal auto-inhibitory receptor function and hence increased presynaptic release of norepinephrine.(50) Although not studied *in vitro*, it is possible that the deletion is associated with beta-blocker response, especially when it is inherited with other genetic variants affecting sympathetic activity. For example, HF patients with ADRB1 Arg389 (with increased agonist-promoted activity) and the ADRA2C deletion (with increased presynaptic release of norepinephrine) could have enhanced beta-adrenergic receptor activity and hence greater response to beta-blockade.

Alpha-2C adrenergic receptor variant: deletion 322-325

Lobmeyer et al investigated the possible interaction between ADRB1 Arg389, the ADRA2C insertion/deletion, and beta-blocker response in 54 HF patients with systolic dysfunction.(51) The deletion carriers had an increased improvement in LVEF compared to insertion homozygotes (+6% versus +1%; p = 0.045). Synergy between the ADRB1 and ADRA2C variants was supported by the magnitude of results, in that patients both homozygous for Arg389 and a deletion carrier exhibited the greatest LVEF response compared to all other genotypes (+12% versus +2% as the greatest change in all other

genotypes; $p < 0.05$ for all comparisons). Nonen et al also investigated ventricular changes (LVFS) in response to beta-blocker and ADRA2C status in 80 IDC patients, but did not find a significant association.(26) However Nonen et al did not test for synergy between ADRB1 Arg389 and the ADRA2C deletion. The discrepancy in results between the Lobmeyer et al and Nonen et al studies could be due to population differences. Lobmeyer et al studied Caucasians and African-Americans with systolic dysfunction due to ischemic and non-ischemic etiologies. Nonen et al studied Japanese patients solely with IDC. Whether the influence of ADRA2C on LVEF is population- specific or only important when inherited in combination with Arg389Gly variants remains unknown.

The complexity of adrenergic regulation through ADRA2C was highlighted in a pharmacogenetic sub-study consisting of 1040 patients from BEST(39) by Bristow et al.(16) Although Lobmeyer et al found that deletion carriers experienced greater LVEF improvement with beta-blocker, Bristow et al found that deletion carriers did not experience survival benefit from beta-blockade ($HR = 1.09$; $p = 0.80$).(16) However Bristow et al found that the insertion homozygous patients experienced survival benefit ($HR = 0.70$; $p = 0.025$). Importantly, the beta-blocker investigated by Bristow et al was bucindolol. Bristow et al previously showed that the marked sympatholysis caused by bucindolol results in increased mortality and HF hospitalizations compared to patients with little or no sympatholytic response.(52) Indeed, this was the case in deletion-carriers. In bucindolol-treated patients, a comparison of homozygous ADRA2C insertion and deletion carriers revealed that deletion carriers had a 3.1-fold greater reduction in norepinephrine ($p = 0.001$). Marked sympatholysis is unique to bucindolol; therefore it is

unclear if the results from Bristow et al can be applied to other beta-blockers, and further study is needed.

G-protein coupled receptor kinase 5

The function of the G-protein receptor kinases is to desensitize ligand-occupied G-protein coupled receptors such as beta-adrenergic receptors.(53) GRK5 is localized to chromosome 10q26.11 - q26.11. Liggett and co-workers studied a variant in G-protein coupled receptor kinase 5 (GRK5) that changes amino acid 41 from glutamine (Gln) to leucine (Leu) both *in vitro* and in association with outcomes in HF patients. The Leu41 allele more effectively desensitizes agonist-stimulated responses.(54) Because patients with Gln41 have more sensitive beta-adrenergic receptors, Liggett et al hypothesized that HF patients with Gln41 would have a greater response to beta-blockade.

G-protein coupled receptor kinase 5 variant: Gln41Leu

Liggett et al examined this potential pharmacogenetic interaction both retrospectively and prospectively in HF patients.(54) In a case-control study, Liggett et al found a significant pharmacogenetic interaction, but only in the African-American sub-group (n = 242), not in European-Americans (n = 568). They then confirmed these findings in a prospective, observational study of a second cohort of 375 African-Americans with HF, where Liggett et al found that only individuals who were homozygous for Gln41 had significantly improved transplant-free survival with beta-blocker treatment (HR = 0.22; $p < 0.001$). There was no difference in this outcome in patients carrying Leu41 with or without beta-blocker (HR = 0.78; p = 0.53). Cresci et al found similar results in a combined cohort of African-American HF patients. (13) In the overall cohort, there was a trend for a beta-blocker treatment effect (HR = 0.698; p =

0.1). However in a sub-group of ADRB1 Gly389 homozygous/GRK5 Gln41 homozygous African-Americans, beta-blockers did provide mortality benefit (HR: 0.385; p = 0.012). When these investigators matched African-Americans and Caucasians by GRK5 genotype and beta-blocker treatment, survival was similar in the two races. These findings must be considered with some caution due to the limited number of events in the first prospective cohort, overlapping composition of the study populations, and the registry design used in these studies. Whether there are differences in beta-blocker treatment effect between Caucasians and African-Americans, has been a subject of controversy.(55) Additional prospective studies are needed, but the work of Liggett and co-workers suggests that genetic variation among African-Americans with heart failure could help explain the heterogeneous efficacy of beta blockade observed in this racial group.

Angiotensin-converting enzyme

The genetic variants discussed to this point are related to the sympathetic adrenergic system, but the renin-angiotensin-aldosterone system (RAAS) also contributes to worsening of the HF syndrome. ACE plays a critical role in the RAAS, where it converts angiotensin I to angiotensin II resulting in downstream effects including sodium and water retention and vasoconstriction. The ACE gene is localized to chromosome 17q23.3 - q23.3, and it comprises 26 exons that are alternately spliced to give two isoforms. The predominant isoform contains exons 1-12 and 14-26 and when translated results in a 1306 amino acid protein. Since its discovery, a 287 base pair insertion/deletion in intron 16 of ACE has been the most studied cardiovascular-relevant variant. The ACE insertion/deletion accounts for half of the variance in serum ACE

levels,(56) with the deletion allele conferring significantly higher levels. Beta-blockers have been shown to decrease RAAS activity in HF,(57) probably via inhibition of ADRB1 present in the kidney, where activation leads to release of renin and ultimately aldosterone.(58) Because the ACE deletion results in higher RAAS activity, it has been hypothesized that HF patients with the ACE deletion would have a greater response to beta-blockers.

Angiotensin-converting enzyme variant: intron 16 insertion/deletion

In 2001, McNamara et al were the first to publish this pharmacogenetic interaction in a cohort of 328 HF patients followed for a median 21 months.(17) In the overall cohort, there was a trend for increased transplant-free survival in patients receiving beta-blockers ($p = 0.065$). However when the ACE insertion/deletion sub-groups were analyzed individually, only patients homozygous for the deletion had a significant improvement in transplant-free survival from beta-blockade (deletion homozygous: $p = .007$; insertion homozygous: $p = 0.74$; heterozygous: $p = 0.59$). These results were validated in another study published by McNamara et al in 2004,(18) when the size of the cohort increased from 328 to 479. de Groote et al also tested this pharmacogenetic interaction in 199 HF patients, but did not yield a significant result with respect to LVEF, peak VO_2, or cardiac survival.(19) However the de Groote et al study was smaller ($n = 199$) than McNamara et al. Therefore based on the results from McNamara et al and the profound functional effects of the ACE insertion/deletion, further study is needed.

ACE inhibitors

The majority of pharmacogenetic literature in HF patients has focused on beta-blockers. Although there have been ten studies from 1998-2010 evaluating pharmacogenetic interactions with ACE inhibitors. A summary of the genetic variants studied for an association with ACE inhibitor response is presented in Table 3. Like the beta-blocker pharmacogenetic literature, the study designs and HF patient populations are diverse. The sample sizes were small, with the majority having less than 200 patients, and the largest study having 479 patients. Not surprisingly, along with a few other genes in the RAAS, ACE was the most commonly studied gene.

Angiotensin-converting enzyme variant: intron 16 insertion/deletion

Because the deletion allele results in significantly higher ACE levels,(56) it has been hypothesized that HF patients possessing the deletion will require a higher dose of ACE inhibitor to achieve the same response as a patient without a deletion allele. Most of the studies investigating intermediate phenotypes such as mean arterial pressure, aldosterone escape, and serum ACE activity support this hypothesis.

In a small (n = 34), double-blind crossover study of captopril and lisinopril, O'Toole et al found that the insertion allele was associated with a greater decrease in mean arterial pressure in patients with HF.(59) However this was only found with captopril and not lisinopril. It is possible that short- and long-acting ACE inhibitors interact with ACE in different ways, with more complete suppression of ACE activity with the longer-acting lisinopril. Cicoira et al(60) addressed the clinical issue of "aldosterone escape," in which up to 38% of patients with HF have elevated plasma levels of aldosterone despite long-term ACE inhibitor therapy.(4) In a study of 132

patients with HF, Cicoira et al prospectively defined aldosterone escape as the presence of aldosterone plasma concentrations above the upper limit of the reference range (>42 nmol/L) after at least 6 months of ACE inhibitor. Thirteen patients had aldosterone escape, and there was a significantly higher frequency of the deletion allele in these patients compared to those who did not experience aldosterone escape (62% vs. 24%; p = 0.005). Of those that experienced aldosterone escape, none were homozygous for the insertion allele. Tang et al also investigated aldosterone escape in a smaller HF cohort (n = 74), but aldosterone escape was not affected by ACE genotype.(61) However Tang et al did find that pre-dose and post-dose ACE activity remained consistently higher in deletion homozygotes.

The relationship between the ACE genotype and the intermediate phenotype of LVEF improvement after ACE inhibitor is not clear. Tiago et al tested this interaction in 107 IDC patients.(62) After 2.5 years of ACE inhibitor therapy, the LVEF improvement was similar among ACE genotypes, but there was a high amount of variability in LVEF changes (deletion homozygotes LVEF change = 9% ± 13%; insertion carriers = 8% ± 13%). Cuoco et al prospectively studied 168 patients with systolic dysfuction.(63) Deletion carriers responded better to ACE inhibitor with respect to LVEF than insertion homozygotes (change in LVEF for deletion carriers = +8.8 %; insertion homozygotes -1.73%; p = 0.01). Perhaps there are population-specific effects of the ACE insertion/deletion on LVEF response. Tiago et al studied IDC patients of African ancestry and Cuoco et al studied Brazilian patients with systolic dysfunction due to ischemic and non-ischemic etiologies.

The relationship between the ACE variant and survival benefit from ACE inhibitors is more clear than the intermediate phenotypes. McNamara et al investigated this pharmacogenetic interaction with the clinical endpoint of death or cardiac transplantation.(18) This was the largest ACE inhibitor pharmacogenetic study in HF patients (n = 479), and investigated the interaction with beta-blockers as well. McNamara et al found a dose-dependent relationship between the ACE insertion/deletion and transplant-free survival. After a median follow-up of 33 months, patients on low-dose ACE inhibitors (≤ 50% of target dose) had poorer transplant-free survival associated with the deletion allele, with a relative risk for deletion homozygotes of 2.07 (p = 0.03). This was exaggerated in patients who were also not receiving a beta-blocker, with a relative risk for deletion homozygotes of 2.75 (p = 0.012). However high-dose ACE inhibitor (>50% of target dose), with or without concomitant beta-blocker, eliminated the adverse effect of the ACE deletion. Although the deletion allele was associated with poorer transplant-free survival, it seemed that deletion homozygotes benefitted the most from ACE inhibitor and beta-blocker therapy. For example, beta-blockers only reduced the event rate in deletion homozygotes (53%; p = 0.004), but not for patients who were heterozygotes (15%; p = 0.46) or insertion homozygotes (3%; p = 0.94).

Wu et al investigated this pharmacogenetic interaction to the endpoint of death from any cause,(64) and this is the only pharmacogenetic study published exclusively analyzing patients with preserved ejection fraction. There was a long follow-up to the primary endpoint, in which the median was 8.7 years for the ACE inhibitor-treated group, and 6.4 years in the non-ACE inhibitor treated group. Similar to the findings by McNamara et al, the deletion allele was associated with all-cause mortality in patients not

receiving an ACE inhibitor (HR = 2.23; p = 0.008), but not for patients receiving an ACE inhibitor (HR = 1.64; p = 0.20). Acknowledging that the McNamara et al and Wu et al studies were performed in observational cohorts in which the patients were not randomized to ACE inhibitor treatment, the ACE data seem to indicate that HF patients with the deletion allele need to be treated with ACE inhibitors to compensate for the increased ACE activity associated with the deletion and also to demonstrate similar outcomes compared to patients with the insertion.

Discussion

The first report of a pharmacogenetic interaction in HF patients was published 13 years ago.(59) Since then, the work of Liggett, Bristow and colleagues concerning the investigational drug bucindolol provides the best evidence yet to support that genetic variation can be associated with differential response to HF pharmacotherapy which can in turn impact the risk of adverse outcomes. Unfortunately, the literature as a whole does not provide sufficient evidence to guide application of available HF drug therapy based on genetic testing.

There are numerous possible explanations for why initial pharmacogenetic associations have failed to be replicated in subsequent studies. Outcome studies are particularly problematic in this field as they almost uniformly lack statistical power due to insufficient event rates (from small sample size and/or short follow-up). The choice of endpoint is also important because studies noted differences in clinical outcome endpoints without detecting differences in surrogates like LVEF or heart rate.(14,16) Publication bias is a common problem in genetic association literature, in which initial

reports of pharmacogenetic associations are false positives due to chance alone or exaggerated due to sampling bias.(32) There are still many gaps in investigation in the HF pharmacogenetic literature. Many of the genetic candidates described to date are common in the population; therefore any given HF patient is likely to possess multiple genetic variants, and the consequences of that have not yet been studied. There is some evidence, such as in the study by Lobmeyer et al(51), that inheriting two genetic variants within the sympathetic adrenergic system has synergistic effects. The literature for ACE inhibitors is not nearly as developed for beta-blockers, and the influence of genetic variants in patients receiving combination anti-neurohormonal therapy is not clear. In an era where the vast majority of HF patients are being treated with combination anti-neurohormonal therapy, teasing out the complex interplay between dual pharmacologic inhibition of the sympathetic adrenergic and renin-angiotensin-aldosterone systems and multiple genetic variants within these systems is difficult. To move the field of HF pharmacogenetics forward, adequately-powered, prospective HF patient cohorts with extensive genotyping and association analyses are needed.

Despite the shortcomings of the current state of HF pharmacogenetic literature, hypothesis-generating studies suggest a number of promising candidates (ADRB1 Ser49Gly, the ADRA2C insertion/deletion, and GRK5 Gln41Leu in African-Americans) for future genetic tailoring of HF pharmacotherapy, and a potential explanation for variable beta-blocker response in African-Americans. The next steps for the potential candidate variants are validation in large, independent HF patient cohorts and then prospective evaluation of interventions based on genotype. In a syndrome that is as fatal

and prevalent as HF, any information that could improve pharmacotherapy decision-making would have profound patient and public health benefit.

Disclosures

The authors have no conflicts of interest to disclose.

Table 1. Genetic variants studied in relation to beta-blocker response in HF patients.

GENE NAME	**GENE SYMBOL**	**VARIANT**	***rsID**	**MOLECULAR PHENOTYPE (Reference)**	**CLINICAL PHENOTYPE (Reference)**
Angiotensin-converting enzyme	*ACE*	Ins/Del	**rs1799752	Higher serum ACE levels with Del(56)	Decreased survival with Del in patients without beta-blocker, but no influence in patients with beta-blocker.(17,18) One study found no association.(19)
Adrenergic receptor alpha-1D	*ADRA1D*	T1848A	rs2236554	Unknown	Greater improvement in LVFS and LVDD with 1848A.(26)
		A1905G	rs709024	Unknown	Greater improvement in LVFS with 1905G.(26)
Adrenergic receptor alpha-2C	*ADRA2C*	Ins/Del	rs61767072	Enhanced release of norepinephrine with Del(50)	Greater LVEF improvement with Del.(51) Studies found no association with LVEF (16,26) and survival.(42) Survival benefit in Ins homozygotes but not Del carriers.(16)
Adrenergic receptor beta-1	*ADRB1*	Ser49Gly	rs1801252	Greater agonist-promoted down-regulation with Gly49(21,22)	Greater LVEF,(24,36) titration,(46) and survival(29,30) response with Gly49, but there are several negative studies. (14,25,26,28,31,37,42)
		Arg389Gly	rs1801253	Greater basal and agonist-simulated activity with Arg389(34)	Greater LVEF,(24,28,35,36,38,51,65) titration,(24) and survival response(31)(30) with Arg389. There are also negative (13,25,26,37,40,42,66) and conflicting studies(67).
Adrenergic receptor beta-2	*ADRB2*	Arg16Gly	rs1042713	Increased agonist-promoted desensitization with Gly16(45)	Several negative studies (25,26,28,38,42,46,47)
		Gln27Glu	rs1042714	Resistance to agonist-promoted desensitization with Glu27(45)	Greater LVEF,(12,38,47) heart rate,(12) and survival response(67) with Glu27. There are also negative studies.(25,26,28,42,46)
		Thr164Ile	rs1800888	Decreased basal and agonist-stimulated activity with Ile164(48)	Decreased survival with Ile164 in patients with beta-blocker.(49) There are also negative studies.(25,26,28)
Cytochrome P450 2D6	*CYP2D6*	EM/IM/PM	n/a	Extensive, intermediate, or poor metabolism.(68)	CYP2D6 phenotype was significantly associated with dose of carvedilol, but not metoprolol.(66) There is also a negative study.(46)
G-protein coupled receptor kinase 5	*GRK5*	Gln41Leu	rs17098707	Increased agonist-promoted desensitization with Leu41(54)	Greater survival response with Gln41(13,54)
Norepinephrine transporter	*NET*	T-182C	rs2242446	Potentially affects transcription(69)	Greater improvement in LVFS with -182T(26)
UDP glucuronosyltransferase 1 family polypeptide A1	*UGT1A1*	EM/IM/PM	n/a	Extensive, intermediate, or poor metabolism(70)	No association with dose of carvedilol or metoprolol.(66)

*The rsID, or reference single nucleotide polymorphisms (SNP) identifier, is a unique number for each genetic variant assigned by the National Center for Biotechnology Information SNP database.

**The ACE insertion/deletion variant has also been assigned the following rsIDs: rs4340, rs13447447, and rs4646994.

Arg = arginine, EM/IM/PM = extensive/intermediate/poor metabolizer, Gln = glutamine, Glu = glutamic acid, Gly = glycine, Ile = isoleucine, Ins/Del = insertion/deletion, LVEF = left ventricular ejection fraction, LVFS = left ventricular fractional shortening, LVDD = left ventricular diastolic diameter, Ser = serine, Thr = threonine

Table 2. Minor allele frequencies of the most commonly studied genetic variants in the beta-blocker and ACE inhibitor HF pharmacogenetic literature

Gene name	Gene symbol	*rsID	Variant	Minor Allele	Caucasian (Reference)	African-American (Reference)
Angiotensin-converting enzyme	*ACE*	**rs1799752	Ins/Del	Ins	44% (71)	43% (71)
Adrenergic receptor alpha-2C	*ADRA2C*	rs61767072	Ins/Del	Del	4% (16)	43% (16)
Adrenergic receptor beta-1	*ADRB1*	rs1801252	Ser49Gly	Gly	17% (30)	25% (72)
		rs1801253	Arg389Gly	Gly	27% (14)	38% (14)
Adrenergic receptor beta-2	*ADRB2*	rs1042713	Gly16Arg	Arg	40% (73)	50% (73) (Africans)
		rs1042714	Gln27Glu	Glu	42% (25)	20% (71)
		rs1800888	Thr164Ile	Ile	2% (25)	<2% (73) (Africans)
G-protein coupled receptor kinase 5	*GRK5*	rs17098707	Gln41Leu	Leu	2% (54)	24% (54)

*The rsID, or reference single nucleotide variant (SNP) identifier, is a unique number for each genetic variant assigned by the National Center for Biotechnology Information SNP database.

**The ACE insertion/deletion variant has also been assigned the following rsIDs: rs4340, rs13447447, and rs4646994.

Arg = arginine, Gln = glutamine, Glu = glutamic acid, Gly = glycine, Ile = isoleucine, Ins/Del = insertion/deletion, Leu = leucine, Ser = serine, Thr = threonine

Table 3. Genetic variants studied in relation to ACE inhibitor response in HF patients. variants studied in relation to beta-blocker response in HF patients.

GENE NAME	**GENE SYMBOL**	**VARIANT**	***rsID**	**MOLECULAR PHENOTYPE (References)**	**CLINICAL PHENOTYPE (References)**
Angiotensin-converting enzyme	*ACE*	Ins/Del	**rs1799752	Higher serum ACE levels with Del(56)	Greater reduction in mean arterial pressure with Ins,(59) higher frequency of aldosterone escape with Del,(60) higher serum ACE activity pre- and post-dose with Del (61), greater improvement in LVEF with Del,(63) poorer survival with Del in low-dose or non-ACE inhibitor treated patients.(18,64) Also a negative LVEF study.(62)
Adrenergic receptor beta-1	*ADRB1*	Arg389Gly	rs1801253	Greater basal and agonist-simulated activity with Arg389(34)	No association with LVEF.(74)
Adrenergic receptor beta-2	*ADRB2*	Arg16Gly	rs1042713	Increased agonist-promoted desensitization with Gly16(45)	No association with LVEF.(75)
		Gln27Glu	rs1042714	Resistance to agonist-promoted desensitization with Glu27(45)	No association with LVEF.(75)
Angiotensinogen	*AGT*	M235T	rs699	Higher plasma angiotensinogen levels with 235T(76,77)	No association with LVEF(62) or survival.(64)
Angiotensin II receptor type 1	*AGTR1*	A1166C	rs5186	Greater responsiveness(78) and sensitivity(79) to angiotensin II with 1166C	Increased all-cause mortality regardless of ACE inhibitor treatment with 1166C.(64)
Cytochrome P450 family 11 subfamily B polypeptide 2	*CYP11B2*	C-344T	rs1799998	Increased aldosterone production with -344C(80)	Greater improvement in LVEF with -344C. (62)
Tumor necrosis factor alpha	*TNF-α*	G-308A	n/a	Increased transcription with -308A(81)	No association with LVEF.(82)

*The rsID, or reference single nucleotide variant (SNP) identifier, is a unique number for each genetic variant assigned by the National Center for Biotechnology Information SNP database.

**The ACE insertion/deletion variant has also been assigned the following rsIDs: rs4340, rs13447447, and rs4646994.

Arg = arginine, Gln = glutamine, Glu = glutamic acid, Gly = glycine, Ins/Del = insertion/deletion, LVEF = left ventricular ejection fraction

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APPENDIX II:

Pharmacogenetics in Chronic Heart Failure: New developments and current challenges

Talameh JA, Lanfear DE. Pharmacogenetics in chronic heart failure: new developments and current challenges. Curr Heart Fail Rep. 2012 Mar;9(1):23-32.

Abstract

The individual patient responses to chronic heart failure (HF) pharmacotherapies are highly variable. This variability cannot be entirely explained by clinical characteristics, and genetic variation may play a role. Therefore, this review will summarize the background pharmacogenetic literature for major HF pharmacotherapy classes (ie, β-blockers, angiotensin-converting enzyme inhibitors, digoxin, and loop diuretics), evaluate recent advances in the HF pharmacogenetic literature in the context of previous findings, and discuss the challenges and conclusions for HF pharmacogenetic data and its clinical application.

Keywords Pharmacogenetics; Pharmacogenomics; Personalized medicine; Heart failure; Polymorphism; Beta-blocker; Angiotensin-converting enzyme inhibitor; Angiotensin receptor blocker; Aldosterone antagonist; Loop diuretic; Digoxin; Hydralazine isosorbide dinitrate

Introduction

Large clinical trials demonstrate, on average, that pharmacotherapy significantly decreases morbidity and/or mortality due to heart failure (HF). However, the individual patient responses to HF pharmacotherapies are highly variable. For example, long-term treatment with angiotensin-converting enzyme (ACE) inhibitors fails to suppress angiotensin II in as many as 15% of HF patients, and aldosterone in 38% (1). Long-term optimal dosing of β-blockers fails to improve left ventricular ejection fraction (LVEF) over 5% in as many as 43% of HF patients (2). The maintenance dose of loop diuretics can range from no diuretic at all to over 400–mg furosemide equivalents (3). Even when dosed according to age, sex, weight, renal function, and concomitant pharmacotherapy, the serum concentration of digoxin can range from 0.5 ng/mL to over 2.0 ng/mL (4).

This wide variability in response to HF pharmacotherapies is not entirely explained by clinical characteristics, which is evident in large clinical trials where there is a similar response among most clinical subgroups (5, 6). Genetic variation may additionally contribute to differences in drug response, the study of which is referred to as “pharmacogenetics” or “pharmacogenomics” (7). Pharmacogenetics has proven successful in other therapeutic areas (8), but whether it can be used to improve the application of pharmacotherapies for HF remains unproven. Therefore, this review will summarize the background pharmacogenetic literature for major classes of HF pharmacotherapy, critically evaluate the most current HF pharmacogenetic literature in this context, and discuss the conclusions and remaining challenges to clinical application of pharmacogenetics in HF.

β-Blocker Pharmacogenetics: Background (Literature Before 2010)

HF pharmacogenetic studies have focused on β-blockers (BBs) much more than any other class of HF pharmacotherapy. Between 2000 and 2009, 25 studies were published. Much BB pharmacogenetic data comes from small (n < 400) observational cohorts of HF patients with systolic dysfunction. These studies are heterogeneous in many aspects (eg, design, size, end point, patient population, specific BB, and the genetic variants tested), making definitive conclusions difficult. A list of genetic variants associated with BB (and other HF pharmacotherapies) response is displayed in Table 1, and the major findings are discussed herein.

Type 1 β-Adrenergic Receptor

Stimulation of the cardiac β-adrenergic receptors results in increased heart rate and contractility. The type 1 β-adrenergic receptor (protein ADRB1; gene *ADRB1*) is the primary target of cardiac BBs and has been the focus of most BB pharmacogenetic literature. A nonsynonymous variant in this gene, Arg389Gly, has been the most-studied (Table 1). This variant is common in the general population, with differences in its frequency among the races (Table 2). Functionally, Arg389 has greater basal and agonist-stimulated activity compared to Gly389 (9). Because Gly389 generally results in less ADRB1 sympathetic stimulation, it raises the question of whether this variant is protective in BB-naïve HF patients, and whether patients with Arg389 would receive greater benefit from BBs.

Consistent in the literature, patients possessing the Arg allele have greater LVEF improvement in response to BBs than those possessing Gly389. This comes from several prospective and retrospective studies totaling 569 patients with a variety of HF etiologies, ethnicities, and BBs (10–13). There is also evidence to support the influence of Arg389Gly on the survival benefit from BBs. The most convincing is a large (n = 1040) pharmacogenetic substudy (14) of the Beta-Blocker Evaluation of Survival Trial (BEST) (15). Patients homozygous for Arg389 had a statistically significant improvement in survival with bucindolol compared to placebo (HR 0.62; P = 0.03), whereas Gly389 carriers did not (HR 0.90; P = 0.57). It is argued whether the results for bucindolol can be applied to other BBs because of its unique pharmacologic properties (16). While these results were replicated in a prospective observational study of 201 HF patients treated with metoprolol or carvedilol (17), other larger cohort studies have not found this association (18). A pharmacogenetic substudy of the Metoprolol CR/XL Randomized Intervention Trial in Congestive Heart Failure (MERIT-HF) (5) also did not find an association of Arg389Gly with survival benefit regardless of treatment (metoprolol CR/XL or placebo) (19). However, this last study did not test BB effect within genotype groups (as was done in BEST), which may help explain the discordant results.

Type 2 β-Adrenergic Receptor

Although not the primary target of BBs, the type 2 β-adrenergic receptor (protein ADRB2; gene *ADRB2*) is present in myocardium, can mediate inotropic response, and while ADRB1 is downregulated, the expression of ADRB2 is unchanged in the failing

heart (20). The most-studied variant in ADRB2 is Gln27Glu. Functionally, Glu27-containing *ADRB2* is resistant to agonist-promoted desensitization compared to Gln27 (21, 22). This suggests that Gln27 genotype is associated with less sympathetic output relative to Glu27, but the clinical pharmacogenetic literature is inconsistent. Several small studies showed a favorable LVEF response for patients carrying Glu27 compared to patients homozygous for Gln27 (23–25). However, four other small studies failed to show a significant association (11, 12, 26, 27), although these included β1-selective BBs, which may limit the ability to detect an interaction with *ADRB2* variants. In terms of survival benefit, one study showed a survival difference by genotype among BB-treated HF patients (28), but several large cohort studies have not demonstrated an association (18, 26, 29). Notably, most patients in these studies were treated with BBs, limiting the ability to examine true pharmacogenetic interactions.

Adrenergic Receptor α-2C

The α-2C adrenergic receptor is presynaptic (protein ADRA2C; gene *ADRA2C*), auto-inhibiting norepinephrine release. A deletion variant in *ADRA2C*, causing a loss of amino acids 322 to 325, results in the loss of normal auto-inhibitory function and increased norepinephrine (30). A potential interaction between *ADRB1* Arg389, the *ADRA2C* deletion, and BB response was prospectively studied in 54 HF patients with systolic dysfunction (31). The deletion carriers had a greater improvement in LVEF compared to insertion homozygotes (+6% vs +1%; $P = 0.045$). Synergy between the *ADRB1* and *ADRA2C* variants was supported by the magnitude of results. No association was found

in 80 patients with idiopathic dilated cardiomyopathy (27), but synergy with *ADRB1* Arg389 was not tested.

G Protein–Coupled Receptor Kinase 5

The function of the G-protein receptor kinases (GRK) is to desensitize ligand-occupied G protein–coupled receptors such as β-adrenergic receptors (32). *GRK5* is abundant in the heart and a Gln41Leu variant in this gene has been studied in vitro, with the Leu41 version more effectively desensitizing agonist-stimulated responses compared to Gln41 (33). Because the Gln41 subtype should have more active β-adrenergic receptors, it has been proposed that Leu41 is protective in BB-naïve HF patients, but these patients may be less responsive to BB.

In an observational study of 375 African-Americans with HF, only patients homozygous for Gln41 had significantly improved transplant-free survival with BB (HR 0.22; $P < 0.001$) (33). There was no difference in outcome for patients carrying Leu41 with or without BB (HR 0.78; $P = 0.53$). Similar results were found in another study featuring 711 African-American HF patients (34). Among all of the African-American patients, the BB treatment effect did not reach statistical significance (HR 0.698; $P = 0.1$). However in a genetic subgroup of *ADRB1* Gly389 homozygous/*GRK5* Gln41 homozygous African-Americans, BBs were associated with marked mortality benefit (HR 0.385; $P = 0.012$). Interestingly, when the African-Americans and Caucasians were matched by *ADRB1* and *GRK5* genotypes and BB treatment, their survival times were similar. This suggests that genetic variants, rather than race, are the major factor

contributing to the apparent differences in BB treatment effect between Caucasians and African-Americans.

B-Blocker Pharmacogenetics: Recent Advances (Publication Year 2010 or Later)

As evidenced by the heterogeneous and observational nature of the background HF BB pharmacogenetic literature, this field is still in an early stage. The four most recent HF BB pharmacogenetic studies support previous insights, but they also demonstrate some unexpected results.

A small but intriguing study in 93 HF patients (35) studied a panel of both pharmacokinetic and pharmacodynamic genetic variants relevant to BBs (*ADRB1* [Arg389Gly], *CYP2D6,* and *UGT1A1*) and assessed them using a multidimensional BB response criteria. CYP2D6 and UGT1A1 are highly polymorphic metabolic enzymes for which carvedilol is a substrate, and metoprolol is mainly metabolized by CYP2D6. They defined BB response as meeting at least three out of five criteria: 1) duration and 2) tolerability of dose titration, 3) an increase in New York Heart Association functional class, 4) LVEF, or 5) 6-minute walk distance. There was no association between the panel of genetic variants and their BB response criterion, but there was a weak relationship between carvedilol dose and Arg389Gly status ($P = 0.012$). Gly389 patients reached higher doses, perhaps indicating greater BB responsiveness in those with Arg389.

A recent prospective study of 183 patients with HF and three previously studied genetic variants (*ADRB1* [Arg389Gly] and *ADRB2* [Arg16Gly and Gln27Glu]) (25) had

findings consistent with the background BB pharmacogenetic literature. The increase in LVEF after carvedilol tended to be greater in *ADRB1* Arg389 homozygous (+7.8 ± 7.6%) and heterozygous patients (+9.0 ± 11.4%) compared to those homozygous for Gly389 (+4.1 ± 7.6%; $P = 0.0847$). Patients homozygous for *ADRB2* Glu27 showed a greater increase in the LVEF (+13.0 ± 12.2%), compared to both heterozygous (+7.1 ± 8.1%) and Gln27 homozygous patients (+8.3 ± 11.4%; $P = 0.022$). In multivariable analysis, cause of HF, systolic blood pressure, dose of carvedilol, and Gln27Glu genotype were significant correlates of LVEF improvement after carvedilol treatment. Notably, the *ADRB1* Arg389Gly genotype was not independently informative in this dataset.

The *ADRA2C* insertion/deletion was tested in the genetic substudy of BEST ($n = 1040$) (36•). In contrast to previous data, which indicated that the *ADRA2C* deletion was associated with improved LVEF response after BB, this large and adequately powered study found no differential effect on LVEF by *ADRA2C* genotype. Interestingly, interaction with *ADRB1* Arg389Gly was not tested. Even more surprising was that this study found that the insertion allele, and not the deletion, was associated with enhanced survival benefit from BB. For the all-cause mortality end point, bucindolol produced a strong tendency toward significance ($P = 0.025$) for a reduction in mortality by 30% in the insertion homozygotes, while there was a nonsignificant ($P = 0.79$) 9% increase in mortality in the bucindolol-treated deletion carriers. There are several possible explanations for the discrepancy in results; the previous small studies could be false-positive associations, or this could be a bucindolol-specific interaction.

A recent retrospective study of 586 HF patients examined differential pharmacogenetic interactions between carvedilol and metoprolol (37). The investigators

combined two genotypes, *ADRB1* Arg389-homozygous and *ADRB2* Gln27-carrier, and compared these patients to all others in terms of time-to-death from any cause. They found a significant interaction between genotype group and carvedilol treatment (P = 0.003), but no interaction with metoprolol (P = 0.61). In patients treated with carvedilol, survival was lower for Arg389/Gln27 group than the remaining patients (HR 2.30). Because two different variants defined these groups and the fact that one is associated with favorable BB response while the other is not, these results are difficult to put in context of the existing literature. Another concern is that there may have been negative results for metoprolol because the genotype groups were partly defined by an *ADRB2* genotype, and metoprolol is β1-specific.

Overall these more recent results, like the preexisting BB pharmacogenetic literature, are provocative but require validation in large, prospective clinical trials of genetic-guided BB therapy. There is sufficient evidence to support this approach for bucindolol, as well as the currently approved agents such as metoprolol and carvedilol, and this represents the most pressing challenge for BB pharmacogenetics in HF. Other areas that remain unclear are whether additional yet unidentified genes should be the focus of future research, whether other genes are relevant to BB effectiveness, and the interaction of race with genetics. The current candidate gene list revolves strictly around the receptor, and whether other genetic loci may directly modify response or interact with the current candidates is unknown. As pointed out above, all of the current candidate variants have frequencies that differ significantly by ancestry (Table 2), which raises the issue of both genetic and nongenetic confounding factors. Therefore, additional

investigation is required for confidence in these associations and the potential application to non-Caucasian populations.

Angiotensin-converting Enzyme Inhibitor Pharmacogenetics: Background (Literature before 2010)

Our extensive searches reveal only six pharmacogenetic studies of ACE inhibitors in HF patients from 1998 to 2008. Like the BB literature, most of the ACE inhibitor pharmacogenetic literature in HF patients comes from small observational studies (n < 200) that are heterogeneous in design. Not surprisingly, the literature has focused on the gene encoding the target of these agents, *ACE*. A 287–base pair insertion/deletion in intron 16 of *ACE* accounts for half of the variance in serum ACE levels (38). The deletion results in significantly higher ACE levels (38); therefore, it was postulated that HF patients possessing the deletion would require a higher dose of ACE inhibitor to achieve the same inhibition. Most of the clinical pharmacogenetic studies are consistent with the functional effects, with respect to mean arterial pressure (39), aldosterone escape (40), serum ACE activity (41), and survival (42), but the association with LVEF is less clear (43, 44).

In 107 patients with idiopathic dilated cardiomyopathy receiving 2.5 years of ACE inhibitor therapy (43), the LVEF improvement was similar among *ACE* genotypes, but another study of 168 HF patients with systolic dysfunction (44) found that deletion carriers had a greater LVEF improvement after ACE inhibitor (deletion carriers: LVEF +8.8 %; insertion homozygotes: −1.73%; P = 0.01). The discordance in results may be

due to population-specific effects. In contrast, the relationship between the *ACE* variant and survival benefit from ACE inhibitors appears more clear based on the largest ACE inhibitor pharmacogenetic study in HF patients (n = 479) (42). There was a dose-dependent relationship between the *ACE* insertion/deletion and transplant-free survival. After a median follow-up of 33 months, patients on low-dose ACE inhibitors (≤ 50% of target dose) had poorer transplant-free survival associated with the deletion allele (RR for deletion homozygotes: 2.07; P = 0.03), and this was exaggerated in patients who were also not receiving a BB. However high-dose ACE inhibitor (> 50% of target dose), with or without concomitant BB, eliminated the adverse effect of the *ACE* deletion. Although the deletion was associated with poorer transplant-free survival, it seemed that deletion homozygotes benefitted the most from ACE inhibitor and BB therapy.

Angiotensin-converting Enzyme Inhibitor Pharmacogenetics: Recent Advances (Publication Year 2010 or Later)

There is little ACE inhibitor pharmacogenetic literature recently, with only one study published (45) within the past 2 years. This study is consistent with the previous findings on survival, and extends this to HF patients with preserved LVEF. This study enrolled 285 HF patients followed for about 7 years for all-cause mortality (45). The deletion allele was associated with higher mortality in patients not receiving an ACE inhibitor (HR 2.23; P = 0.008), but this impact was reduced among patients receiving an ACE inhibitor (HR 1.64; P = 0.20). Acknowledging the limitation that the *ACE* pharmacogenetic literature comes entirely from observational cohorts, the sum of these

data suggest that HF patients that are deletion carriers may need higher dose ACE inhibition to achieve similar outcomes compared to insertion homozygotes. While at this point it seems unlikely that renewed interest will come to ACE inhibitor pharmacogenetics, a variety of intriguing points remain, such as whether other genes are important, whether adverse events (eg, angioedema and hyperkalemia) can be predicted based on genetics, or whether genetics can help guide combinations of therapies (eg, BB + ACE inhibition vs BB alone vs ACE inhibition alone).

Pharmacogenetics of Loop Diuretics: Background (Literature before 2010)

There have been six studies investigating the association between genetic variation and loop diuretic response published from 2004 to 2008. Five of the studies were performed in healthy volunteers (HV), and one small study included patients with HF. Collectively, these studies demonstrated that genetic variants involved in the metabolism (*CYP2C9*), uptake (*SLC22A11* and *SLCO1B1*), and action (*SLC12A3*, *SCNN1B,* and *SCNN1G*) of the loop diuretics can influence their pharmacokinetics and pharmacodynamics.

In 10-mg torsemide single-dose HV studies, the decreased function *CYP2C9**3 allele had significant effects on torsemide pharmacokinetics and modest effects on pharmacodynamics (46). The total oral clearance of torsemide was about threefold lower in *CYP2C9* *3/*3 patients compared to *1/*1 patients. Sodium and chloride excretion were about 25% higher in carriers of one *CYP2C9**3 allele after torsemide administration (46). HVs homozygous for the two most frequent haplotypes of *SLC22A11* (gene encoding the organic anion transporter 4 [OAT4]) had an 80% difference in the renal

clearance of torsemide (47), and HVs homozygous for 521T, and heterozygous and homozygous for 521C in *SLCO1B1* (gene encoding the organic anion transporting polypeptide 1B1 [OATP1B1]) had torsemide oral clearances estimated as 62, 46, and 41 mL/min, respectively ($P < 0.001$) (48). Taken together, variants in *CYP2C9* and the genes forOATP1B1, OAT1, and OAT4 explain nearly 50% of torsemide pharmacokinetic variation (49).

Three renal sodium reuptake transporters are the primary targets of the loop diuretics: NKCC2 (gene *SLC12A1),* NCC (gene *SLC12A3),* and ENaC (genes *SCNN1A*, *SCNN1B*, and *SCNN1G)* (50). A three-period crossover study was performed in 97 HVs using single oral doses of bumetanide (2 mg), furosemide (80 mg), and torsemide (10 mg) to evaluate the influence of variation in these on diuretic response (50). There were three significant associations with the 24-hour excretion, and this was consistent among the three loop diuretics: 1) patients homozygous for Ala264 in *SLC12A3* excreted an average 32% more chloride and 42% more potassium compared to those homozygous for Gly264; 2) patients homozygous for the most frequent haplotype in *SCNN1B* excreted 24% more volume, 13% more sodium, and 13% more chloride compared to patients without the most frequent haplotype; and 3) patients homozygous for G4 at a synonymous C4G substitution in *SCNN1G* excreted 23% less volume and 24% less calcium compared to patients homozygous for the C4 allele.

The loop diuretic pharmacogenetic data discussed to this point involve single-dose studies performed in HVs, but the data in HF patients receiving steady-state dosing appear consistent with the HVs. In a small, open-label, pharmacokinetic study of 24 patients receiving stable doses of 10-mg daily torsemide (n = 18 with arterial

hypertension and *n* = 6 with HF) (51), the primary end point was area under the plasma concentration-time curve during the 24-hour dosing interval at steady state ($AUC_{24,ss}$). *CYP2C9* genotype, *SLCO1B1* genotype, and sex independently predicted $AUC_{24,ss}$. Similar to HVs, HF patients with the *CYP2C9* *1/*3 genotype had a mean $AUC_{24,ss}$ 46% greater than those with the *1/*1. Patients heterozygous for T521C in *SLCO1B1* had a 38% increase in $AUC_{24,ss}$ compared to patients homozygous for 521T (no 521C homozygotes found).

Pharmacogenetics of Loop Diuretics: Recent Advances (Publication Year 2010 or Later)

Despite the interesting associations above, whether these differences in pharmacokinetic parameters across genotypes translate into clinically meaningful differences in drug effectiveness in patients with HF remains unknown. One recent study attempted to answer this question. This was a randomized, single-blind, three-arm, triple-crossover study in 95 HVs (52) who received a single oral dose of bumetanide (2 mg), furosemide (80 mg), and torsemide (10 mg) at 2-week intervals. Together, eight genetic variants had an impact of 20%, 15%, 10%, and 23% on the variation in the urinary excretion of sodium, volume, potassium, and calcium. Thus, genetic variation seems to importantly impact not only pharmacokinetics of loop diuretics but also their clinical effect. The incorporation of genetic data may help in determining diuretic dosing, though the clinical situation where this would be necessary is not obvious. Important questions that remain

to be addressed are whether genetic variation can predict worsening renal function associated with chronic diuretic therapy, or even the risk of recurrent hospitalization.

Digoxin Pharmacogenetics: Background (Literature before 2010)

Digoxin is a narrow therapeutic index drug, with recommended serum digoxin concentration (SDC) being 0.7 to 0.9 ng/mL (53). The pharmacokinetics, and therefore SDC, may be affected by genetic variation. Indeed, a study in monozygotic and dizygotic twins estimated the genetic component contributing to digoxin $AUC_{0\text{-}12}$ to be 89% (54). The seminal paper investigating the influence of genetic variation on digoxin pharmacokinetics was published in 2000 (55), and it has been followed by 12 more studies with inconsistent results.

Most of the digoxin pharmacogenetic literature has focused on a common C3435T variant in *ABCB1* (Table 2). *ABCB1* encodes for P-glycoprotein (P-gp), an efflux protein for which digoxin is a substrate. In the seminal paper, patients homozygous for 3435T (n = 5) had over twofold lower expression of P-gp in the duodenum compared to patients homozygous for 3435C (n = 6; P = 0.056). As would be expected, patients homozygous for 3435T had 38% higher SDC than patients homozygous for 3435C (P = 0.006). Acknowledging this small sample size, these results have been consistent with six subsequent studies in HVs (56–61), as well as a population-based study of 195 “digoxin-users” (HF status not reported) (62). Despite this seemingly consistent line of evidence, some contrasting data has arisen. Another study of 39 HF patients found no difference in steady-state SDC among C3435T genotypes, which was consistent with another study in

50 HVs (63) as well as a meta-analysis (64). Unfortunately, this small HF study did not control for differences in renal function, which can vary widely among patients with HF.

Overall, the preponderance of evidence favors an impact of genetic variation on digoxin pharmacokinetics. Despite this, whether the difference in digoxin pharmacokinetics by C3435T genotypes is clinically meaningful in patients with HF is not established. Adding to this complexity is that the association of the C3435T genotype with digoxin pharmacokinetics may depend on ethnicity. Two studies in Japanese patients found a reverse association, in which the 3435C genotype had higher SDC (65, 66).

Digoxin Pharmacogenetics: Recent Advances (Publication Year 2010 or Later)

Only a single study, with a unique postmortem SDC design (n = 112), was identified in our searches regarding digoxin pharmacogenetics in the past 2 years. This study's results are consistent with the notion that *ABCB1* 3435T confers higher SDC (67), but adds to the existing data by suggesting that the interactions with C3435T may be sex-specific. There was a relationship between the frequency of 3435T allele and postmortem SDC, but surprisingly, the results were driven by females. If true, this relationship could help explain previous data demonstrating higher mortality in women treated with digoxin versus placebo due to differences in SDC between women and men (68). Validation of this finding in adequately sized, prospective, sex-specific cohorts is needed. If validated, one could envision using genotype to identify patients at higher risk of toxicity who

should not receive digoxin, receive reduced dosing, or receive more intense drug-level monitoring.

Conclusions

While there are many gaps in the HF pharmacogenetic knowledge base, limiting its current clinical application, we have already learned a great deal from the relatively modest body of HF pharmacogenetic literature. For example, while genetic variant functional/mechanistic effects need to be demonstrated to truly establish causation, this is not sufficient as these associations do not always translate into clinical effects. Another important insight is that there can be synergy or interaction between multiple genetic variants, as is the case for *ADRB1* Arg389Gly and *ADRA2C* insertion/deletion with LVEF response to BBs. Even more daunting is the complexity and specificity of some pharmacogenetic associations. HF pharmacogenetic associations may be race-specific (eg, *GRK5* Gln41Leu and BB response in African-Americans), dose-specific (eg, *ACE* insertion/deletion and low- and high-dose ACE inhibitors), sex-specific (eg, *ABCB1* C3435T and SDC in women), and drug-specific (eg, *ADRB1/ADRB2* and response to carvedilol but not metoprolol).

Despite the fact that pharmacogenetics has been in existence for decades, the number of studies on HF therapies are still relatively small and we are yet in the early stages of this part of the field. This foundation has yielded the important insights above and also provided numerous improvements in approach both in terms of genotyping and analysis. This has set the stage for accelerated advances moving forward. At this point,

some of the key knowledge gaps include 1) lack of foundational pharmacogenetic data regarding angiotensin-receptor blockers, aldosterone antagonists, and hydralazine/isosorbide dinitrate; 2) investigation into acute or intravenous HF pharmacotherapies; and 3) better understanding of multi-variant/multi-drug combinations on pharmacogenetics. Most importantly, what are broadly needed to make real progress are prospective intervention clinical trials where a genetic-guided approach is compared to empiric therapy. These are required to validate proposed associations and establish that a specific response to the genetic information can improve treatment outcomes. Because HF is a fatal and common disease requiring polypharmacy, any information (even genetic) that could improve HF pharmacotherapies would give profound patient and public health benefit.

Disclosures

No potential conflicts of interest related to this article were reported.

Table 1 Genetic variants associated with HF pharmacotherapy response

Gene	Variant	rsID	Beneficial allele	Study
β-Blockers				
ACE	Ins/Del	rs1799752	Del	(42, 69)
ADRA1D	T1848A	rs2236554	A	(27)
	A1905G	rs709024	G	(27)
ADRA2C	Ins/Del	rs61767072	Ins	(36)
			Del	(31)
ADRB1	Ser49Gly	rs1801252	Gly	(11, 13, 70–72)
	Arg389Gly	rs1801253	Arg	(2, 10, 11, 13, 14, 17, 25, 31, 35, 37, 71–73)
			Gly	(37)
ADRB2	Gln27Glu	rs1042714	Glu	(23–25, 37)
	Thr164Ile	rs1800888	Thr	(74)
EDN1	G/A (IVS)-4	rs2071942	G	(75)
	Lys198Asn	rs5370	Lys	(75)
GRK5	Gln41Leu	rs17098707	Gln	(33, 34)
NET	T-182C	rs2242446	T	(27)
ACE inhibitors				
ACE	Ins/Del	rs1799752	Ins	(39–42, 45)
AGTR1	A1166C	rs5186	A	(45)
CYP11B2	T-344C	rs1799998	C	(43)
Aldosterone antagonists				
ACE	Ins/Del	rs1799752	Ins	(76)
Angiotensin-receptor blockers				
AGTR1	A1166C	rs5186	C	(77)
Hydralazine/isosorbide dinitrate				
CYP11B2	T-344C	rs1799998	C	(78)
NOS3	Glu298Asp	rs1799983	Glu	(79)
Loop diuretics[a]				
ACE	Ins/Del	rs1799752	Del	(52)
ADD1	Gly460Trp	rs4961	Trp	(52)
ANP	Val32Met	rs5063	Val	(52)
	Ter152Arg	rs5065	Arg	(52)
CYP2C9	CYP2C9*1/*2/*3	n/a	*3	(46)
GNB3	C825T	rs5443	C	(52)
SCNN1B	Most frequent haplotype vs others	N/A	Most frequent haplotype	(50)
SCNN1G	C4G	rs5723	C	(50)
SLC12A3	Gly264Ala	rs1529927	Ala	(50)

a. Data from healthy volunteers

ACE/*ACE*—angiotensin-converting enzyme; *ADD1*—α-adducin 1; *ADRA1D*—α-1d adrenergic receptor; *ADRA2C*—α-2c adrenergic receptor; *ADRB1*—β-1 adrenergic receptor; *ADRB2*—β-2 adrenergic receptor; *AGTR1*—angiotensin II receptor type 1; Arg—arginine; *ANP*—atrial natriuretic peptide precursor; Asn— asparagine; *CYP11B2*— cytochrome P450, family 11, subfamily B, polypeptide 2; *CYP2C9*—cytochrome P450, family 2, subfamily C, polypeptide 9; Del—deletion; *EDN1*—endothelin-1; Gln—glutamine; Glu—glutamic acid; Gly—glycine; *GNB3*—guanine nucleotide binding protein-β polypeptide 3; *GRK5*—G-protein coupled receptor kinase 5; HF—heart failure; Ile— isoleucine; Ins—insertion; IVS—intervening sequence; Leu—leucine; Lys—lysine; Met—methionine; N/A—not available; *NET*—norepinephrine transporter; *NOS3*—nitric oxide synthase 3; rsID—Reference Sequence Identification; *SCNN1B*—sodium channel non–voltage-gated 1-β; *SCNN1G*—sodium channel non–voltage-gated 1-γ; Ser—serine; *SLC12A3*—solute carrier, family 12, member 3; Ter—termination; Thr— threonine; Trp—tryptophan; Val—valine

Table 2 Frequencies of genetic variants associated with heart failure pharmacotherapy response

Gene	Variant	rsID	Minor allele	Caucasian	African-Americans
ABCB1	C3435T	rs1045642	C	45%	87% (Africans)
ACE	Ins/Del Intron 16	rs1799752	Ins	44%	43%
ADD1	Gly460Trp	rs4961	Trp	17%	11%
ADRA1D	T1848A	rs2236554	A	46%	12% (Africans)
	A1905G	rs709024	A	38%	70% (Africans)
ADRA2C	Ins/Del 322-325	rs61767072	Del	4%	43%
ADRB1	Ser49Gly	rs1801252	Gly	17%	25%
	Arg389Gly	rs1801253	Gly	27%	38%
ADRB2	Gly16Arg	rs1042713	Arg	40%	50% (Africans)
	Gln27Glu	rs1042714	Glu	42%	20%
	Thr164Ile	rs1800888	Ile	2%	< 2% (Africans)
AGTR1	A1166C	rs5186	C	25%	5%
ANP	Val32Met	rs5063	Met	4%	2%
	Ter152Arg	rs5065	Ter	14%	43% (Africans)
CYP11B2	T-344C	rs1799998	C	43%	30%
CYP2C9	*2	N/A	N/A	14%	< 1%
	*3	N/A	N/A	11%	< 1%
EDN1	G/A (IVS)-4	rs2071942	A	29%	17% (Africans)
	Lys198Asn	rs5370	Asn	30%	17%
GNB3	C825T	rs5443	T	39%	91% (Africans)
GRK5	Gln41Leu	rs17098707	Leu	2%	24%
NET	T-182C	rs2242446	C	25%	15% (Africans)
NOS3	Glu298Asp	rs1799983	Asp	22%	7% (Africans)
SCNN1B	Most frequent haplotype	N/A	N/A	65%	N/A
SCNN1G	C4G	rs5723	G	28%	24% (Africans)
SLC12A3	Gly264Ala	rs1529927	Ala	8%	< 1% (Africans)

A—adenine; *ABCB1*—P-glycoprotein (ATP-binding cassette, subfamily B, member 1); *ACE*—angiotensin-converting enzyme; *ADD1*—α-adducin 1; *ADRA1D*—α-1d adrenergic receptor; *ADRA2C*—α-2c adrenergic receptor; *ADRB1*—β-1 adrenergic receptor; *ADRB2*—β-2 adrenergic receptor; *AGTR1*—angiotensin II receptor type 1; Arg—arginine; *ANP*—atrial natriuretic peptide precursor; Asn—asparagine; Asp—aspartic

acid; C—cytosine; *CYP11B2*— cytochrome P450, family 11, subfamily B, polypeptide 2; *CYP2C9*—cytochrome P450, family 2, subfamily C, polypeptide 9; Del—deletion; *EDN1*—endothelin-1; G—guanine; Gln—glutamine; Glu—glutamic acid; Gly—glycine; *GNB3*—guanine nucleotide binding protein–β polypeptide 3; *GRK5*—G protein–coupled receptor kinase 5; Ile— isoleucine; Ins—insertion; IVS—intervening sequence; Leu—leucine; Lys—lysine; Met—methionine; N/A—not available; *NET*—norepinephrine transporter; *NOS3*—nitric oxide synthase 3; rsID—Reference Sequence Identification; *SCNN1B*—sodium channel non–voltage-gated 1-β; *SCNN1G*—sodium channel non–voltage-gated 1-γ; Ser—serine; *SLC12A3*—solute carrier, family 12, member 3; T—thymine; Ter—termination; Thr— threonine; Trp—tryptophan; Val—valine

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APPENDIX III:

Beta-1 adrenergic receptor genotype Ser49Gly is associated with beta-blocker Survival benefit in patients with heart failure

Talameh J, Garrand A, Ghali J, Oren RM, Dunlap S, Bakel AV, et al. Beta-1 adrenergic receptor genotype Ser49Gly is associated with beta-blocker survival benefit in patients with heart failure. J Am Coll Cardiol 2012 3/27;59(13, Supplement):E861.

Summary: Although beta-blockers (BB) reduce mortality in patients (Pts) with heart failure (HF), genetic differences in neurohormonal activation may limit effectiveness. Systematic review identified 3 adrenergic receptor SNPs as primary candidates. We report on the beta-1 receptor Gly49 variant that results in agonist-induced receptor down-regulation in vitro. We hypothesized Gly49-carriers would have reduced response to BB compared to Ser49-homozygotes. This hypothesis was tested in the UNITE-HF DNA Registry, a prospective, observational, multicenter study of the genomics of HF in US Pts seen in HF specialty clinics. Data on BB use and survival (death index) were examined in Pts mostly enrolled from 2000-2002. Adjusted Cox models (including race) assessed BB*Ser49Gly interaction and the association between BB and survival stratified by Ser49Gly genotype. Data were available in 715 Pts on baseline BB use, vital status and Ser49Gly genotype (68% Ser49-homozygous and 32% Gly49-carriers). The cohort was 63% male, 38% African-American (AA) and 44% ischemic etiology. Age of the cohort was 57±13 years (mean±SD), LVEF 32±16%, NYHA class 2.5±0.6 and SBP 119±22 mmHg. Baseline drug use included ACEI or ARB (89%), diuretics (89%) and spironolactone (24%). BB utilization was 68% consistent with the enrollment period. Baseline characteristics were similar by genotype except AA race (33% Ser49-homozygotes vs 49% Gly49-carriers, $p<0.001$). There were 348 deaths (52% Gly49-carriers, 47% Ser49-homozygotes) at an average follow-up of 6.9±3.6 years. BB use was associated with reduced mortality in the overall study population (adjusted HR=0.72, 95% CI 0.56 − 0.91, $p=0.004$) and Ser49-homozygotes (adjusted HR=0.56, 95% CI 0.42 − 0.75, $p<0.001$) but not Gly49-carriers (adjusted HR=1.28, 95% CI 0.80 − 2.04, $p=0.31$). The adjusted interaction between Ser49Gly genotype and BB association with overall survival was $p=0.004$. These findings were independent of race (AA vs Non-AA). In conclusion, BB use was associated with prolonged survival in Ser49 homozygotes, but not in Gly49-carriers. This novel hypothesis-generating finding warrants replication in a prospective trial.

APPENDIX IV:

PON1 Q192R and Clopidogrel: A case of the winner's curse or inadequate replication?

PON1 Q192R and clopidogrel: a case of the winner's curse or inadequate replication? Talameh JA, McLeod HL. Clin Pharmacol Ther. 2011 Dec;90(6):771-4.

75-word introduction (in place of an abstract)

The anti-platelet drug clopidogrel is one of the most commonly prescribed drugs in the world, but among patients there is wide variability in its anti-platelet effects. The majority of this variation is due to genetic effects, but there is controversy over which genetic variants are important and their relative contribution. This controversy may stem from the genetic association research paradigm, which casts the "winner's curse."

Coronary artery disease (CAD) is the leading cause of morbidity and mortality in the United States. Dual anti-platelet therapy with aspirin and clopidogrel is currently the standard of care for patients experiencing an acute coronary syndrome (ACS) who are managed either medically or with percutaneous coronary intervention (PCI). Clopidogrel inhibits adenosine diphosphate (ADP)–stimulated platelet activation by irreversibly binding the P2Y12 receptor, but it must first be enzymatically activated. Inadequate platelet inhibition from clopidogrel occurs in approximately 25% of the population and leads to recurrent cardiovascular events. This has been attributed to inadequate formation of clopidogrel active metabolites, in contrast to pharmacodynamic issues with binding to the P2Y12 receptor. However, the issues related to clopidogrel failure are controversial.

The majority of pharmacogenetic studies for clopidogrel have observed that CYP2C19 inactivating variations are associated with decreased clopidogrel activation, a decreased anti-platelet effect, and an increased likelihood of a cardiovascular event.(1,2) These observations have been confirmed in a genome-wide association study.(1) More recently, Bouman et al demonstrated the importance of genetic variation in paraoxonase-1 (PON1), an esterase synthesized in the liver, in determining clopidogrel efficacy.(3) Using a microsomal expression system of metabolizing enzymes, Bouman et al determined PON1 to be the rate-limiting step in the formation of the active metabolite of clopidogrel. A genetic variant in PON1 resulting in an amino acid change of glutamine (Q) to arginine (R) at position 192 (Q192R; A576G; rs662) more efficiently activated clopidogrel. This variant is common and varies across population groups, with Q192 occurring in 60-75% of European-derived groups and 30-40% of Asian or African-derived populations.(4) In a case-cohort study of 41 CAD patients receiving clopidogrel with stent thrombosis and 71 well-matched CAD controls without stent thrombosis, PON1 Q192R was significantly associated with PON1 enzyme activity, clopidogrel pharmacokinetics, platelet response, and the risk of stent thrombosis. This was confirmed in a prospective evaluation of 1,982 ACS patients followed for 12 months with respect to several clinical endpoints (Table 1).

In this issue of Clinical Pharmacology & Therapeutics, two studies failed to replicate the PON1 Q192R and clopidogrel findings (Table 1). Shuldiner et al followed 227 patients for 12 months that underwent non-emergent PCI and did not find an association between Q192R and post-discharge ischemic events. Simon et al followed 2,170 patients for 12 months after admission for definite myocardial infarction (MI) and

did not find an association between Q192R and several in-hospital outcomes or 1-year outcome of death, MI, or stroke in all patients or the hospital survivors. In fact, none of the replication studies to date have been able to replicate the finding of an influence of PON1 on patient outcome (Table 1). The reason for this is not entirely clear, but it may be a case of the winner's curse.

The problem of the winner's curse

The winner's curse is a common phenomenon in genetic association literature, in which the initial reported genotype-phenotype association is exaggerated relative to the estimated effect in follow-up studies or cannot be subsequently replicated at all.(5) Failed replication can be due to issues in the initial studies and/or the attempted replication studies. Bouman et al, in reporting the initial PON1 Q192R findings, used both biologic plausibility and an independent replication cohort to support their findings. However, the biologic plausibility of PON1 Q192R, and all other genetic associations, must be carefully considered. The use of *in vitro* experiments remains challenging in the context of a complex human physiologic milieu, such as clopidogrel metabolism. Bouman et al used supra-physiologic drug concentrations in *in vitro* experiments to assess biologic impact, which might have given false security of a meaningful finding. The use of existing positive variables was not able to be used to build confidence in the experimental system, in that CYP2C19 was not confirmed as a player in clopidogrel metabolism. There was also the assumption that any PON1 effect would be via clopidogrel metabolism, when PON1 has anti-oxidant and atheroprotective effects (6), and Q192R is independently associated with all-cause mortality in cardiovascular disease patients.(7)

These possible independent mechanisms of PON1 would not be evident in the subsequent biologic replication studies, such as the platelet aggregation tests in Shuldiner et al, Trenk et al(8), and Sibbing et al(9), which were well-powered compared to clinical endpoint analyses.

In addition to demonstrating biologic plausibility, initial reports of genotype-phenotype associations can be strengthened by replication. This method was also pursued by Bouman et al, so is the use of the term 'failed replication' justified by the studies of Shuldiner et al and Simon et al? The first issue to consider is study power. The study by Shuldiner et al was much smaller than Bouman et al. Simon et al had a similar sample size and length of follow-up as Bouman et al, but the number of actual patients undergoing PCI (n = 1538) was smaller than Bouman et al. Both Shuldiner et al and Simon et al were able to confirm the CYP2C19 association, but this doesn't rule out a Type II error for the replication of PON1 Q192R findings.

The second issue is study heterogeneity. As shown in Table 1, the studies evaluating clopidogrel clinical response and PON1 Q192R are diverse with respect to design, patient population, and clinical endpoints. Shuldiner et al included patients undergoing non-emergent PCI, which are a lower risk group compared to the ACS patients studied by Bouman et al. Thirty-seven percent of the patients in the Shuldiner et al study were African-American, compared to Bouman et al that recruited their entire patient cohort among the white population of the Netherlands and Northwest Germany. Simon et al also studied a white European cohort, but underlying population genetic differences may still exist. Specifically, Q192R deviated from Hardy-Weinberg equilibrium in the Simon et al patient population, and there was a higher frequency of the

QQ192 genotype. Because of the potential anti-oxidant and atheroprotective effects of Q192R, there could be pre-existing selective pressure on patients prior to ever receiving clopidogrel. The ischemic stroke endpoint was not significantly associated with Q192R in Bouman et al, so it is interesting that Simon et al and Shuldiner et al chose to include it in their composite endpoints. Stent thrombosis was not a clinical endpoint considered in the Simon et al study's 1-year composite endpoint, nor do they indicate how many of the patients received stents. Stent thrombosis has the closest pathophysiologic link to platelet aggregation compared to other endpoints such as death, MI, or stroke.

One of the greatest challenges in pharmacogenetics is the replication of a therapeutic predictor. Exact replication among genetic association studies is difficult, as there is rarely the luxury of multiple study cohorts for the same clinical scenario. This leads to a lack of clarity on whether a subsequent replication failure is due to correction of an initial false positive result or represents significant deviations in study design, statistical power, and clinical endpoint. The challenges with identifying replication datasets has also strengthened the use of *in vitro* studies to demonstrate biologic plausibility. However, as demonstrated in the case of PON1 Q192R, it may not always translate. Another lesson that can be learned from the PON1 Q192R story is the importance of evaluating "new" genetic markers (i.e. PON1 Q192R) in the context of "old" genetic markers (i.e. CYP2C19); all of the replication studies in Table 1 failed to replicate PON1 Q192R, but each confirmed the association with CYP2C19. The presence of a previously observed predictor anchors the results, giving confidence that the patient population and clinical context is likely to be consistent with previous reports.

While PON1 does not have a clear clinical use, the PON1 Q192R and clopidogrel story is not over. It is possible that all of the variants in the Simon et al and Shuldiner et al studies (i.e. PON1, CYP2C19, and ABCB1) are "winners," with each contributing a modest to small effect. This would not be clearly observed in the study sample sizes present in the reports to date. However to robustly assess genetic markers and optimize the use of resources, collaboration among researchers is needed to form well-powered and well-characterized clinical cohorts. A strategy of periodic meta-analyses of genetic association studies must be performed to take into account variation in replication study design and to find the true winners.(10)

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Conflicts of Interest

The authors have no conflicts of interest to disclose.

Table 1. PON1 Q192R and clopidogrel studies with clinical outcomes

Study (reference)	Design	Patient population	Race/ethnicity	n	Clinical endpoints	Length of follow-up	Number of events (%)	HR/OR (95% CI; p-value)
Bouman et al 2011(3)	case-cohort	Clinical presentation of stable angina pectoris or ACS, who had undergone PCI with stent implantation.	White population of the Netherlands and Northwest Germany	112	Nonfatal stent thrombosis	18 months	41 cases 71 controls	(RR reference) QR = 4.41 (1.89–10.20; p = 0.001) QQ = 12.82 (4.74–90.91; p<0.001)
	prospective cohort	Clinical presentation of ACS who had undergone PCI with stent implantation	Self-reported European ancestry	1,982	1) non/fatal stent thrombosis 2) MI 3) composite of vascular death, nonfatal MI and nonfatal ischemic stroke 4) ischemic stroke 5) nonvascular death 6) major bleeding	12 months	1) 44 (2.2%) 2) 142 (7.2%) 3) 216 (10.9%) 4) 26 (1.3%) 5) 15 (0.8%) 6) 57 (2.9%)	(RR reference) QQ = 1) 10.20 (4.39–71.43; p < 0.001) 2) 4.93 (2.16–11.24; p < 0.001) 3) 3.89 (2.10–7.19; p < 0.001) 4) 2.38 (0.54–10.42; p = 0.250) 5) 0.98 (0.20–4.72; p = 0.981) 6) 0.36 (0.18–0.75; p = 0.006)
Trenk et al 2011(8)	prospective cohort	Patients undergoing elective PCI with stent placement	Caucasian patients in Germany	760	1) death or MI 2) stent thrombosis	12 months	1) 24 (3.2%) 2) 16 (2.1%)	(RR reference) QQ = 1) 0.61 (0.20-1.88; p= 0.390) 2) 0.28 (0.08-1.01; p= 0.051)
Sibbing et al 2011(9)	*post hoc* analysis of prospective cohort and registry	Patients undergoing PCI with stent placement	Enrolled in Germany	1,566	Stent thrombosis within 30 days	n/a	127 cases 1439 controls	(RR reference) QQ = 1.53 (0.77-3.05; p =

								0.22) (R-carrier reference) QQ = 0.46 (0.20-1.06; p = 0.07)
Shuldiner et al Clinical Pharmacology and Therapeutics 2011	prospective cohort	Patients undergoing non-emergent PCI	Enrolled in Baltimore, MD, USA; 140 (62%) were Caucasian, 83 (37%) were African American, and 4 (2%) were of other race/ethnicity. (self-reported)	227	Composite of MI, ischemic stroke, stent thrombosis, unplanned target vessel revascularization, hospitalization for coronary ischemia without revascularization, or cardiovascular death	12 months	Not reported	
Simon et al Clinical Pharmacology and Therapeutics 2011	registry	Patients admitted to the ICU for definite acute MI	Enrolled in France; 99% Caucasian	2,170	1) in-hospital outcomes (several categories) 2) 1-year outcomes death, MI, or stroke in all patients 3) 1-year outcomes death, MI, or stroke in hospital survivors	12 months	1) (several categories) 2) 296 (13%) 3) 191 (10%)	(RR reference) QQ = 1) NS 2) 1.03 (0.66-1.61; p = 0.41) 3) 0.86 (0.52-1.44; p = 0.12)

ACS = acute coronary syndromes, HR = hazard ratio, ICU = intensive care unit, MI = myocardial infarction, NS = not significant, OR = odds ratio, PCI = percutaneous coronary intervention

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