MOLECULAR ANALYSIS AND PHARMACOGENETIC ASSESSMENT OF CALCIUM SIGNALING PATHWAY VARIATION

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2012

To my parents, Collette and Robert Davis;
my sisters Kim Cheeseman and Margo Feaser;
my aunt Eileen Larkin;
and my boyfriend Justin Marquand

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Julie Johnson for her commitment and guidance in my professional development. I would also like to thank Dr. Taimour Langaee for his gracious assistance, enthusiasm for teaching, and infectious optimism. I owe much gratitude to Dr. Yan Gong for her expertise and advice. I would also like to acknowledge Dr. Haojie Zhu for always taking the time to answer my questions and making me feel welcome in his lab. Additionally, I owe many thanks to Dr. Jorg Bungert for his advice, expertise, and for being available and prepared to help me with last-minute questions about everything molecular biology.

Members of the Vascular Surgery Department, Dr. Adam Beck, Dr. Peter Nelson, Dr. Thomas Huber, Dr. Robert Feezor, Dr. Salvatore Scali, Dr. Timothy Flynn, Nancy Hanson, the department residents and support staff all provided invaluable assistance in the creation and development of our vascular tissue bank, and for this I owe them much gratitude. Additionally, I would like to thank all of the patients who chose to participate in our clinical study.

Current and former members of the department, including Dr. Mariellen Moore, Dr. Caitrin McDonough, Dr. Jaekyu Shin, Dr. Maximilian Lobmeyer, Dr. Elvin Price, Dr. Julio Duarte, Dr. Abdolreza Davoodi-Semiromi, Dr. Rhonda Cooper-DeHoff, Ben Burkley, Lynda Stauffer, Cheryl Galloway and Azadeh Hassanzadeh all provided assistance throughout the course of this project for which I have much appreciation.

I would also like to thank my family for standing behind me throughout this endeavor. My mother, father, sisters and in-laws have all been encouraging, patient and kind. Finally, I would like to thank my boyfriend Justin and our dog Deuce, for making the whole experience much more colorful.

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Requirements for the Degree of Doctor of Philosophy

MOLECULAR ANALYSIS AND PHARMACOGENETIC ASSESSMENT OF CALCIUM SIGNALING PATHWAY VARIATION

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May 2012

Chair: Julie A. Johnson
Major: Pharmaceutical Sciences

One in three American adults has hypertension. Less than half of those individuals have achieved blood pressure control despite the availability of efficacious pharmacotherapy. The current study aimed to determine whether genetic variation within Ca^{2+} signaling pathway genes may provide insight into the variability in responses observed to commonly used antihypertensives. Additionally, we sought to investigate the functional mechanisms of a clinically associated polymorphism found within this pathway.

Blood pressure (BP) response to atenolol and hydrochlorothiazide was evaluated relative to genetic variation among Ca^{2+} signaling pathway candidate genes among uncomplicated hypertensives. We identified several significant and novel associations within *CACNA1C* (calcium channel, voltage-dependent, L type, alpha 1c subunit) and BP response to atenolol among blacks.

We then evaluated the relationship between genetic variation of the Ca^{2+} signaling pathway and clinical susceptibility to adverse cardiovascular outcomes in a high-risk cohort of hypertensives with coronary artery disease. Herein we identified several significant and novel pharmacogenetic treatment interactions among three different

racial/ethnic groups. Additionally, several polymorphisms within *CACNA1C* significant for BP response among uncomplicated hypertensives also demonstrated significant associations with adverse cardiovascular outcomes in our high-risk cohort.

Finally, we used human vascular tissue to evaluate whether differential expression of mRNA, protein, or transcription factor binding could be observed relative to the clinically associated *CACNB2* (calcium channel, voltage-dependent, beta 2 subunit) rs2357928 polymorphism. The current data show promising trends for an association with differential expression of mRNA, but definitive conclusions cannot be made due to a limiting sample size. Additional investigation is warranted, as the genetic associations reported here have yet to be replicated in an independent study population and a functional evaluation of molecular mechanisms would benefit greatly from a larger sample cohort.

CHAPTER 1
BACKGROUND AND SIGNIFICANCE

Hypertension

An estimated 76 million Americans, or roughly one in three American adults has hypertension (HTN). Elevated blood pressure (BP) is a primary risk factor for myocardial infarction (MI), heart failure, stroke and kidney diseases, the incidence of which increase proportionately with BP value.[1] Of Americans treated for HTN, as few as 48% have achieved BP control in the face of widely available and efficacious antihypertensive agents.[2] The pathophysiology of essential HTN involves numerous interrelated environmental, biologic and genetic factors that contribute to elevated BP, and their effects differ between individuals.[3] Successful BP control relies largely upon a "trial-and-error" approach to identify optimal drug therapy as any particular antihypertensive may only be efficacious in 40-60% of patients.[4]

Given the heterogeneous nature of HTN, response to any single antihypertensive agent is characterized by marked variability between individuals and traditional predictors of BP response such as race, age and gender are of limited usefulness in efforts to identify optimal therapy for a particular patient.[5] The lack of rationale for drug selection and the time lag associated with identifying an effectual regimen to achieve BP control is associated with poor therapeutic outcomes.[6] The principle aim of pharmacogenetic research is to identify genetic factors for use as a screening tool to aid in the selection of drug therapy that would be optimal for an individual patient. Targeting the genetic component of HTN pathophysiology may enhance efficacy and improve overall benefit from therapy.[7] This tailored approach could decrease time to optimal BP control and avoid the lengthy "trial-and-error" phase of initial treatment.[5]

Calcium Current (I_{Ca})

In its vital role as an intracellular second messenger, Ca^{2+} regulates a number of diverse physiological processes including excitation-contraction coupling, electrical signaling, hormone secretion and gene transcription.[8,9] Voltage-gated Ca^{2+} channels (Ca_v) belong to a gene superfamily of transmembrane ion channel proteins that also includes both voltage-gated sodium (Na^+) and potassium (K^+) channels.[9,10] Ca_v contain between four to five subunits, of which the α_1 pore-forming and voltage-sensing subunit is the largest.[11] Ten human genes comprising three distinct subfamilies encode the α_1 subunits and Ca_v channels are identified by the subfamily of α_1 subunit they contain (Ca_v1 to Ca_v3).[9] L-type Ca^{2+} current ($I_{Ca,L}$) is conducted by Ca_v1 channels and characterized by high-voltage activation as well as sensitivity to Ca^{2+} channel blockers (CCBs) widely used in the treatment of HTN and cardiac arrhythmia.[9] $Ca_v1.3$ (α_{1D}) is found at high levels in pacemaker cells of the heart where it controls cardiac rhythm.[9,12] $Ca_v1.2$ (α_{1C}), found in vascular smooth muscle and ventricular myocytes, provides the majority of $I_{Ca,L}$ within the myocardium, where channel activation carries the plateau phase of the cardiac action potential.[8,10]

Excitation-Contraction Coupling

Ca^{2+} influx through depolarization activated $Ca_v1.2$ on the sarcolemma produces near simultaneous activation of numerous ryanodine receptors (RyR, *RYR2*) and the coordinated release of large amounts of Ca^{2+} from the sarcoplasmic reticulum (SR, Figure 1-1). In this process best known as Ca^{2+}-induced Ca^{2+} release, myocardial RyRs function to increase free intracellular Ca^{2+} ($[Ca^{2+}]_i$) within the myocyte, allowing Ca^{2+} to bind and activate contractile myofilaments.[8] Relaxation arises from declining $[Ca^{2+}]_i$, as Ca^{2+} reuptake into the SR occurs via activation of the SR Ca^{2+}-ATPase 2a pump

(SERCA2a, *ATP2A2*) and cellular extrusion of Ca^{2+} occurs across the sarcolemma via the Na^{+}/ Ca^{2+} exchanger (NCX, *SLC8A1*).[11] In arterial smooth muscle each Ca^{2+} spark also activates between 10-100 Ca^{2+} activated potassium (K^{+}) channels (K_{Ca} or BK) on the plasma membrane. K_{Ca} activation causes K^{+} efflux and subsequent membrane hyperpolarization, a process that deactivates Ca_v. Found in vascular smooth muscle, K_{Ca} functions through this negative feedback mechanism and restores resting membrane potential in vascular smooth muscle following Ca^{2+} induced contraction.

Calcium Signaling in Cardiovascular Disease

CACNA1C

Located on chromosome 12p13.3, *CACNA1C* is a large, complex gene with limited linkage disequilibrium encoding the 210-240 kDa α_{1C} subunit of $Ca_v1.2$ channels. Mutations in this gene have been associated with lethal cardiac arrhythmias and sudden cardiac death.[13]

Located in the coding sequence of *CACNA1C*, the synonymous single nucleotide polymorphism (SNP) rs1051375 has been identified as having a significant interaction with treatment strategy in a cohort of high-risk hypertensive patients with coronary artery disease (CAD).[14] Individuals homozygous for the major allele (A/A) within the INternational VErapamil SR Trandolapril STudy GENEtic Substudy (INVEST-GENES) case-control cohort randomized to a verapamil-SR based CCB treatment strategy experienced a 45% reduced risk of primary outcome (first occurrence of all-cause mortality, nonfatal myocardial infarction (MI) or nonfatal stroke; OR 0.54, 95% CI 0.32-0.92) compared to individuals randomized to an atenolol-based β-blocker (βB) strategy.[14] In contrast, individuals homozygous for the minor allele (G/G) randomized to the CCB treatment strategy experienced a 4.5-fold increase in the primary outcome

compared to G/G homozygotes randomized to the βB treatment strategy (OR 4.59, 95% CI 1.67-12.67). The evidence suggests subjects carrying the *CACNA1C* rs1051375 A/A genotype may experience better outcomes if treated with a CCB, whereas G/G carriers would likely benefit most from βB therapy.[14] Subsequent analyses showed A/A carriers randomized to the βB treatment strategy were more likely to require four or more drugs to achieve BP control than those carriers randomized to the CCB treatment strategy.[14] The data suggest that differences in BP response may influence the effect observed in treatment outcomes.[14] These data from a large, multicenter, randomized hypertensive treatment outcomes trial have incited additional investigation of the association of *CACNA1C* variants with cardiovascular disease phenotypes.

CACNA1D

Located on chromosome 3p14.3, *CACNA1D* encodes the α_{1D} subunit of $Ca_v1.3$ responsible for $I_{Ca,L}$ within pacemaker cells of the heart. Genetic variations in *CACNA1D* have been associated with BP response to dihydropyridine CCB in a small cohort of Japanese hypertensives.[15]

CACNB2

CACNB2 encodes the β_2 regulatory subunit of $Ca_v1.2$ involved in targeting the α_1-subunit to the plasma membrane where the channel exerts its function. Via hydrophobic interactions the β_2 subunit is tightly bound to a highly conserved motif in the cytoplasmic linker between homologous repeating motifs I and II of the α_1-subunit, termed the α-interaction domain (AID). This I-II loop of the α_1 subunit contains a retention signal for the endoplasmic reticulum that restricts cell surface expression of the channel. Interaction with the β_2 subunit reverses the inhibition associated with the retention signal leading to cell surface expression of $Ca_v1.2$.[16] As $Ca_v1.2$ must be on

the cell surface for CCBs to bind, the β_2 subunit may be a functionally important mediator of response to CCBs. Genetic variation in *CACNB2* has been associated with Brugada syndrome, cardiac conduction disease and hypertrophic cardiomyopathy.[17,18]

A genome-wide association study of BP and HTN within the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium found the *CACNB2* rs11014166 intronic variant to be significantly associated with the phenotypes of systolic BP (SBP), diastolic BP (DBP), and HTN.[19] Within the INVEST-GENES case-control cohort, Hispanic carriers of the minor *CACNB2* rs11014166 T allele randomized to the CCB treatment strategy had greater risk for the primary outcome than those randomized to the βB treatment strategy (adjusted HR [CCB versus βB], 3.13; 95% CI, 1.39-7.06; p = 0.006).[20] Additional analyses showed the pharmacogenetic effect of this SNP was primarily driven by all-cause mortality (adjusted HR, 22.0; 95% CI, 2.63-184.17; p = 0.0043).[20]

A SNP within an alternative promoter of *CACNB2*, rs2357928, was also found to have a significant interaction with treatment strategy in the INVEST-GENES white population (p for interaction, 0.002). Minor allele homozygotes (G/G) randomized to the CCB treatment strategy were at an increased risk for adverse cardiovascular outcomes when compared to those randomized to the βB treatment strategy (adjusted HR [CCB versus βB] 2.35; 95% CI, 1.19-4.66; p = 0.014).[20] No similar associations were found for carriers of the major allele (A/A, A/G).[20] The rs2357928 promoter SNP of *CACNB2* has also been associated with decreased transcriptional activity in a reporter gene assay, which lends support to the hypothesis that the observed pharmacogenetic

interaction is driven by a functional mechanism affecting $Ca_v1.2$ β_2 subunit gene expression.[20]

KCNMB1

Encoded by *KCNMB1*, the K_{Ca} β_1 regulatory subunit, greatly enhances Ca^{2+} sensitivity of the K_{Ca} channel complex in vascular smooth muscle. K_{Ca} opening restores resting cell membrane potential by coupling intracellular Ca^{2+} sparks to membrane potential hyperpolarization and is thus a critical regulator of arterial tone.[21]

The *KCNMB1* Glu65Lys variant was evaluated within a Spanish cohort that compared normotensive subjects to those with differing levels of diastolic HTN.[22] The genotype frequency of the *KCNMB1* Glu65Lys mutation decreased with increasing DBP values, an association that suggested a protective effect of the Lys65 allele and a progressively deleterious effect of the Glu65Glu genotype.[22] Expression of the Lys65 β_1 subunit in HEK-293 cells alone or in combination with the wild-type β_1 subunit showed enhanced K_{Ca} channel activity, suggesting the variant provides a gain of function.[22]

The *KCNMB1* Glu65Lys variant was also investigated in association with variable BP response to CCB treatment within the INVEST-GENES cohort. Among subjects receiving verapamil SR monotherapy, *KCNMB1* Lys65 variant carrier status was significantly associated with the need for fewer drugs to achieve BP control (OR, 0.48; 95% CI, 0.23-0.99), and shorter median time to achieve BP control compared to carriers of the Glu65Glu genotype (1.47 months v. 2.83 months; $p = 0.01$).[23] Additionally within INVEST-GENES, the *KCNMB1* valine to leucine polymorphism at codon 110 (Val110Leu) was associated with a 33% risk reduction for the primary outcome within the nested case-control cohort (OR, 0.66; 95% CI, 0.43-1.01).[23] Overall, these data

suggest genetic variation within *KCNMB1* may contribute to the heterogeneous response to verapamil SR observed in this high-risk hypertensive cohort.

Calcium Signaling and Antihypertensive Therapy

In addition to the CCB class of antihypertensive agents, a number of commonly prescribed antihypertensives intercede in pathways in which Ca^{2+} signaling is intimately involved.

β-blockers inhibit the binding of endogenous catecholamines to β_1-/β_2- adrenergic receptors. β-adrenergic receptors couple to the stimulatory G protein (G_s) which initiates a signaling cascade with subsequent activation of adenylyl cyclase, increase in cyclic adenosine monophosphate, and activation of protein kinase A (PKA).[24] PKA phosphorylates a number of substrates important in intracellular Ca^{2+} regulation, including the Ca_v1 channels, RYR and phospholambam (PLN, Figure 1-1).[11] Additionally, a proposed mechanism for the antihypertensive effects of thiazide and thiazide-like diuretics is enhancing K_{Ca} channel sensitivity, thereby enhancing vasodilation mediated by vascular smooth muscle.[25]

Conclusion

In light of evidence supporting the association of variants within the Ca^{2+} signaling pathway with adverse cardiovascular outcomes in treated hypertensives, we have developed the following hypotheses:

1) Variation present in genes comprising the activation pathway of Ca^{2+} signaling and Ca_v1 are associated with BP response to antihypertensive therapy.

2) Genetic variation within the Ca^{2+} signaling pathway is associated with clinical susceptibility to adverse CV outcomes in treated hypertensives with CAD.

To address the first hypothesis, we will identify genetic associations with BP response within the Pharmacogenomic Evaluation of Antihypertensive Responses (PEAR) study, a population of uncomplicated hypertensives, in a group of Ca^{2+} pathway candidate genes: *ATP2A2*, *CACNA1C*, *CACNA1D*, *CACNB2*, *CASQ2*, *KCNMB1*, *PLN*, *RYR2*, *SLC8A1*.

We will also broaden the scope of the previous investigations within INVEST-GENES to test the second hypothesis that genetic variation within interrelated genes of the Ca^{2+} signaling pathway (*ATP2A2, CACNA1C, CACNA1D, CACNB2, CASQ2, KCNMB1, PLN, RYR2 and SLC8A1*) are associated with adverse cardiovascular outcomes in treated hypertensives with CAD.

A final hypothesis addresses evidence of altered transcriptional activity documented with the clinically associated rs2357928 promoter SNP of *CACNB2.*

3) Quantifiable differences in gene expression may contribute to clinically significant genetic associations found in regulatory sequences.

To investigate the underlying functional mechanism for this clinically associated promoter SNP we will utilize techniques in molecular biology to ascertain how this variant influences the expression profile of the $Ca_v1.2$ β_2 subunit in human vascular tissue.

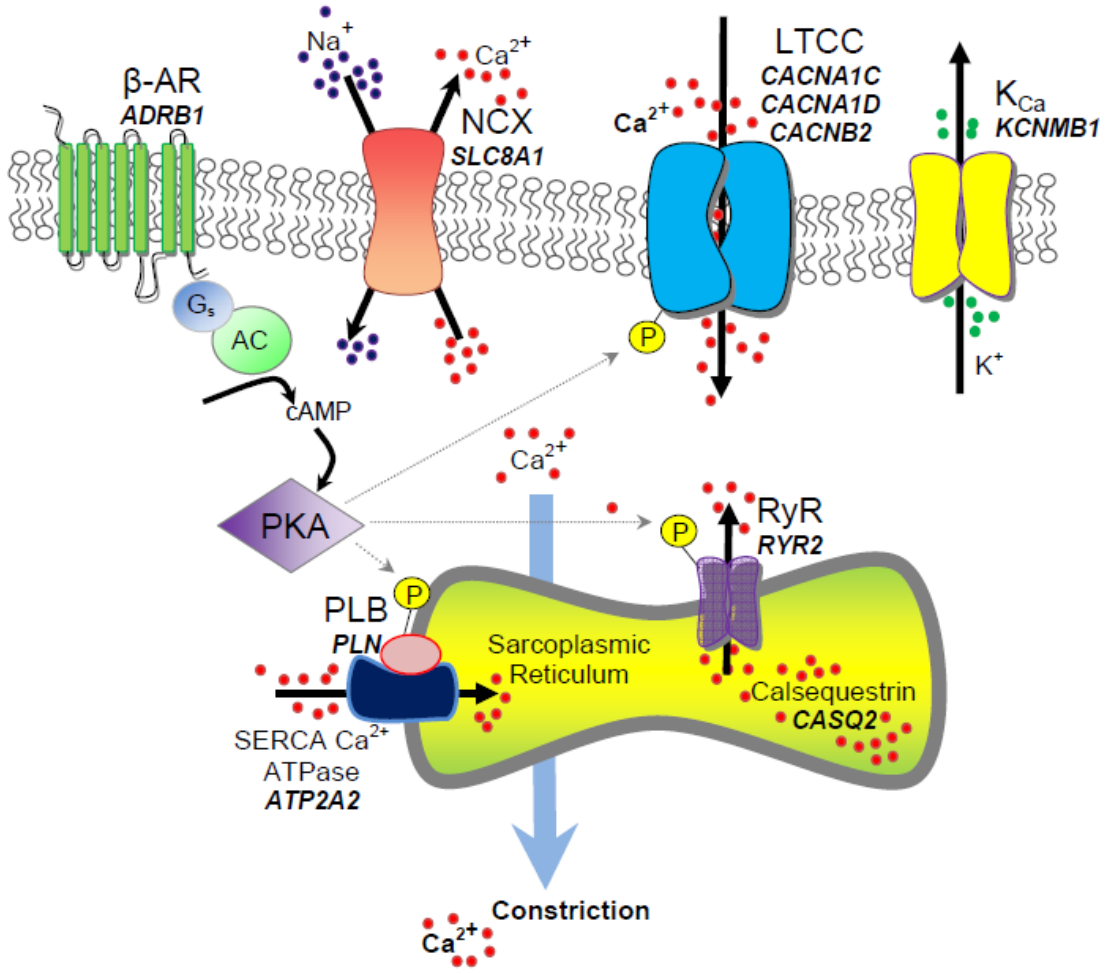

Figure 1-1. Schematic of the calcium signaling pathway

CHAPTER 2
ASSOCIATION OF CALCIUM SIGNALING PATHWAY VARIATION WITH BLOOD PRESSURE RESPONSE WITHIN THE PHARMACOGENOMICS OF ANTIHYPERTENSIVE RESPONSE (PEAR) STUDY

Introduction

Thiazide-type diuretics are current Joint National Committee (JNC) guideline recommended initial therapy for hypertensives without compelling indications.[1] Despite being the third most commonly prescribed antihypertensive in the United States in 2010, the mechanism by which hydrochlorothiazide (HCTZ) produces long-term BP lowering has yet to be elucidated. Current evidence suggests the vasorelaxant effects of HCTZ may be generated by opening of the K_{Ca} channel, either directly, or via pH activation through the inhibition of carbonic anhydrase.[25-27] Additional mechanistic evidence indicates thiazide-like diuretics may cause Ca^{2+} desensitization via the Rho-Rho kinase pathway to moderate agonist-induced vasoconstriction.[28]

Also an appropriate first-line therapy for the treatment of hypertension, β-blockers (βBs) as a class share a mechanism of action that has long been established, which is intimately linked to the actions of Ca^{2+} signaling as previously described. Given the heterogeneous nature of hypertension pathophysiology and the widely variable responses observed to commonly used antihypertensives, pharmacogenetic analyses may prove valuable in informing therapeutic decisions that are currently guided by a "trial and error" approach. Associations between variants within Ca^{2+} signaling pathway genes and BP have been illuminated in recent literature.[14,19,23,29] The current study aimed to determine whether genetic variation within Ca^{2+} signaling pathway genes may provide insight into the variability in responses to two commonly used antihypertensive drugs (Chapter 1).

Methods

Patient Population

Pharmacogenomic Evaluation of Antihypertensive Responses (PEAR) was a multi-center, prospective, open label, randomized study designed to address whether genetic predictors of BP lowering in response to a thiazide diuretic, a βB, or their combination could be identified.[30] PEAR recruited hypertensive individuals aged 17-65 years with DBP 90-110 mm Hg, who were without cardiovascular or renal disease, diabetes or any of the traditional exclusions to βBs.[30] Subjects were enrolled at the University of Florida (Gainesville, FL), Emory University (Atlanta, GA) and Mayo Clinic (Rochester, MN). Potential subjects were those with newly diagnosed, untreated or known hypertension treated with one or two antihypertensive drugs. Eligible subjects currently treated with antihypertensive therapy had their therapy tapered (as necessary) and discontinued, with a minimum antihypertensive-free period of 18 days, and a preferred washout period of 4 to 6 weeks. Subjects not meeting any exclusion criteria were further screened for BP inclusion based on untreated home and office BP. Following baseline studies that included the collection of home and 24 hour ambulatory BP data, PEAR subjects were then randomized to HCTZ 12.5 mg daily or atenolol 50 mg daily in an unblinded fashion. Subjects returned after 3 weeks on the initial dose, and proceeded through a dose titration protocol to 25 mg and 100 mg, respectively, based on a threshold BP >120/70 mm Hg. Those subjects with BPs ≤120/70 mm Hg were held at their current treatment step. Response to therapy was assessed after a minimum of 6 weeks on the target dose, after which those subjects with BP >120/70 mm Hg had the second drug added with a similar dose titration and response assessment procedure.[30]

Genotype Determination

PEAR genotypes for Ca^{2+} signaling pathway candidate genes were obtained from the Illumina® HumanCVD and HumanOmni1-Quad BeadChips. The HumanCVD BeadChip is a custom array of over 50,000 markers designed to capture genetic diversity across approximately two thousand genes thought to be involved in a range of cardiovascular, metabolic and inflammatory syndromes.[31] Normalized DNA (50 ng/µL) prepared at the University of Florida (UF) Center for Pharmacogenomics was transferred to the UF Interdisciplinary Center for Biotechnology Research (UF-ICBR), where the HumanCVD BeadChip was genotyped on Illumina®'s iScan System using the Infinium® II Assay (Illumina, San Diego, CA). Genotypes were called using GenomeStudio® Software version 2011.1 and the Genotyping Module version 1.9 calling algorithm (Illumina, San Diego, CA).

The HumanOmni1-Quad BeadChip covers over one million loci sourced from HapMap Phases 1-3 and the 1000 Genomes Project with coverage for minor allele frequency (MAF) >0.05. HumanOmni1-Quad BeadChip genotyping was run by Illumina®, Inc. (San Diego, CA) coordinated by the Brown Foundation Institute of Molecular Medicine for the Prevention of Human Diseases at the University of Texas, Houston.

Calcium signaling pathway candidate genes were selected for analysis as previously described in Chapter 1. Genotypes for SNPs within genomic regions 10 kb up and downstream of candidate genes were obtained. SNPs with MAF less than 5% in both race groups were not included in the final analysis due to the limited power to detect associations at that threshold. The putative functionality of SNPs meeting criteria for significance was assessed with PupaSuite 3.1 (http://pupasuite.bioinfo.cipf.es).

Statistical Analyses

For quality control, Hardy-Weinberg equilibrium was tested via χ^2 analysis performed separately by principal component analysis confirmed race, SNPs with Hardy-Weinberg p-values less than 10^{-5} had all associations ignored. Associations between genotype and BP responses were tested by linear regression following adjustment for covariates that included age, gender and baseline BP. Baseline characteristics were compared by treatment strategy using χ^2 or *t*-test as appropriate. Statistical analyses were performed in JMP® Genomics 5 and SAS® 9.2 (SAS Institute Inc, Cary, NC). Demographic variables recorded at baseline and BP measured by home BP monitoring at the end of the baseline period and the assessment following monotherapy were used to determine BP response to monotherapy. Response to monotherapy combined with add-on therapy in the opposite treatment arm was adjusted for BP at baseline in the monotherapy group and BP at the first response assessment for the add-on therapy group as well as controlling for the order of drug initiation. Response to add-on therapy was determined as the home BP observed at the second response assessment adjusted for the home BP value from the first (monotherapy) response assessment.

All SNPs were tested for an association with monotherapy, add-on therapy in the opposite treatment arm, and the combination of monotherapy with add-on therapy from the opposite treatment arm. For *ATP2A2*, *CASQ2*, *KCNMB1* and *PLN* a prespecified α level of p = 0.01 was used to identify SNPs of interest upon initial assessment of monotherapy and add-on therapy. There were relatively fewer SNPs tested for these genes and thus the more liberal threshold for significance (Table 2-1). For *CACNA1C*,

CACNA1D, *CACNB2*, *RYR2* and *SLC8A1*, given the large number of SNPs tested, a $p < 0.005$ was the required threshold for SNPs of interest upon initial assessment. Subsequent analysis consisted of evaluating for the consistency of the association between monotherapy and add-on therapy, requiring a nominal threshold in the alternate assessment ($p < 0.05$), along with testing for directional consistency, when testing for the evaluations of the same drug, or an opposite effect for the other study drug ($p = 0.5$). Those SNPs achieving an initial p-value of interest and surviving additional criteria for significance are considered the strongest association signals. This approach greatly reduces the probability of type I error, as demonstrated with the largest genes evaluated in this study, an initial $p = 0.005$ (of interest) combined with a consistent association in the alternate assessment $p < 0.05$, and an additional test for directional consistency yields an α level of 1.25×10^{-4} (Table 2-1). SNPs previously reported in the literature for an association with BP, hypertension or antihypertensive drug response were considered if they achieved nominal significance.

A strict Bonferroni correction was not used because of the differences in numbers of SNPs per gene (i.e. from 11 to 639), and because the Bonferroni correction assumes independence of all tests, which these were not, due to linkage disequilibrium (LD). However, the required p-value to identify SNPs of interest was reduced from the nominal p of 0.05 to try to balance too strict a p-value requirement resulting in dismissal of true positives, while minimizing the false positives for which we will pursue replication in the future.

Power calculations were generated using G*Power version 3.1.3, and tested the hypothesis that SNPs within candidate genes were associated with BP lowering in

response to HCTZ or atenolol.[32] For simplicity, the power analysis is based on two groups, those subjects with at least one copy of the variant allele and the group homozygous for the other, with tests for significance at α of 0.01 and 0.005 performed separately by race (Table 2-2). Effect sizes were based off of diastolic BP lowering response data from PEAR. In whites (n=461), we had 80% power to detect a 4.7 mmHg change in diastolic BP for a SNP with MAF of 0.05 at α = 0.005. The same analysis in blacks (n=304) demonstrated we had 80% power to detect a 5.9 mmHg change in diastolic BP for a SNP with MAF of 0.05. Linkage disequilibrium was assessed with PEAR genotypes in the HapMap PHASE format (http://stephenslab.uchicago.edu/software.html) uploaded to Haploview 4.2.[33] Haplotypes were inferred for each racial/ethnic group using PHASE version 2.1 software and coded according to the number of copes (zero, one, or two).[33]

Results

Genotyping

PEAR baseline demographics are included in Table 2-3. There was a significant difference in baseline BP by treatment strategy, although this is of little relevance as comparisons between treatment arms were not made. HumanCVD and HumanOmni1-Quad data were collected on 768 and 767 PEAR subjects, respectively. Patients were excluded if sample genotype call rates were below 95% and SNPs were excluded if genotype call rates were below 90%. 81 blind duplicates were included in genotyping and had a concordance rate of 99.992%. Gender was confirmed from X chromosome genotype data, and those who were discordant were excluded (*n*=1). Cryptic relatedness was estimated by pairwise identity-by-descent (IBD) analysis implemented using PLINK (http://pngu.mgh.harvard.edu/purcell/plink/).[34] One pair of monozygotic

twins was identified in this analysis, and confirmed by the study coordinator, and one twin was removed (n=1). Five pairs of samples were identified as first degree relatives, these individuals were kept for the analysis. Heterozygosity was assessed using PLINK, by estimating the inbreeding coefficient, F. One subject had F values > 4 negative standard deviations from the mean and was excluded. The final dataset consisted of 765 subjects. Quality control included removing monomorphic SNPs and those with a missing data rate >10%. Additional SNPs were removed from the HumanOmni1-Quad dataset with a Hardy-Weinberg $p < 10^{-6}$. No subjects were removed for a missing data rate >5% from either dataset. Four additional subjects identified as repeats were removed from the HumanOmni1-Quad dataset. Data were concordant for HumanOmni1-Quad BeadChip quality control repeat genotyping (n=15). Following quality control, final analyses were conducted on 761 PEAR subjects with HumanOmni1-Quad data.

Genetic Associations with Blood Pressure Response

Four SNPs within *CACNA1C* met prespecified criteria for significance to atenolol therapy among PEAR blacks. The LD structure of the significant associations is detailed in Figure 2-1, and highlights very limited LD between these SNPs. The rs2239101 SNP of *CACNA1C* recently identified as associated with systolic BP in a candidate gene analysis of BP and hypertension in 6 European cohorts comprising the Cohorts for Heart and Aging Research in Genomic Epidemiology Consortium (CHARGE) was nominally associated with BP response to atenolol monotherapy, as well as atenolol add-on, and overall significantly associated with the response to atenolol monotherapy combined with atenolol add-on from the opposite treatment arm among blacks (Figure 2-2).[29] As reported in CHARGE, increasing copies of the

CACNA1C rs2239101 minor allele were associated with a decrease in BP, within PEAR this association corresponded to a greater BP response to atenolol therapy, observed for both diastolic and systolic BP responses (Table 2-4).

The association between the conserved rs12425032 intronic SNP of *CACNA1C* and BP response to antihypertensive therapy in PEAR (Table 2-5) is considered the strongest as BP response to atenolol therapy met criteria for significance among blacks, and a directionally opposite, nominal association was also observed for BP response to HCTZ in the same race group (Table 2-5). Increasing copies of the *CACNA1C* rs12425032 minor allele were significantly associated with a lower BP response to atenolol add-on therapy in blacks. Directionally consistent associations were also observed for BP response to atenolol monotherapy as well as for the combined assessment of atenolol monotherapy with atenolol add-on from the opposite treatment arm (Figure 2-3). Response to HCTZ add-on therapy showed a directionally opposite association, as increasing copies of the rs12425032 minor allele were nominally associated with greater BP response (Table 2-5). A similar trend was also observed for the response to HCTZ monotherapy, and the combined assessment of HCTZ monotherapy with HCTZ add-on from the opposite treatment arm (Figure 2-3).

The association between the *CACNA1C* rs2238078 intronic SNP and BP response to atenolol monotherapy was consistent with atenolol add-on, where increasing copies of the minor allele were associated with greater BP response to therapy (Table 2-6). Overall, the combined assessment of atenolol monotherapy with atenolol add-on from the opposite treatment arm was also significant (Table 2-6). Also of note, *CACNA1C* rs2238078 was nominally associated with a directionally opposite effect on BP response

to HCTZ monotherapy as well as the combined assessment of HCTZ monotherapy with HCTZ add-on from the opposite treatment arm in whites, where increasing copies of the minor allele corresponded to lower BP response to HCTZ (Table 2-7).

Finally, the rs11831085 intronic SNP of *CACNA1C* was also significantly associated with BP response to atenolol add-on therapy in blacks. Increasing copies of the rs11831085 minor allele were associated with lower BP response to atenolol, an effect which was consistent between atenolol add-on for diastolic and systolic BP responses and systolic BP response to atenolol monotherapy (Table 2-8). For the combined response assessment of atenolol monotherapy with atenolol add-on from the opposite treatment arm, the association was highly significant for systolic BP response ($p = 6.3x10^{-5}$), and nominally associated with diastolic BP ($p = 0.017$; Table 2-8, Figure 2-4). No associations meeting prespecified criteria for significance were observed for whites in *CACNA1C*, or for either race group among other candidate genes included in the study analyses. A summary of the observed associations and their direction are detailed in Table 2-12. However, additional associations showing a strong trend for significance are reported in Appendix A.

Haplotype Analysis

Haplotypes were imputed for PEAR blacks incorporating the 4 *CACNA1C* SNPs demonstrating significant associations with BP response. The initial haplotype analysis revealed a strong association for two relatively common 4 SNP haplotypes (Appendix A). Because the greatest amount of LD was observed between *CACNA1C* rs11831085 and rs12425032, haplotypes incorporating the two SNPs alone were also assigned. Analysis of the 2 SNP haplotypes proved equally as informative as the original 4 SNP haplotype, and more informative than the single SNP associations alone.

Among PEAR blacks, increasing copies of the common *CACNA1C* AT haplotype were associated with greater BP response to atenolol, whereas a directionally opposite association was observed for BP response to HCTZ (Table 2-10; Figure 2-5). Additionally, carriers of the alternate GC haplotype had a significantly lower BP response to atenolol (Table 2-11; Figure 2-6).

Discussion

The PEAR study provided a unique opportunity to assess the influence of genetic variation on BP response to two commonly used BP therapies in a racially diverse population of uncomplicated hypertensives. Our study demonstrated genetic variants within genes critical to the regulation of Ca^{2+} signaling are significantly associated with BP response to atenolol and HCTZ. Interestingly, the strongest associations between genetic variation and BP response observed in this study were among blacks, in response to atenolol therapy.

A SNP previously identified as related to systolic BP in a large candidate gene cohort study was found to be nominally associated with BP response to atenolol monotherapy as well as atenolol monotherapy combined with atenolol add-on from the opposite treatment arm in blacks.[29] The association in PEAR is directionally consistent with the allelic effect on systolic BP observed in the CHARGE cohort, where the minor allele is associated with a decrease in systolic BP. While this finding does not represent a true replication of the previously reported data, it certainly lends credence to the validity of the observed association. Three additional novel genetic associations with BP response were observed within *CACNA1C*, the strongest of which was the intronic rs12425032. Associated with lower BP response to atenolol therapy in all response

assessments among blacks, *CACNA1C* rs12425032 was also associated with greater BP response to HCTZ in the same race group.

Finally, the common *CACNA1C* AT haplotype comprised of the conserved rs11831085 and rs12425032 SNPs was significantly associated with BP response to both atenolol and HCTZ, in a directionally opposite manner.

Because βB and thiazide diuretics affect different extremities of the Ca^{2+} signaling pathway we might expect to see directionally alternate BP response associations to functionally relevant SNPs. β-adrenergic stimulation enhances the probability of functional $Ca_v1.2$ channel activation and stimulates a shift of channels from a null gating inactivable pool to an activable pool.[35] βB attenuate the increase in $I_{Ca,L}$ associated with β-adrenergic stimulation. Conversely, HCTZ may enhance the voltage-dependent inactivation of $Ca_v1.2$ channels via K_{Ca} channel opening which hyperpolarizes the cell membrane.[25]

A SNP within a conserved intronic region of *CACNA1C* may confer lower transcriptional efficiency or stability, or code for an alternative splice site. Such a change could manifest in a decreased $I_{Ca,L}$, which could contribute to a lower BP response to βB therapy. A decrease in $I_{Ca,L}$ might also exacerbate the BP response observed to HCTZ, if membrane hyperpolarization effectively blunted a Ca^{2+} current that was comparatively weaker among variant carriers.

The effect of genetic variation within the Ca^{2+} signaling pathway on BP response in the current study appears be to context dependent. It is generally accepted that single gene effects on antihypertensive drug response tend to be small, and it may be the genetic associations observed presently are distinguishable because they're in

response to a therapy which does not directly target the underlying genetic mechanisms of hypertension in the racial population from which they're observed.[5] Hence, the lack of a consistent genetic effect among PEAR whites may be attributable to an overriding BP response that is driven by a decrease in renin secretion and influenced less by any effect on Ca^{2+} signaling.

The PEAR study recruited uncomplicated hypertensives, those subjects without concomitant disease states which might confound the initial response observed to antihypertensive therapies, and included a temporary washout period prior to randomization.[30] Reported here are novel associations between variants within genes critical to the pathway of Ca^{2+} signaling and BP response to two common antihypertensive agents. Despite careful study design, we do accept the limitation that we may have failed to capture associations between genetic variation and BP response due to a limited power to detect effects at lower minor allele frequencies. Our approach to hypothesis testing utilizing a relatively liberal p-value initially was an attempt to balance the detection of SNPs with small effects while minimizing the likelihood of Type I error by testing associations between separate populations, one receiving a drug as monotherapy and another receiving a drug as add-on therapy in the opposite treatment arm. Additional testing for BP associations in the opposite treatment arm as well as accounting for a directional effect yielded p-values often more stringent than a Bonferroni corrected analysis.

Overall, the associations reported here may provide some insight into the heterogeneity of BP response observed to two commonly used antihypertensive therapies in a racially diverse population with mild to moderate hypertension. Further

study is warranted, however, as the associations reported here have yet to be replicated in an independent study population.

Table 2-1. Illumina® BeadChip SNP Coverage and p-value thresholds

Gene	Human CVD	Human Omni1-Quad	Total	Initial p-value	Additional p-value	Direction of effect*	Product p-value
ATP2A2	11	11	19	0.01	0.05	0.5	$2.5x10^{-4}$
CACNA1C	258	279	428	0.005	0.05	0.5	$1.25x10^{-4}$
CACNA1D	0	142	142	0.005	0.05	0.5	$1.25x10^{-4}$
CACNB2	0	242	242	0.005	0.05	0.5	$1.25x10^{-4}$
CASQ2	17	58	66	0.01	0.05	0.5	$2.5x10^{-4}$
KCNMB1	19	40	54	0.01	0.05	0.5	$2.5x10^{-4}$
PLN	3	11	14	0.01	0.05	0.5	$2.5x10^{-4}$
RYR2	324	459	639	0.005	0.05	0.5	$1.25x10^{-4}$
SLC8A1	0	303	303	0.005	0.05	0.5	$1.25x10^{-4}$

*Direction as hypothesized for additional p-value

Table 2-2. Power to detect diastolic BP differences across MAF ranges by race, PEAR

Black n=304	α=0.01			α=0.005		
	5%	10%	25%	5%	10%	25%
4 mmHg DBP	47%	80%	99%	37%	73%	>99%
7 mmHg DBP	96%	>99%	>99%	94%	99%	>99%
White n=461	α=0.01			α=0.005		
	5%	10%	25%	5%	10%	25%
4 mmHg DBP	70%	95%	>99%	61%	93%	99%
7 mmHg DBP	>99%	>99%	>99%	96%	>99%	>99%

Table 2-3. PEAR Baseline Demographics

Characteristic	All (n=765)	ATEN (n=385)	HCTZ (n=380)	p-value
Age	48.8 ± 9.2	48.6 ± 9.2	49.0 ± 9.2	0.58
Sex (% female)	403 (52.7)	216 (56.1)	187 (49.2)	0.06
Race				
Black (%)	304 (39.7)	152 (39.5)	152 (40.0)	
White (%)	461 (60.3)	233 (60.5)	228 (60.0)	0.88
Home SBP (mm Hg)	145.8 ± 10.3	145.0 ± 9.9	146.6 ± 10.7	0.03
Home DBP (mm Hg)	93.7 ± 6.0	93.3 ± 5.9	94.2 ± 6.0	0.03
Home heart rate (bpm)	77.5 ± 9.5	77.8 ± 9.4	77.2 ± 9.7	0.37
History of hypertension				
Duration of hypertension (yr)	6.5 ± 7.2	6.7 ± 7.0	6.4 ± 7.2	0.42
Family history of hypertension (%)	586 (76.4)	298 (77.4)	286 (75.5)	0.77
Never taken an antihypertensive drug (%)	93 (12.1)	47 (12.2)	46 (12.0)	0.95
Taking antihypertensive drug at entry (%)	672 (87.8)	338 (87.8)	334 (87.8)	0.96
Smoking status				
Current smoker (%)	111 (14.5)	50 (13.0)	61 (16.0)	0.23
Ex-smoker (%)	531 (82.7)	276 (84.7)	252 (80.5)	0.17
BMI (kg/m^2)	30.8 ± 5.5	30.8 ± 5.9	30.8 ± 5.1	0.97
Waist circumference (cm)	97.8 ± 13.3	97.6 ± 13.1	98.1 ± 13.5	0.57

Mean ± SD unless otherwise noted, p-value by *t* test for continuous variables and χ^2 test for categorical variables between treatment groups.

Table 2-4. Associations between *CACNA1C* rs2239101 genotype and blood pressure response to atenolol among PEAR blacks

Response	MAF	ΔDBP Major Hmz. (mmHg)	ΔDBP Het. (mmHg)	ΔDBP Minor Hmz. (mmHg)	*P*[†]	ΔSBP Major Hmz. (mmHg)	ΔSBP Het. (mmHg)	ΔSBP Minor Hmz. (mmHg)	*P*[†]
Atenolol Monotherapy	0.05	-3.8	-8.0	-	*0.04*	-12.0	-12.1	-	*0.026*
Atenolol Add-On	0.05	-5.6	-8.4	-	*0.05*	-6.0	-9.5	-	*0.04*
Atenolol Monotherapy + Add-On	0.05	-4.7	-8.2	-	*0.0065*	-4.2	-9.7	-	*0.0036*

[†]Adjusted for age, gender, and baseline BP. Response to monotherapy + add-on was adjusted for BP at baseline in the monotherapy group and BP at the first response assessment for the add-on therapy group as well as controlling for the order of drug initiation.

Table 2-5. Associations between *CACNA1C* rs12425032 genotype and blood pressure response to atenolol and HCTZ among PEAR blacks

Response	MAF	ΔDBP Major Hmz. (mmHg)	ΔDBP Het. (mmHg)	ΔDBP Minor Hmz. (mmHg)	$P^{\dagger}$	ΔSBP Major Hmz. (mmHg)	ΔSBP Het. (mmHg)	ΔSBP Minor Hmz. (mmHg)	$P^{\dagger}$
Atenolol Monotherapy	0.25	-5.2	-3.2	-2.1	*0.046*	-5.1	-1.2	0.0	*0.03*
Atenolol Add-On	0.25	-7.1	-5.9	0.68	*0.003*	-8.3	-6.0	0.9	$9.6x10^{-4}$
Atenolol Monotherapy + Add-On	0.25	-6.2	-4.5	-0.7	$6.3x10^{-4}$	-6.7	-3.5	0.4	$3.2x10^{-4}$
HCTZ Monotherapy	0.25	-6.8	-6.5	-10.7	*0.59*	-11.1	-11.3	-19.8	*0.38*
HCTZ Add-On	0.25	-6.1	-8.0	-11.8	*0.05*	-10.4	-14.9	-20.2	*0.007*
HCTZ Monotherapy + Add-On	0.25	-6.4	-7.2	-11.2	*0.069*	-10.8	-13.0	-20.0	*0.014*

†Adjusted for age, gender, and baseline BP. Response to monotherapy + add-on was adjusted for BP at baseline in the monotherapy group and BP at the first response assessment for the add-on therapy group as well as controlling for the order of drug initiation.

Table 2-6. Associations between *CACNA1C* rs2238078 genotype and blood pressure response to atenolol among PEAR blacks

Response	MAF	ΔDBP Major Hmz. (mmHg)	ΔDBP Het. (mmHg)	ΔDBP Minor Hmz. (mmHg)	$P^{\dagger}$	ΔSBP Major Hmz. (mmHg)	ΔSBP Het. (mmHg)	ΔSBP Minor Hmz. (mmHg)	$P^{\dagger}$
Atenolol Monotherapy	0.13	-3.7	-5.9	-	*0.013*	-2.1	-7.1	-	*0.002*
Atenolol Add-On	0.13	-5.5	-8.0	-	*0.016*	-6.3	-8.6	-	*0.018*
Atenolol Monotherapy + Add-On	0.13	-4.6	-7.1	-	*0.001*	-4.1	-8.0	-	$2.8x10^{-4}$

†Adjusted for age, gender, and baseline BP. Response to monotherapy + add-on was adjusted for BP at baseline in the monotherapy group and BP at the first response assessment for the add-on therapy group as well as controlling for the order of drug initiation. DBP = diastolic BP; SBP = systolic BP; Hmz. = homozygote; Het. = heterozygote.

Table 2-7. Associations between *CACNA1C* rs2238078 genotype and blood pressure response to HCTZ among PEAR whites

Response	MAF	ΔDBP Major Hmz. (mmHg)	ΔDBP Het. (mmHg)	ΔDBP Minor Hmz. (mmHg)	*P*[†]	ΔSBP Major Hmz. (mmHg)	ΔSBP Het. (mmHg)	ΔSBP Minor Hmz. (mmHg)	*P*[†]
HCTZ Monotherapy	0.39	-4.9	-4.1	-2.6	*0.01*	-8.0	-8.1	-5.6	*0.12*
HCTZ Add-On	0.39	-3.6	-2.5	-2.3	*0.34*	-7.0	-5.5	-4.1	*0.07*
HCTZ Monotherapy + Add-On	0.39	-4.3	-3.3	-2.4	*0.008*	-7.5	-6.8	-4.8	*0.02*

[†]Adjusted for age, gender, and baseline BP. Response to monotherapy + add-on was adjusted for BP at baseline in the monotherapy group and BP at the first response assessment for the add-on therapy group as well as controlling for the order of drug initiation. DBP = diastolic BP; SBP = systolic BP; Hmz. = homozygote; Het. = heterozygote.

Table 2-8. Associations between *CACNA1C* rs11831085 genotype and blood pressure response to atenolol among PEAR blacks

Response	MAF	ΔDBP Major Hmz. (mmHg)	ΔDBP Het. (mmHg)	ΔDBP Minor Hmz. (mmHg)	$P^{\dagger}$	ΔSBP Major Hmz. (mmHg)	ΔSBP Het. (mmHg)	ΔSBP Minor Hmz. (mmHg)	$P^{\dagger}$
Atenolol Monotherapy	0.32	-4.1	-4.6	-2.1	*0.35*	-4.1	-3.9	5.2	*0.006*
Atenolol Add-On	0.32	-7.2	-5.3	-4.1	*0.007*	-8.8	-5.1	-4.6	*0.001*
Atenolol Monotherapy + Add-On	0.32	-5.8	-4.9	-3.1	*0.017*	-6.7	-4.4	0.2	$6.3x10^{-5}$

†Adjusted for age, gender, and baseline BP. Response to monotherapy + add-on was adjusted for BP at baseline in the monotherapy group and BP at the first response assessment for the add-on therapy group as well as controlling for the order of drug initiation. DBP = diastolic BP; SBP = systolic BP; Hmz. = homozygote; Het. = heterozygote.

Table 2-9. *CACNA1C* rs11831085, rs12425032 haplotype frequency among PEAR blacks

Haplotype	Frequency (%)
GT	15.6
GC	16.8
AT	59.7
AC	7.8

Table 2-10. Associations between *CACNA1C* rs11831085, rs12425032 AT haplotype and blood pressure response among PEAR blacks

Response	Frequency	ΔDBP, 0 copy (mmHg)	ΔDBP, 1 copy (mmHg)	ΔDBP, 2 copies (mmHg)	$P^{\dagger}$	ΔSBP,0 copy (mmHg)	ΔSBP, 1 copy (mmHg)	ΔSBP, 2 copies (mmHg)	$P^{\dagger}$
Atenolol Monotherapy	0.60	-2.0	-4.6	-4.6	*0.14*	3.6	-4.4	-3.9	*0.014*
Atenolol Add-On	0.60	-2.8	-5.6	-8.0	$1.3x10^{-4}$	-3.9	-5.6	-9.4	$3.8x10^{-4}$
Atenolol Monotherapy + Add-On	0.60	-2.4	-5.0	-6.5	$3.1x10^{-4}$	-0.1	-4.9	-7.1	$6.9x10^{-5}$
HCTZ Monotherapy	0.60	-8.9	-7.0	-6.3	*0.31*	-15.5	-12.1	-10.1	*0.14*
HCTZ Add-On	0.60	-10.0	-7.4	-5.1	*0.028*	-19.0	-12.5	-10.0	*0.024*
HCTZ Monotherapy + Add-On	0.60	-9.5	-7.2	-5.8	*0.02*	-17.2	-12.3	-10.0	*0.0095*

†Adjusted for age, gender, and baseline BP. Response to monotherapy + add-on was adjusted for BP at baseline in the monotherapy group and BP at the first response assessment for the add-on therapy group as well as controlling for the order of drug initiation.

Table 2-11. Associations between *CACNA1C* rs11831085, rs12425032 GC haplotype and blood pressure response among PEAR blacks

Response	Frequency	ΔDBP, 0 copy (mmHg)	ΔDBP, 1 copy (mmHg)	ΔDBP, 2 copies (mmHg)	$P^{\dagger}$	ΔSBP,0 copy (mmHg)	ΔSBP, 1 copy (mmHg)	ΔSBP, 2 copies (mmHg)	$P^{\dagger}$
Atenolol Monotherapy	0.17	-4.8	-3.1	-	*0.08*	-4.8	0.0	-	*0.0027*
Atenolol Add-On	0.17	-6.6	-5.2	-	*0.07*	-7.8	-4.4	-	*0.002*
Atenolol Monotherapy + Add-On	0.17	-5.7	-4.1	-	*0.02*	-6.3	-2.1	-	$4.3x10^{-5}$

†Adjusted for age, gender, and baseline BP. Response to monotherapy + add-on was adjusted for BP at baseline in the monotherapy group and BP at the first response assessment for the add-on therapy group as well as controlling for the order of drug initiation.

Table 2-12. Summary of significant *CACNA1C* SNP associations with BP response

Treatment	SNP	Race	MAF	Allele	BP response*
Atenolol	rs2239101	Black	0.05	T/**C**	↑
	rs2238078	Black	0.13	**G**/T	↑
	rs12425032	Black	0.25	**C**/T	↓
	rs11831085	Black	0.32	A/**G**	↓
HCTZ	rs12425032	Black	0.25	T/**C**	↑
	rs2238078	White	0.39	**G**/T	↓

*Minor allele effect on BP response, minor allele shown in bold

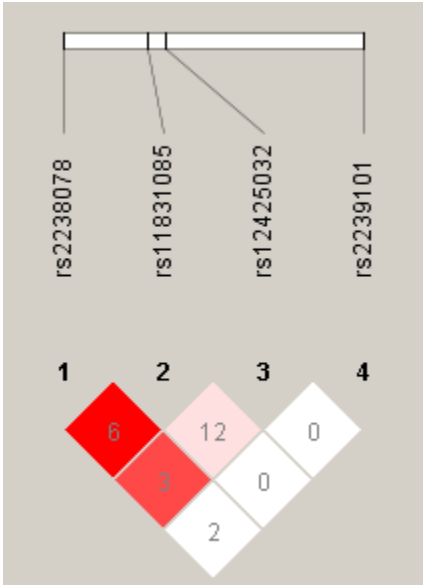

Figure 2-1. LD structure of *CACNA1C* significant associations within PEAR blacks, r^2 values shown

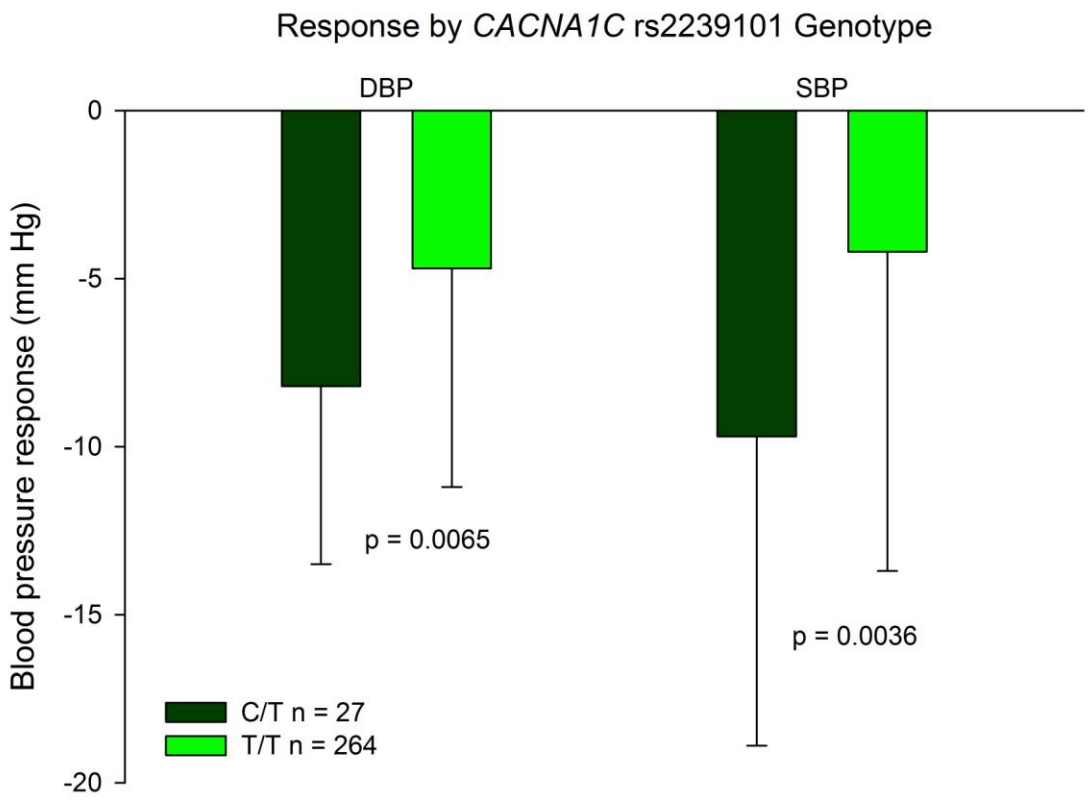

Figure 2-2. Blood pressure response to the combined assessment of atenolol monotherapy with atenolol add-on from the opposite treatment arm by *CACNA1C* rs2239101 genotype among PEAR blacks

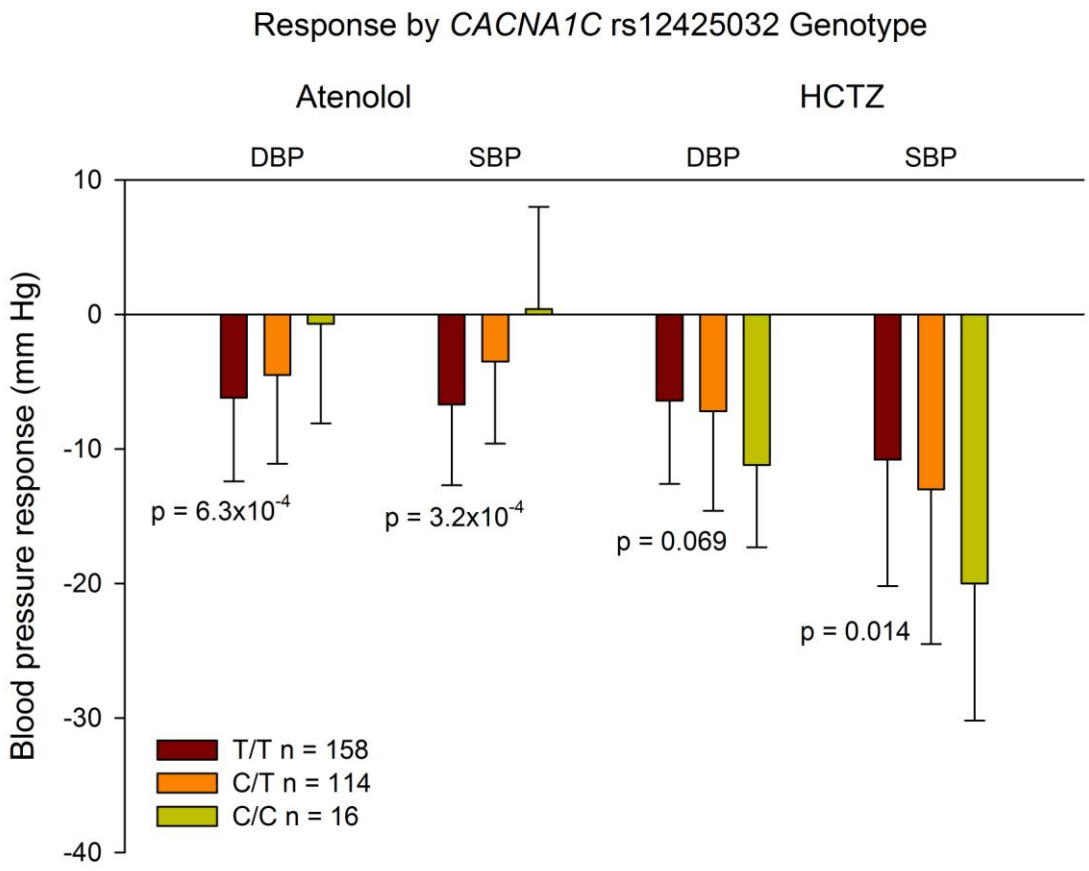

Figure 2-3. Blood pressure response to the combined assessment of monotherapy with add-on from the opposite treatment arm by *CACNA1C* rs12425032 genotype among PEAR blacks

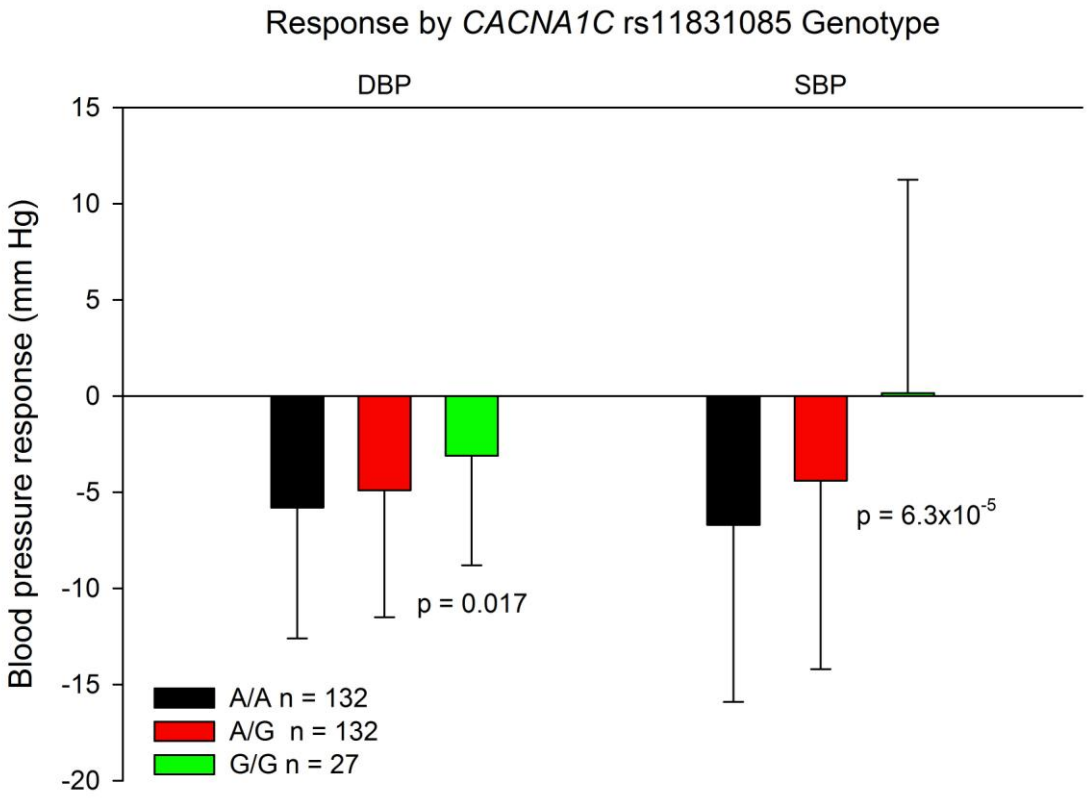

Figure 2-4. Blood pressure response to the combined assessment of atenolol monotherapy with atenolol add-on from the opposite treatment arm by *CACNA1C* rs11831085 genotype

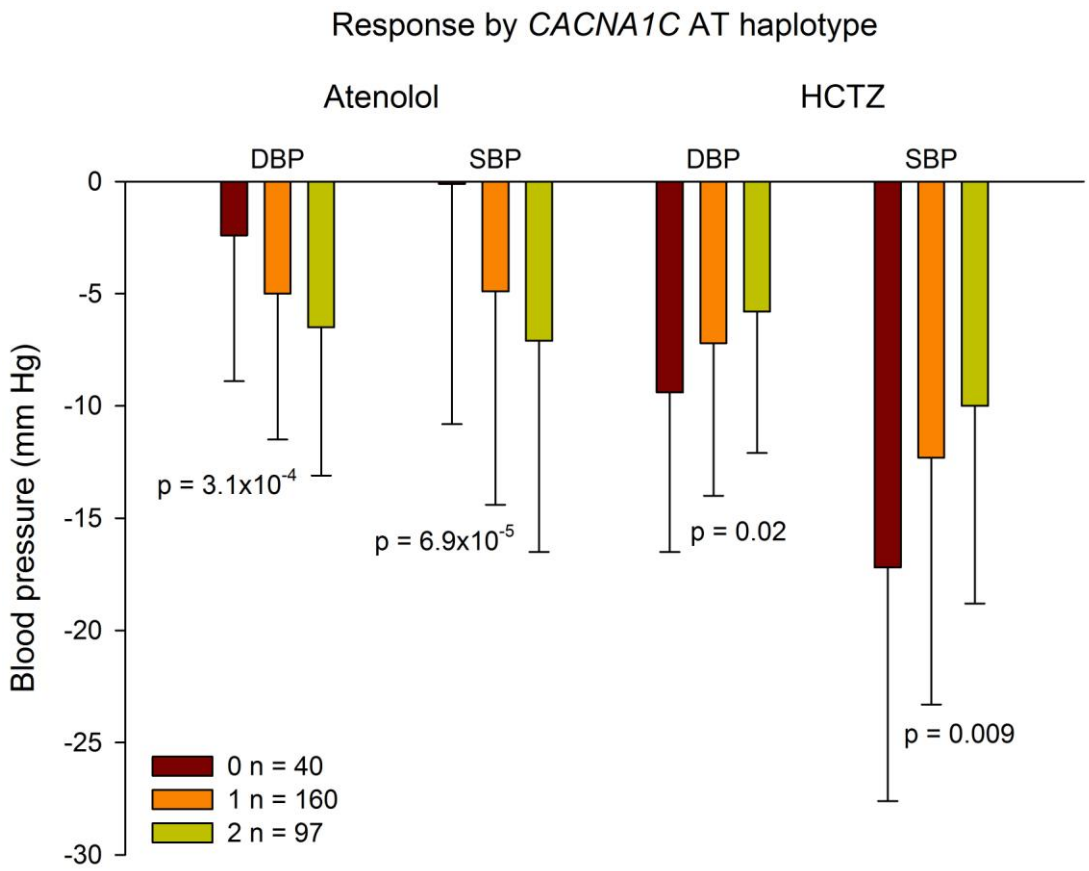

Figure 2-5. Blood pressure response to the combined assessment of monotherapy with add-on from the opposite treatment arm by *CACNA1C* AT haplotype among PEAR blacks

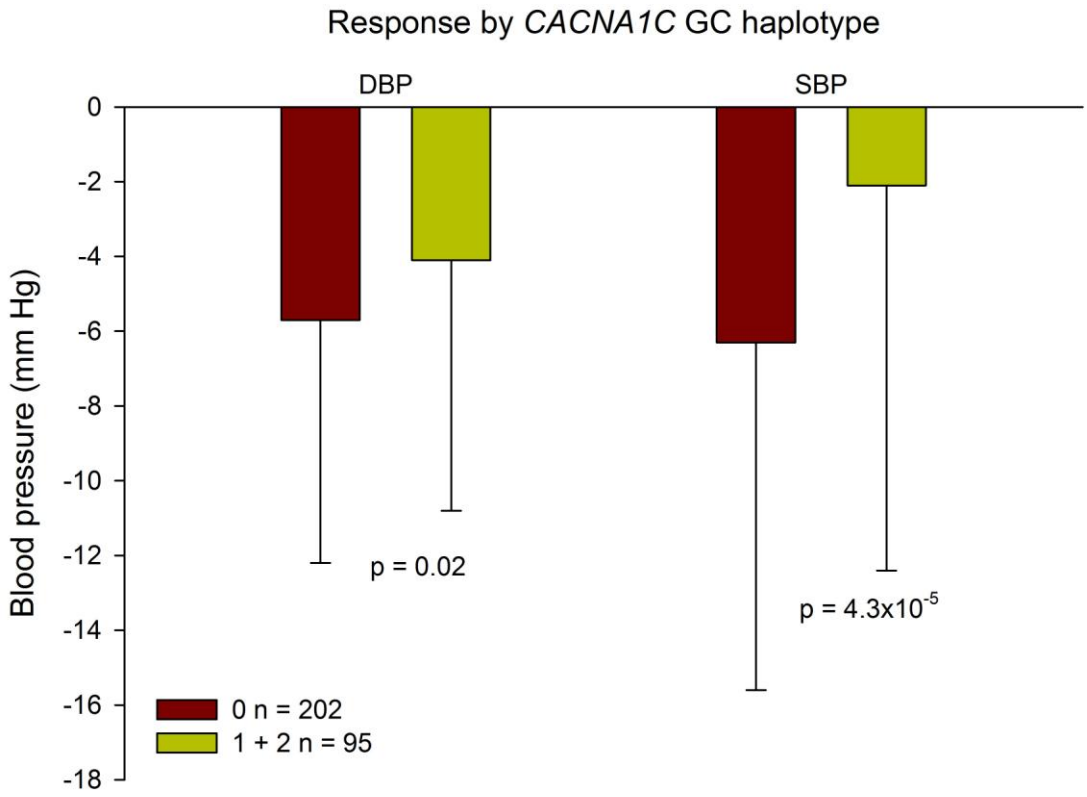

Figure 2-6. Blood pressure response to the combined assessment of atenolol monotherapy with atenolol add-on from the opposite treatment arm by *CACNA1C* GC haplotype among PEAR blacks

CHAPTER 3
ASSOCIATION OF CALCIUM SIGNALING PATHWAY VARIATION WITH CARDIOVASCULAR OUTCOMES IN THE INTERNATIONAL VERAPAMIL SR TRANDOLAPRIL STUDY – GENETIC SUBSTUDY (INVEST-GENES)

Introduction

Genetic variation within the Ca^{2+} signaling pathway has previously been associated with adverse outcomes dependent upon treatment strategy within the International Verapamil SR-Trandolapril Study Genetic Substudy (INVEST-GENES) population (Chapter 1). INVEST was a large, multicenter, randomized hypertensive treatment outcomes trial utilizing both CCB and βB treatment strategies. The INVEST-GENES cohort provides a rare opportunity to investigate associations between variation within genes critical to the hypertensive treatment response and adverse cardiovascular outcomes among high-risk hypertensive patients with coronary artery disease (CAD).

Methods

INVEST-GENES Clinical Cohort

INVEST evaluated BP response and adverse outcomes occurring with either an atenolol-based βB or a verapamil SR-based CCB treatment strategy in 22,576 patients with documented CAD and hypertension.[36] The design, protocol, and primary outcome data have been published in detail elsewhere.[36,37] To summarize, the protocol required patients to be seen at baseline, 6, 12, 18 and 24 weeks, and then every 6 months thereafter until 2 years after the last patient was enrolled. At each study visit, patients had BP and heart rate measured, clinical assessment performed and additional antihypertensive medications added as needed to meet the Joint National Committee VI BP goals.[38] Blood pressure control and cardiovascular outcomes were similar between the treatment strategies in the main trial.[36]

The INVEST-GENES cohort consisted of 5,979 patients from 184 sites in the United States and Puerto Rico who provided DNA samples and additional written informed consent for genetic studies. Using the 5,979 patients with genetic samples, a nested case-control cohort was studied consisting of patients who experienced the primary outcome (first occurrence of all cause death, nonfatal myocardial infarction, or nonfatal stroke) matched to patients who did not have a primary outcome event during study follow-up. Controls were matched to cases by age (in decades), sex, and race/ethnicity in a ratio of approximately 4:1. The nested case-control approach has previously been documented to yield similar results to analyses of the entire genetic cohort.[23,39] Secondary outcome measures were defined as the individual components of the primary outcome.[36]

Genotype Determination

Buccal tissue samples were obtained by mouthwash and genomic DNA was isolated using the Gentra Systems PureGene® kit. INVEST-GENES genotypes for Ca^{2+} signaling pathway candidate genes were obtained from the Illumina® HumanCVD BeadChip (Table 2-1). 250ng to 500ng of genomic DNA from each patient of the INVEST-GENES case-control cohort was hybridized to a HumanCVD BeadChip custom SNP array (IBC array, version 2; Illumina®, Inc) on Illumina®'s iScan System according to manufacturer specified protocols. A description of the strategy for gene and SNP selection on the Illumina® HumanCVD BeadChip is provided in detail elsewhere.[31] SNP interrogation was performed with Illumina®'s Infinium® II chemistry. Genotypes were called using GenomeStudio® Software version 2011 and the Genotyping Module version 1.9 calling algorithm (Illumina, San Diego, CA). The putative functionality of

SNPs meeting criteria for significance was assessed with PupaSuite 3.1 (http://pupasuite.bioinfo.cipf.es).

A Principal Component Analysis (PCA) was performed in all subjects using an LD pruned dataset using the EIGENSTRAT method implemented through JMP® Genomics version 5.0 (SAS®, Cary, NC). INVEST included primarily white, Hispanic, and black race/ethnic groups, determined by patient report with interaction by the study investigator. Race/ethnic groups were confirmed with PCA clustering results. If race/ethnic category disagreed strongly with PCA, patients were re-categorized to reflect the PCA result, considered to better reflect genetic ancestry. PCA was then performed in each race/ethnic group. To adjust for ancestry in the final analyses, principal components 1 through 3 were included as covariates for whites and Hispanics and principal components 1 through 4 were included as covariates for blacks as these provided the best separation of ancestry clusters and were chosen as covariates in analysis to adjust for ancestry.

Statistical Analyses

Hardy-Weinberg equilibrium was tested for each PCA-defined racial/ethnic group using χ^2 analyses. All statistical analyses were performed using SAS® version 9.2 (Cary, NC) or JMP® Genomics 5. Baseline characteristics were compared by treatment strategy using χ^2 or analysis of variance, as appropriate. Primary and secondary outcome adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using logistic regression by PCA defined race, and adjusted for prespecified baseline covariates that were found to influence prognosis. These baseline covariates included age, sex, INVEST treatment strategy, previous myocardial infarction, history of

heart failure and diabetes. Significant p values for the main effect primary outcome or secondary outcome of death were $p = 2.5x10^{-4}$ for *CACNA1C* and *RYR2*, and p = 0.005 for *ATP2A2*, *CASQ2*, *KCNMB1* and *PLN*. A strict Bonferroni correction was not used for reasons described in Chapter 2. For analysis of pharmacogenetic interactions, a p = 0.005 was considered significant for *CACNA1C* and *RYR2*, and the threshold set for *ATP2A2*, *CASQ2*, *KCNMB1* and *PLN* was p = 0.01. For those SNPs demonstrating significant associations with BP response in PEAR, a nominal association of $p < 0.05$ was considered significant for main effect, secondary outcome and pharmacogenetic interaction analyses. SNPs previously represented in the literature for associations with cardiovascular outcomes had a $p < 0.05$ considered significant. Power calculations were performed with QUANTO version 1.2.[40] Within the case-control cohort we had 95% power to detect an OR of 2.5 with an α of 0.005 and 80% power to detect an OR of 2.5 with an α of $2.5x10^{-4}$ for the primary outcome (Table 3-1). LD was assessed with INVEST-GENES genotypes in the HapMap PHASE format (http://stephenslab.uchicago.edu/software.html) uploaded to Haploview 4.2.[33] To assess LD with SNPs not included on the HumanCVD BeadChip, Seattle SNPs Genome Variation Server (http://gvs.gs.washington.edu/GVS/index.jsp) was utilized incorporating genotypes from HapMap populations.

Results

Baseline characteristics and medical history for the INVEST-GENES case-control cohort are shown in Table 3-2. Characteristics of the case-control cohort are consistent with the original INVEST-GENES cohort, consisting of an elderly and racially diverse hypertensive CAD population. Patients in the verapamil-based CCB treatment strategy

and atenolol-based βB treatment strategy were similar in baseline characteristics, medical history and nonstudy medication use (Table 3-2).

Genotyping and Quality Control

Of the INVEST-GENES subjects genotyped, patients were excluded if sample genotype call rates were below 95% and SNPs were excluded if genotype call rates were below 90%. 87 blind duplicates were included in genotyping and had a concordance rate of 99.997%. Gender was confirmed from X chromosome genotype data, and those who were discordant were excluded. Cryptic relatedness was estimated by pairwise identity-by-descent (IBD) analysis implemented using PLINK (http://pngu.mgh.harvard.edu/purcell/plink/). Forty-one pairs of samples were identified as first degree relatives, these individuals were kept for the analysis. Heterozygosity was assessed using PLINK, by estimating the inbreeding coefficient, F. Six subjects had F values > 4 standard deviations from the mean. One of these subjects also had a high missing genotype rate of > 4% and this subject was excluded. Outliers in the by race/ethnic group Principal Component Analysis were also removed (n=4). The final INVEST dataset consisted of 2,214 subjects. Genotype data were complete for 1345 patients of the case-control cohort, comprised of 269 patients who experienced a primary outcome event during study follow-up, and 1,076 patients who did not.

Genetic and Pharmacogenetic Associations With Adverse Cardiovascular Outcomes

CACNA1C

The *CACNA1C* rs2239101 and rs12425032 polymorphisms significantly associated with BP response to atenolol among blacks in the PEAR population were also associated with adverse cardiovascular outcomes within INVEST-GENES. The

rs2239101 SNP previously identified as associated with systolic BP in a candidate gene analysis of BP and hypertension from CHARGE, and also associated with greater BP response to atenolol among PEAR blacks (Table 2-4) was associated with an increased risk of death among whites within the INVEST-GENES case-control cohort (Table 3-3).[29] The association within the INVEST-GENES case-control cohort was consistent among whites randomized to both CCB and βB treatment strategies (main effect p = 0.004). No similar associations were observed among black or Hispanic racial/ethnic groups.

The *CACNA1C* rs12425032 SNP significantly associated with lower BP response to atenolol and greater BP response to HCTZ among PEAR blacks also demonstrated a nominally significant association with the primary outcome among INVEST-GENES case-control cohort blacks. *CACNA1C* rs12425032 black minor allele carriers randomized to the CCB treatment strategy were at an almost 10-fold greater risk of experiencing the primary outcome (OR, 9.97; 95% CI, 1.33-74.3; p = 0.025; Appendix B). Consistent with and driven by these data, the rs12425032 SNP also demonstrated a significant pharmacogenetic association with the primary outcome among INVEST-GENES case-control cohort blacks (Figure 3-1; SNP*treatment interaction p = 0.005). Minor allele carriers randomized to the CCB treatment strategy were at a comparatively increased risk for experiencing the primary outcome relative to rs12425032 minor allele carriers randomized to the βB treatment strategy (Table 3-4). No associations were observed among white or Hispanic racial/ethnic groups.

Finally, the *CACNA1C* rs2299567 intronic SNP associated with greater BP response to HCTZ among minor allele white carriers in PEAR (Appendix A) was also

associated also associated with a decreased risk for the primary outcome among INVEST-GENES case-control cohort blacks independent of treatment strategy (OR, 0.17; 95% CI, 0.05-0.57; p = 0.004; Table 3-3). The rs2299657 SNP was also associated with increased odds of death among INVEST-GENES case-control cohort whites randomized to the CCB treatment strategy (OR, 2.53; 95% CI, 1.56-4.10; p = $1.6x10^{-4}$; Appendix B). No associations were observed between adverse outcomes and *CACNA1C* rs2299657 genotype among Hispanics.

CASQ2

Among INVEST-GENES case-control cohort whites, minor allele carriers of the conserved intronic *CASQ2* rs3811003 SNP demonstrated a significant interaction with INVEST treatment strategy for both the primary outcome and death (SNP*treatment interaction p = 0.003, Figure 3-2). Among blacks, the rs3811003 minor allele was nominally associated with an increased risk for the outcome of death, independent of treatment strategy (OR death, 3.71; 95% CI, 1.20-11.4; p = 0.02; Table 3-3).

Another intronic *CASQ2* SNP in high LD with rs3811003 among INVEST-GENES case-control cohort whites, rs7355132 (r^2 = 0.57) also demonstrated a significant and directionally consistent pharmacogenetic interaction with treatment strategy for the outcome of death (p = 0.001; Table 3-4). An additional, nominally significant pharmacogenetic interaction with treatment strategy was also observed among blacks, where randomization to the CCB treatment strategy was associated with an increased risk for the primary outcome, and a comparatively neutral effect was observed among those randomized to the βB treatment strategy (p for interaction = 0.048; Table 3-4). The *CASQ2* rs3811003 and rs7355132 SNPs among INVEST-GENES case-control cohort blacks share a lower degree of LD relative to whites (r^2 = 0.18). Notably, the

pharmacogenetic associations observed for both *CASQ2* rs3811003 and rs7355132 are directionally opposite between blacks and whites (Table 3-4). No associations between adverse outcomes and *CASQ2* genotype were observed among Hispanics within the INVEST-GENES case-control cohort.

KCNMB1

The Lys65 variant (rs11739136) of *KCNMB1* was observed to be associated with greater odds for the primary outcome among INVEST-GENES case-control cohort blacks independent of randomized treatment strategy (OR, 5.58; 95% CI 1.66-18.7; p = 0.005; Table 3-3). An additional, nominal association for greater odds of death among Hispanics for carriers of the Lys65 variant randomized to the βB treatment strategy was also observed (OR, 3.65; 95% CI, 1.38-9.67; p = 0.009; Appendix B). No consistent associations were observed among whites of the INVEST-GENES case-control cohort.

Another *KCNMB1* SNP, the conserved intronic rs2075612 was significantly associated with the outcome of death independent of treatment strategy among INVEST-GENES case-control cohort blacks (OR, 4.98; 95% CI 1.67-14.8; p = 0.003; Table 3-3). There was no LD (r^2 = 0.0) observed between *KCNMB1* rs11739136 and rs2075612 in this race group. Additionally, a nominally significant pharmacogenetic association was observed with the primary outcome among Hispanics, wherein a lower risk was observed for patients randomized to the CCB treatment strategy relative to the βB treatment strategy (p = 0.04; Appendix B). There were no associations between rs2075612 and adverse cardiovascular outcomes among whites.

Finally, Hispanic carriers of the *KCNMB1* Leu110 variant (rs2301149) randomized to the βB treatment strategy were at an increased risk of experiencing the primary outcome (OR, 3.45; 95% CI, 1.20-9.96; p = 0.02; Appendix B). In an analysis of

combined race groups in the full INVEST-GENES cohort, differences by genotype among patients randomized to the βB treatment strategy were not observed.[23] Although, when treatment strategy was not considered, Leu110 carriers had a decreased risk of experiencing the primary outcome. No additional associations were observed among the other racial/ethnic groups for the *KCNMB1* Val110Leu polymorphism in the current analyses.

RYR2

The intronic *RYR2* rs961121 polymorphism was significantly associated with a more than 3-fold increased risk for death among white minor allele carriers independent of treatment strategy (OR, 3.26; 95% CI, 1.89-5.61; $p = 2.1 \times 10^{-5}$; Table 3-3). Separated by treatment strategy, analyses reveal approximately equivalent risk among whites in each treatment strategy (p = 0.003; Appendix B). No additional associations were observed among the black or Hispanic racial/ethnic groups.

There were several significant pharmacogenetic interactions observed within the cardiac ryanodine receptor gene. A relatively protective effect of CCB treatment strategy against the outcome death was observed among white minor allele carriers of the rs2485584 SNP, whereas those randomized to the βB treatment strategy experienced a neutral to increased risk for this outcome (p = 0.004; Table 3-4). No consistent associations were observed among the other racial/ethnic groups.

Among the Hispanic ethnic group, the *RYR2* rs16834782 SNP was associated with an increased risk of death among carriers of the minor allele randomized to the CCB treatment strategy, whereas randomization to the βB treatment strategy was associated with a neutral to decreased risk (p = 0.003; Table 3-4). The opposite was true for Hispanic minor allele carriers of *RYR2* rs3766884, for whom the CCB treatment

strategy was associated with an 83% reduced risk of death, whereas minor allele carriers randomized to the βB treatment strategy were at a comparatively increased risk (p = 0.005; Table 3-4). No similar associations for either *RYR2* SNPs were observed among blacks or whites in the INVEST-GENES case-control cohort.

PLN

The intronic rs9489438 SNP within the gene encoding phospholamban demonstrated a significant pharmacogenetic interaction with treatment strategy for the primary outcome among INVEST-GENES case-control cohort whites (p = 0.007; Table 3-4). Additionally, a nominal association with decreased risk for death among Hispanics randomized to the CCB treatment strategy was also observed (p = 0.02; Appendix B). The rs9489438 SNP of *PLN* is in complete LD (r^2 = 1.0) with a nonsynonymous SNP of chromosome 6 open reading frame 204 (*C6orf204*) encoding an amino acid change of proline to threonine. Found in the same chromosomal region as *PLN*, genetic variation within *C6orf204* has been identified as associated with QRS interval duration in a genome-wide meta-analysis in individuals of European descent.[41]

Discussion

CACNA1C encodes the α_{1C} pore-forming subunit of $Ca_v1.2$ channels, the drug target of CCBs. The present study has identified several *CACNA1C* variant alleles that associate with an increased risk for adverse cardiovascular outcomes among INVEST-GENES case-control cohort blacks and whites randomized to the CCB treatment strategy.

The identification of the *CACNA1C* rs2239101 variant as a risk allele among INVEST-GENES case-control cohort whites is counter to previous associations indicating a phenotype of lower systolic BP. *CACNA1C* rs2239101 has previously been

associated with lower systolic BP in a large cohort candidate gene study, as well as nominally associated with a directionally consistent effect among PEAR blacks, whom demonstrated greater BP response to atenolol (Chapter 2).[29]

The rs12425032 SNP of *CACNA1C* demonstrated a significant interaction with treatment strategy for the primary outcome among INVEST-GENES case-control cohort blacks, where minor allele carriers randomized to the CCB treatment strategy demonstrated a significantly increased risk for the primary outcome. Although the rs12425032 minor allele was significantly associated with lower BP response to atenolol among PEAR blacks, within the INVEST-GENES case-control cohort, minor allele carriers randomized to the βB treatment strategy had a comparatively neutral risk for experiencing the primary outcome.

Calsequestrin 2, a high capacity Ca^{2+} storage protein found in the sarcoplasmic reticulum of cardiac muscle is encoded by *CASQ2*. Genetic variants within the coding sequence of *CASQ2* have previously been associated with catecholamine-induced polymorphic ventricular tachycardia, a potentially fatal arrhythmia.[42-44] The *CASQ2* rs3811003 minor allele associated with decreased odds of adverse cardiovascular outcomes among whites randomized to the CCB treatment strategy in the INVEST-GENES case-control cohort is in high LD with two *CASQ2* SNPs previously associated with an increased risk of sudden cardiac death among patients from a separate cohort with coronary artery disease (rs7536370 $r^2 = 0.96$; rs3010396 $r^2 = 0.47$).[45] The pharmacogenetic interaction between *CASQ2* rs3811003 and INVEST-GENES treatment strategy for the primary outcome as well as death indicates a comparatively increased risk for adverse outcomes among white minor allele carriers randomized to

the βB treatment strategy. Within the INVEST-GENES population, it appears the CCB treatment strategy modulates the risk associated with the rs3811003 minor allele in this race group.

Several significant associations were observed between SNPs present in *KCNMB1* and adverse cardiovascular outcomes in the INVEST-GENES case-control cohort. A prior INVEST-GENES case-control cohort investigation of the *KCNMB1* gene revealed no significant difference in the occurrence of the primary outcome by codon 65 genotype.[23] The current analysis performed by PCA defined race revealed an over 5-fold increased risk for the primary outcome among black rs11739136 (Glu65Lys) minor allele carriers. An additional nominally associated risk for increased odds of death was also observed among Hispanics randomized to the βB treatment strategy. The *KCNMB1* Lys65 allele has previously been associated with a protective effect against more severe phenotypes of diastolic hypertension, and functional data support the Lys65 variant as a gain-of-function to the K_{Ca} channel, resulting in a negative feedback loop with enhanced efficiency.[22] Additional evidence indicates a Lys65 allelic dose dependent lowering of both systolic and diastolic BP in men.[46] Despite these data indicating a beneficial effect of the Lys65 allele on hypertension phenotypes, outcomes data within the black and Hispanic racial/ethnic groups of the INVEST-GENES case-control cohort indicate the Lys65 may be a risk allele for adverse cardiovascular outcomes.

Among INVEST-GENES case-control cohort Hispanics, Leu110 variant allele carriers randomized to the βB treatment strategy were at an increased risk of experiencing the primary outcome. Previously genotyped in the entire INVEST-GENES

cohort (n = 5486), Leu110 variant carriers among combined racial/ethnic groups were found to have a reduced risk of the primary outcome, an association which was more pronounced among those subjects randomized to the CCB treatment strategy.[23] The current finding is perhaps consistent with the previous subgroup analysis, which indicated a neutral risk among those subjects randomized to the βB treatment strategy. No additional associations between Val110Leu genotype and adverse cardiovascular outcomes were observed among other racial groups of the INVEST-GENES case-control cohort.

Encoded by *PLN*, phospholamban is a protein inhibitor of the cardiac sarcoplasmic reticulum Ca^{2+}-ATPase responsible for reuptake of Ca^{2+} into the organelle. Protein kinase A phosphorylation of the phospholamban relieves the inhibitory effect of the protein, which thereby regulates both the rate of cardiomyocyte relaxation and Ca^{2+} storage within the sarcoplasmic reticulum.[47] The *PLN* rs9489438 SNP in complete LD with a non-synonymous SNP of *C6orf204* is also in moderate LD ($r^2 = 0.37$) with several additional SNPs associated with higher resting heart rate and greater LV internal diastolic dimensions in two separate genome-wide association analyses performed in individuals of European descent.[48,49] The current association between *PLN* rs9489438 and treatment strategy in the INVEST-GENES case-control cohort demonstrating lower risk of the βB treatment strategy among whites adds to an accumulating wealth of associations recently reported between the chromosome 6q22 locus and cardiovascular phenotypes.

Finally, we report herein several novel associations between intronic SNPs of the cardiac ryanodine receptor gene and adverse cardiovascular outcomes. Of interest, the

RYR2 rs961121 minor allele was identified as a risk allele for the outcome of death among whites, an effect which is consistent between INVEST-GENES treatment strategies.

Most of the associations reported in one racial/ethnic group of the INVEST-GENES case-control cohort failed to reach nominal significance among the other populations represented. There may be no consistent associations within the alternate populations, or there may be a lack of power to detect the genetic effect due to differing sample sizes. Additionally, the associations reported herein may represent tagSNPs for functional SNPs that would not be observed due to the difference in LD between the racial/ethnic populations studied. Furthermore, despite the compelling nature of several associations reflecting the significance of previously reported yet alternate cardiovascular phenotypes, the associations reported here have yet to be replicated in a separate hypertension outcomes cohort. Overall, we have reported herein several novel associations between genetic variation within genes essential in the response to the antihypertensive treatment strategies utilized in this large, randomized hypertensive treatment outcomes trial and adverse cardiovascular outcomes.

Table 3-1. Power to detect differences across MAF, INVEST-GENES

		INVEST-GENES n=1345			Whites n=795	
MAF	OR	Main effect α		Interaction α	Main effect α	
		0.005	0.00025	0.05	0.005	0.00025
0.05	1.5	16%	3%	5%	11%	2%
	2.0	68%	35%	7%	51%	20%
	2.5	95%	80%	8%	86%	58%
	3.0	>99%	97%	10%	97%	85%
	3.5	>99%	>99%	13%	>99%	97%
0.25	1.5	53%	21%	15%	37%	12%
	2.0	98%	89%	36%	92%	71%
	2.5	>99%	>99%	57%	>99%	97%
	3.0	>99%	>99%	74%	>99%	>99%
	3.5	>99%	>99%	85%	>99%	>99%

Table 3-2. INVEST-GENES baseline characteristics

Characteristic	INVEST-GENES cohort (n=5,598)	Case control cohort (n = 1,345)	CCB (n = 679)	BB (n = 666)	*p*-value[†]
Demographics and social history					
Age, mean (SD), years	66.1 ± 9.6	70.8 ± 9.5	70.3 ± 9.5	71.3 ± 9.5	0.07
Women	3090 (55.2)	675 (50.2)	345 (51.1)	330 (48.9)	0.64
Race/ethnicity					
White	2315 (41.4)	795 (59.1)	398 (58.6)	397 (59.6)	
Black	657 (11.7)	170 (12.6)	82 (12.1)	88 (13.2)	
Hispanic	2589 (46.3)	380 (28.3)	199 (29.3)	181 (27.2)	0.62
BMI, mean (SD), kg/m2	29.4 ± 5.5	28.6 ± 5.4	28.6 ± 5.3	28.7 ± 5.4	0.77
BP, mean (SD), mmHg					
Systolic	148.0 ± 18.4	148.9 ± 18.3	148.0 ± 19.0	149.8 ± 17.5	0.07
Diastolic	85.4 ± 10.7	83.3 ± 10.5	83.1 ± 10.5	83.5 ± 10.4	0.43
Heart rate (beats per min)	74.8 ± 9.6	74.9 ± 9.3	75.1 ± 9.3	74.7 ± 9.3	0.48
Smoking history	2319 (41.1)	572 (42.5)	295 (43.4)	277 (41.6)	0.49
Past Medical History					
Myocardial infarction	1317 (23.5)	376 (27.9)	180 (26.5)	196 (29.4)	0.23
Heart Failure (NYHA class I-III)	193 (3.5)	78 (5.8)	42 (6.2)	36 (5.4)	0.54
Stroke or TIA	395 (7.1)	117 (8.7)	59 (8.7)	58 (8.7)	0.99
Arrhythmia	187 (7.0)	127 (9.4)	71 (10.5)	56 (8.4)	0.19
Left Ventricular Hypertrophy	842 (15.0)	213 (15.8)	105 (15.5)	108 (16.2)	0.70
Peripheral Vascular Disease	620 (11.1)	159 (11.8)	79 (11.6)	80 (12.0)	0.83
Diabetes	1594 (28.5)	301 (22.4)	148 (21.8)	153 (22.9)	0.60
Hypercholoesterolemia	3068 (54.8)	819 (60.9)	426 (62.7)	393 (59.0)	0.16
Renal Insufficiency	88 (1.6)	38 (2.8)	23 (3.4)	15 (2.3)	0.21
Cancer	230 (4.1)	81 (6.0)	39 (5.7)	42 (6.3)	0.66
Medication					
Aspirin/other antiplatelet agent	2590 (46.3)	781 (58.1)	402 (59.2)	378 (56.9)	0.39
Antidiabetic medication	1392 (24.9)	260 (19.3)	126 (18.6)	134 (20.1)	0.47
Lipid Lowering Drugs	2034 (36.3)	565 (42.0)	300 (44.2)	265 (39.8)	0.10
Nitrates	1568 (28.0)	366 (27.2)	183 (26.9)	183 (27.5)	0.83

Data are presented as no. (%) or mean ± SD. NYHA indicates New York Heart Association; TIA, transient ischemic attack. [†]By *t* test for continuous variables and χ^2 test for categorical variables.

Table 3-3. Associations between SNPs of the Ca^{2+} signaling pathway and adverse cardiovascular outcomes in INVEST-GENES[a]

Gene	SNP	Race	MAF	Outcome	OR (95% CI)	*p-value*
CACNA1C	*rs2239101	White	0.12	Death	2.00 (1.24-3.25)	*0.004*
CACNA1C	*rs2299657ǂ	Black	0.29	PO	0.17 (0.05-0.57)	*0.004*
CASQ2	rs3811003ǂ	Black	0.56	Death	3.71 (1.20-11.4)	*0.02*
KCNMB1	rs11739136	Black	0.08	PO	5.58 (1.66-18.7)	*0.005*
KCNMB1	rs2075612	Black	0.46	Death	4.98 (1.67-14.8)	*0.003*
RYR2	rs961121	White	0.05	Death	3.26 (1.89-5.61)	$2.1x10^{-5}$

[a]Adjusted for age, sex, history of MI, heart failure, diabetes, ancestry; ǂDominant model; *Previous association with BP response to atenolol or HCTZ in PEAR; PO = primary outcome

.

Table 3-4. Significant SNP-treatment interactions[a]

Gene	SNP	Race	MAF	Outcome	OR (95% CI) CCB	OR (95% CI) βB	*p-value*
CACNA1C	*rs12425032	Black	0.22	PO	6.03 (0.83-43.9)	0.26 (0.04-1.56)	*0.005*
CASQ2	**rs3811003**	**White**	**0.31**	**PO**	**0.44 (0.26-0.76)**	**1.47 (0.82-2.64)**	***0.003***
CASQ2	rs3811003	White	0.31	Death	0.28 (0.14-0.60)	1.23 (0.55-2.78)	*0.01*
CASQ2	**rs7355132**	**White**	**0.25**	**Death**	**0.35 (0.16-0.75)**	**2.20 (0.96-5.04)**	***0.001***
CASQ2	rs7355132	White	0.25	PO	0.51 (0.29-0.89)	1.29 (0.73-2.26)	*0.02*
CASQ2	rs7355132	Black	0.19	PO	32.0 (2.19-468)	1.84 (0.42-8.05)	*0.048*
PLN	rs9489438	White	0.25	PO	1.63 (0.96-2.76)	0.49 (0.26-0.91)	*0.007*
RYR2	rs2485584	White	0.47	Death	0.40 (0.20-0.79)	2.19 (0.79-6.04)	*0.004*
RYR2	rs16834782	Hisp	0.16	Death	4.74 (1.85-12.1)	0.22 (0.04-1.13)	*0.003*
RYR2	rs3766884	Hisp	0.21	Death	0.17 (0.05-0.55)	1.80 (0.59-5.49)	*0.005*

[a]Shows the dominant model adjusted for age, sex, history of MI, heart failure, diabetes, ancestry; strongest single associations shown in bold
*Previous association with BP response to atenolol or HCTZ in PEAR; PO = primary outcome

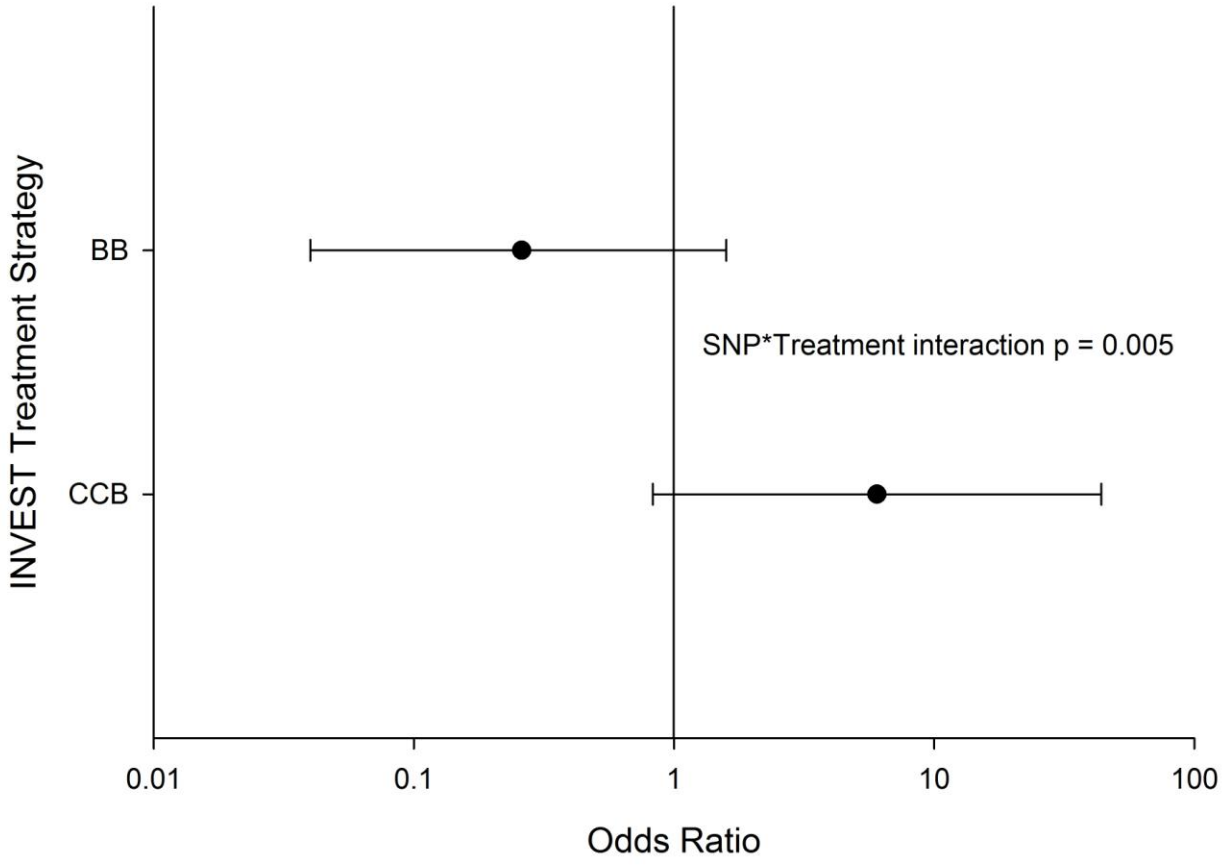

Figure 3-1. Primary outcome adjusted odds ratios (log scale shown) and 95% confidence intervals by treatment strategy among INVEST-GENES case-control cohort black *CACNA1C* rs12425032 minor allele carriers.

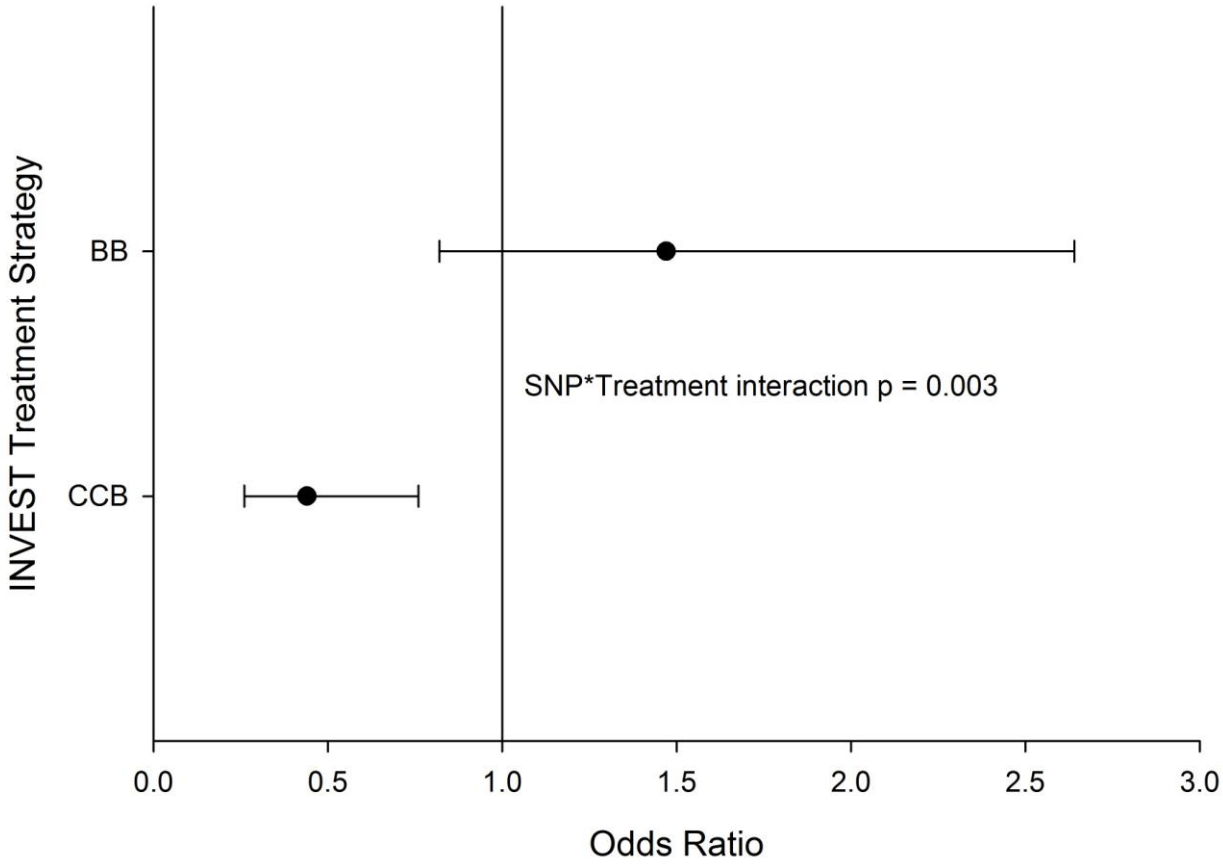

Figure 3-2. Primary outcome adjusted odds ratios and 95% confidence intervals by treatment strategy among INVEST-GENES case-control cohort white *CASQ2* rs3811003 minor allele carriers.

CHAPTER 4
INFLUENCE OF THE RS2357928 PROMOTER POLYMORPHISM ON THE EXPRESSION PROFILE OF THE $CA_V1.2$ BETA 2 SUBUNIT IN HUMAN VASCULAR TISSUE

Introduction

$Ca_v1.2$ channels are the fundamental machinery in maintenance of vascular smooth muscle tone and myocardial contraction. Data from genome wide association studies of BP and hypertension have implicated variation in the β2 regulatory subunit (*CACNB2*) of the $Ca_v1.2$ channel as related to these cardiovascular phenotypes (Chapter 1).[19,29] Additionally, we have shown *CACNB2* to contain SNPs that also associate with adverse cardiovascular outcomes in a treatment specific manner within the INVEST-GENES.[20] The A allele of the *CACNB2* rs2357928 SNP has been associated with a significantly greater risk for experiencing a primary outcome event in patients randomized to the βB treatment strategy (OR 1.74, 95% CI: 1.25-2.43, p=0.001). This finding was not observed among patients randomized to the CCB treatment strategy (OR 0.90, 95% CI: 0.67-1.20, p=0.47). Furthermore, we have previously documented the rs2357928 promoter SNP of *CACNB2* to be associated with decreased transcriptional activity in a reporter gene assay.[20]

The goal of the current study is to further investigate the underlying functional mechanism of the clinically associated *CACNB2* rs2357928 promoter SNP, utilizing techniques in molecular biology to ascertain how this variant influences the expression profile of the $Ca_v1.2$ β_2 subunit in human vascular tissue. To achieve this end, a genetic database of subjects matched to vascular tissue samples and clinical data was developed.

Methods

Study Protocol

The study utilized remnant specimens of vascular tissue that would normally be discarded after surgery from patients undergoing lower limb amputation or other vascular procedure at UF/Shands Hospital. Men and women aged greater than 21 years requiring surgery generating vascular tissue waste were enrolled at Shands at the University of Florida (UF) in Gainesville, FL. The study was approved by the UF Institutional Review Board (IRB#579-2010). Study participants provided written informed consent following recommendation or consultation for surgery. Baseline data including age, gender, weight and self-reported race/ethnicity was obtained. Additional clinical information pertaining to the subject's disease state and medication history were also collected. Exclusion criteria included documented HIV, hepatitis B or C infection, or active infection within the tissue to be collected (i.e. gangrenous).

During the surgical procedure, excised tissue was placed aside in a sterile environment prior to collection or immediately placed into sterile containers containing RNA*later*® (Ambion®) reagent for RNA preservation. A 5 to10-mL blood sample was also collected by the anesthesiologist via a central line in surgery, or by the surgeon via aspiration from removed limb. If a blood sample was unavailable for collection, a mouthwash sample was collected for the purpose of extracting genetic material.

Collected samples were transported to the UF Center for Pharmacogenomics Core laboratory, where tissue samples were weighed to ensure submersion in 5 to 10 times greater volume of RNA*later*® reagent. Vascular tissue samples were then incubated at 2-8^{o}C at least overnight, but no more than 3 days, then removed from RNA*later*® reagent and placed in a freezer at -80^{o}C.

DNA

Genomic DNA was isolated from blood lymphocytes using a commercially available kit (Qiagen DNA Blood Isolation Kit) according to the manufacturer specified protocol.

Genotype determination. *CACNB2* rs2357928 genotype was determined by TaqMan® SNP genotyping assay. Allelic discrimination was performed with a made to order probe for rs2357928 (C__2740542_10). Genotype accuracy was verified by duplicate genotyping for study participants, and also included additional samples from a separate study population with known *CACNB2* rs2357928 genotype.

RNA

50 mg of arterial tissue was homogenized in 1 mLTRIzol® (Ambion®) reagent on ice with the Fisher Scientific TissueMiser homogenizer. 200 µL of chloroform was added following room temperature incubation. Subsequent centrifugation allowed for the separation of an aqueous phase containing RNA, an interphase containing DNA and a lower phenol-chloroform phase containing protein. The RNA containing aqueous phase was processed using TRIzol® Plus RNA Purification Kit (Ambion®) per manufacturer specified protocol. The interphase and phenol-chloroform phase were further processed with 300 µL of 100% ethanol prior to separation and storage at -80^{o}C for downstream processing. Isolated RNA was quantified in duplicate on the NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific Inc, Rockford, IL).

RT-PCR. 500 ng of RNA for n = 10 samples were converted to cDNA using the Applied Biosystems High Capacity RNA-to-cDNA Kit. 1 µL 20x TaqMan® *CACNB2* Gene Expression Assay (Hs00167861_m1) was combined with 2 µL cDNA, 10 µL 2X TaqMan® Gene Expression Master Mix and 7 µL RNase-free water to generate a 20 µL

reaction. N = 10 samples were analyzed in triplicate along with reactions for endogenous controls to *18S* (Hs03003631_g1), *B2M* (Hs00984320_m1) and *GAPDH* (Hs99999905_m1) on an ABI 7300 Real-Time PCR System (Applied Biosystems).

Protein

Proteins were precipitated with isopropanol from the phenol-ethanol supernatant previously aliquoted from tissue homogenization in TRIzol® reagent. The resultant protein pellet was washed with 0.3M guanidine hydrochloride 95% ethanol and resuspended in 1% SDS buffer.

Nuclear extract

40 mg of arterial tissue was washed with PBS prior to processing with the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific Inc) per manufacturer specified protocol. Halt™ Protease Inhibitor Cocktail and 0.5M EDTA were added to the 400 µL CER I reagent used in sample homogenization. The cytoplasmic extract was removed following subsequent incubation and centrifugation steps with 22 µL CER II reagent. The remaining insoluble pellet fraction was resuspended in NER reagent, and stored at -80^{o}C until use.

Quantitation

Protein concentrations were determined using the Pierce® BCA Protein Assay kit. 1% SDS diluent was used to construct the standard curve using serial dilutions of bovine serum antigen per manufacturer instructions. Absorbance was read at 562nm on the Biotek® Synergy™ HT (Biotek Instruments, Inc, Winooski, VT). All concentrations were measured in duplicate.

Antibodies

Anti- CACNB2 mouse monoclonal antibody (ab54920, abcam®) was used at a dilution of 1:1000. Anti-GAPDH rabbit polyclonal antibody (sc-257778, Santa Cruz Biotechnology, Inc.) was used as a loading control at a dilution of 1:1000, and was a gift from Dr. Jorg Bungert (Department of Biochemistry and Molecular Biology, University of Florida).

Western blot

Approximately 20 µg protein extract were combined in a 2:1 volume of laemmli sample buffer (62.5 mM, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue, 5% β-mercaptoethanol) and heated to 95^{o}C in a water bath for 5 minutes. Samples were loaded onto a 4-20% Mini-PROTEAN® TGX™ precast gel (Bio-Rad Laboratories, Inc) with 1X Tris/Glycine/SDS buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) and Bio-Rad Precision Plus Protein™ standards were included as the ladder. Electrophoresis commenced at 120 V for 60 minutes. Proteins were then transferred onto a 0.45 µm nitrocellulose membrane in 1X Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.6) under ice at 15 mA overnight.

Following transfer, the membranes were processed according to the instructions provided in the Pierce® Fast Western Blot Kit. Membranes were washed in 1X Wash Buffer and incubated in a 1:1000 dilution of primary antibody for 30 minutes at room temperature with shaking. The membrane was then incubated with the Optimized HRP Reagent Working Dilution for 15 minutes at room temperature with shaking, and washed 3 x 5 minutes in 1X Wash Buffer. The SuperSignal West Pico Working Solution was added, and the membrane incubated for an additional 5 minutes. Bands were then imaged on Kodak® X-Omat LS film with a Fisher Biotech FB-XC-810 Autoradiography

cassette. Band intensity was calculated with Quantity One® data Analysis Software (Bio-Rad Laboratories, Inc).

Electrophoretic mobility shift assay (EMSA)

DNA corresponding to the sequence 24 base pairs (bp) upstream and 25 bp downstream of each *CACNB2* rs2357928 allele (A/G) and reverse complement were produced by Eurofins MWG Operon. Two sequences of single stranded oligonucleotides, 5'-TAACAGTGAA TCCAGGATGC TGCT(G/A)TCAGC CTGTGCATTG TGAAGAAGGC-3' and the reverse complement 3'-ATTGTCACTT AGGTCCTACG ACGA(C/T)AGTCG GACACGTAAC ACTTCTTCCG-5' and a nonconsensus sequence 5'-GCATCATCGC TATCGGATCT ATTGTATCGA CACATGAGTC TAGACTAGAT-3' and 3'-CGTAGTAGCG ATAGCCTAGA TAACATAGCT GTGTACTCAG ATCTGATCTA-5' were annealed at a 1:1 molar concentration in pH 7.6 20 mM Tris-Acetate, 5 mM $MgAc_2$ buffer at 95^{o}C for 5 min, then 70 cycles of 1 min 95^{o}C (-1^{o}C/cycle) and subsequently held at 4^{o}C on a Applied Biosystems Veriti™ Thermal Cycler. Annealing efficiency was confirmed on a 1% agarose gel stained with ethidium bromide and visualized with UV light on the Molecular Imager® Gel Doc™ XR system (Bio-Rad Laboratories, Inc).

Nuclear protein extract was treated with the TURBO DNA-free™ Kit (Ambion®) according to manufacturer instructions prior to the EMSA binding reaction. EMSA was performed with the Molecular Probes™ Electrophoretic Mobility Shift Assay (EMSA) Kit (Molecular Probes, Inc, Eugene, OR) according to manufacturer specified protocol. 30 ng of annealed oligonucleotide sequences corresponding to the *CACNB2* A allele, G allele and nonconsensus sequence was incubated with a range of 9.5 – 19.5 µg crude nuclear protein extract in 1X binding buffer containing 750 mM KCl, 0.5 mM

dithiothreitol, 0.5 mM EDTA, 50 mM Tris, pH 7.4 in a final reaction volume of 20 µl. Additional control reactions for DNA and protein were also prepared with deionized water and 1X binding buffer. Reactions were incubated at 24^{o}C for 35 minutes prior to loading on a 4-20% Mini-PROTEAN® TBE precast gel (Bio-Rad Laboratories, Inc, Hercules, CA) with 1X TBE buffer (89 mM Tris base, 89 mM boric acid, 1 mM EDTA, pH~8.0). Electrophoresis ran for 3 hours at 4^{o}C, after which the gel was incubated for 20 minutes in 50 mL 1x SYBR® Green EMSA staining solution. Stained nucleic acids were visualized with UV transillumination on the Gel Doc™ XR system (Bio-Rad Laboratories, Inc). The gel was subsequently stained with 50 mL SYPRO® Ruby EMSA protein gel stain for a minimum of 3 hours. The gel was destained in a 10% methanol, 7% acetic acid solution for 60-90 minutes prior to visualization of the stained proteins with UV transillumination on the Gel Doc™ XR system (Bio-Rad Laboratories, Inc).

Analysis

The $2^{-\Delta\Delta Ct}$ method was used for the relative quantification (RQ) of *CACNB2* mRNA expression between *CACNB2* rs2357928 genotype groups, where the ancestral homozygous G/G genotype was treated as the control. RQ analysis was performed with DataAssist™ Software v3.0 (Applied Biosystems). A two-tailed Student's t-test comparing ΔC_T values (Average C_T – endogenous control) between *CACNB2* rs2357928 genotype groups was performed with SAS® version 9.2 (Cary, NC) and a $p < 0.05$ was considered significant. For western blot analysis, Student's t-test of mean band intensity was compared between genotype groups, and a $p < 0.05$ was considered significant. Due to a limited sample size, a nonparametric Wilcoxon rank sum (WRS) test was also utilized to evaluate differential RNA and protein expression for both RT-PCR and western blot analyses.

Results

Demographic and clinical information for study participants is outlined in Table 4-1. Patients were predominately male, young (age < 65 years), with hypertension and peripheral vascular disease. The most common study medications were aspirin, βBs, and statins.

RT-PCR was successful for n = 9 samples, and n = 1 A/G heterozygote sample was eliminated prior to analysis due to a variably high concentration of cDNA transcript. *B2M* demonstrated the lowest average pairwise variation relative to *GAPDH* and *18S* (calculated by DataAssist^{TM} Software v3.0), and was selected as the endogenous control with which to calculate ΔC_T.[50] Relative quantitation of *CACNB2* expression is displayed in Figure 4-1. Mean ΔC_T was significantly different between *CACNB2* rs2357928 A/G and G/G genotype groups (*t*-test p = 0.02, WRS p = 0.21; Table 4-2). A similar trend for significance was also observed between A/A and G/G genotype groups (*t*-test p = 0.07, WRS p = 0.54).

For the western blot, GAPDH loading control expression was significantly different between rs2357928 G/G and A/G genotype groups (*t*-test p = 0.03, WRS p = 0.08; Table 4-3, Figure 4-2). Interestingly, CACNB2 protein expression in arterial tissue was not significantly different between *CACNB2* rs2357928 genotype groups (trend test for G allele carriers *t*-test, WRS p = 0.22; Table 4-3).

A post-hoc power calculation was performed G*Power version 3.1.3, utilizing the means and standard deviations derived from both RT-PCR and western blot experiments. These data indicate at least one additional rs2357928 G/G individual is required to detect significant differences in mRNA expression between A/A and G/G genotype groups, and n = 3 additional individuals of both A/A and G/G genotypes would

provide 86% power to detect a 30% difference in mean band intensity between the homozygote genotype groups.

EMSA analysis did not reveal differences in nucleic acid and protein binding between the alternate *CACNB2* rs2357928 alleles (Figure 4-3). Bands visualized with SYBR® Green nucleic acid stain do indicate an interaction between nucleic acid and nuclear protein in both *CACNB2* oligonucleotide sequences that are not observed for reactions containing the nonconsensus, mutant sequence. Unfortunately, the current results are confounded by a high background of nonspecific interactions, due to the use of crude nuclear cell extract, and the absence of poly-dIdC to prevent nonspecific protein binding.

Discussion

We have previously presented evidence of the association between the rs2357928 polymorphism within an alternative promoter of *CACNB2* and increased risk for adverse cardiovascular outcomes among patients randomized to a βB treatment strategy in our INVEST-GENES cohort.[20] Presently, we evaluated whether quantifiable differences in *CACNB2* gene expression might be ascertained in human arterial tissue relative to rs2357928 genotype.

Despite the limited sample size for inclusion in the molecular analysis of the clinically associated *CACNB2* rs2357928 promoter SNP, significant differences in mRNA expression were observed between genotype groups. RT-PCR analysis revealed greater *CACNB2* mRNA expression within an arterial tissue sample of a *CACNB2* rs2357928 G/G homozygote compared to samples derived from A/G heterozygous individuals. These data are consistent with our previously published

association demonstrating greater promoter activity of the rs2357928 G allele in a human cell line.[20]

Western blot analysis revealed inconclusive results, however, as CACNB2 protein expression among G/G individuals was not found to be significantly different than the expression observed among A allele carriers. Although it is difficult to draw conclusions based on our limited data set, there are several factors to consider that might aid the understanding of these preliminary results.

Protein expression is subject to a wide range of variability in mRNA post-transcriptional modification and degradation. Likewise, variation within the cellular machinery responsible for the nuclear export, translation or degradation of mRNA as well as cis-acting sequences are also a potential source of differential protein expression.[51] Additionally, use of arterial tissue from patients with advanced vascular disease, concomitant disease states and complex pharmacotherapy may further confound these results.

Finally, an EMSA analysis was used to evaluate whether differences in transcription factor binding could be observed between the alternate *CACNB2* rs2357928 alleles. Although several putative transcription factor binding sites have been identified in the proximal region surrounding the rs2357928 SNP, presently, no qualitative change in protein-DNA interaction could be observed between alleles.

Overall, the evaluation of *CACNB2* mRNA expression in human arterial tissue relative to rs2357928 genotype appears promising given the consistency observed with our previously published data from a reporter gene assay. However, due to a limited

sample size, additional experiments incorporating a larger sample cohort will be required to delineate the validity the results herein.

Table 4-1. Baseline demographics of vascular tissue bank participants

Characteristic	Vascular Tissue Bank (n = 12)	*CACNB2* genotype G/G (n = 2)	A/G (n = 8)	A/A (n = 2)
Demographics and Social History				
Age, mean (SD), years	60.4 ± 13.7	55.5 ± 6.4	62.1 ± 15.9	58.5 ± 13.4
Women	4 (33.3)	1 (50.0)	2 (25.0)	1 (50.0)
Race				
White	9 (75.0)	2 (100.0)	6 (75.0)	1 (50.0)
Black	3 (25.0)	0 (0.0)	2 (25.0)	1 (50.0)
Weight (kg)	77.1 ± 30.5	60.7 ± 11.8	73.6 ± 13.5	52.0 ± 0.0
BP, mean (SD), mmHg				
Systolic	134.8 ± 22.7	114.0 ± 7.1	143.1 ± 23.5	122.5 ± 4.9
Diastolic	68.3 ± 10.0	58.5 ± 16.2	69.0 ± 7.5	75.5 ± 12.0
HR (beats per minute)	88.8 ± 13.8	84.0 ± 16.9	86.3 ± 13.4	102.5 ± 9.2
Smoking history	10 (83.3)	1 (50.0)	7 (66.7)	2 (100.0)
Medical history				
Hypertension	11 (91.0)	2 (100.0)	7 (87.5)	2 (100.0)
Myocardial infarction	2 (16.7)	0 (0.0)	1 (12.5)	1 (50.0)
Heart failure	4 (33.3)	0 (0.0)	3 (37.5)	1 (50.0)
Diabetes	7 (58.3)	2 (100.0)	5 (62.5)	0 (0.0)
Hypercholesterolemia	7 (58.3)	2 (100.0)	4 (50.0)	1 (50.0)
Peripheral vascular disease	11 (91.7)	2 (100.0)	7 (87.5)	2 (100.0)
Autoimmune/genetic disorder	2 (16.7)	1 (50.0)	1 (8.3)	0 (0.0)
Medication use				
Aspirin	11 (91.7)	2 (100.0)	7 (66.7)	2 (100.0)
βB	10 (83.3)	2 (100.0)	6 (75.0)	2 (100.0)
CCB	4 (33.3)	0 (100.0)	3 (37.5)	1 (50.0)
ACE inhibitor	4 (33.3)	1 (50.0)	3 (37.5)	0 (0.0)
Diuretic	4 (33.3)	0 (0.0)	3 (37.5)	1 (50.0)
Statin	10 (83.3)	2 (100.0)	6 (75.0)	2 (100.0)
Warfarin	5 (41.7)	2 (100.0)	2 (25.0)	1 (50.0)

Data are presented as no. (%) or mean ± SD; HR = heart rate.

Table 4-2. Analysis of RT-PCR mean ΔC_T by *CACNB2* rs2357928 genotype

CACNB2 rs2357928	Sample size	Mean ΔC_T	SD	*t*-test p-value	WRS p-value
G/G	1	5.23	-	-	
A/G	6	7.63	0.69	0.02	0.21
A/A	2	8.18	0.27	0.07	0.54

p-values represent *t*-test or nonparametric Wilcoxon rank sum (WRS) of mean ΔC_T relative to G/G; trend test for G allele carriers *t*-test p = 0.31, WRS p = 0.30.

Table 4-3. Net intensity of CACNB2 and GAPDH expression by *CACNB2* rs2357928 genotype

CACNB2 Genotype	Net Intensity ± SD CACNB2	Net Intensity ± SD GAPDH	*t*-test p-value CACNB2	WRS p-value CACNB2	*t*-test p-value GAPDH	WRS p-value GAPDH
G/G	473 ± 91	599 ± 118	-	-	-	-
A/G	552 ± 161	364 ± 88	0.55	0.56	0.03	0.08
A/A	675 ± 86	510 ± 52	0.15	0.24	0.43	0.69

t-test or nonparametric Wilcoxon rank sum (WRS) of mean net intensity relative to G/G; trend test for G allele carriers CACNB2 *t*-test, WRS p = 0.22; GAPDH *t*-test p = 0.48, WRS p = 0.33.

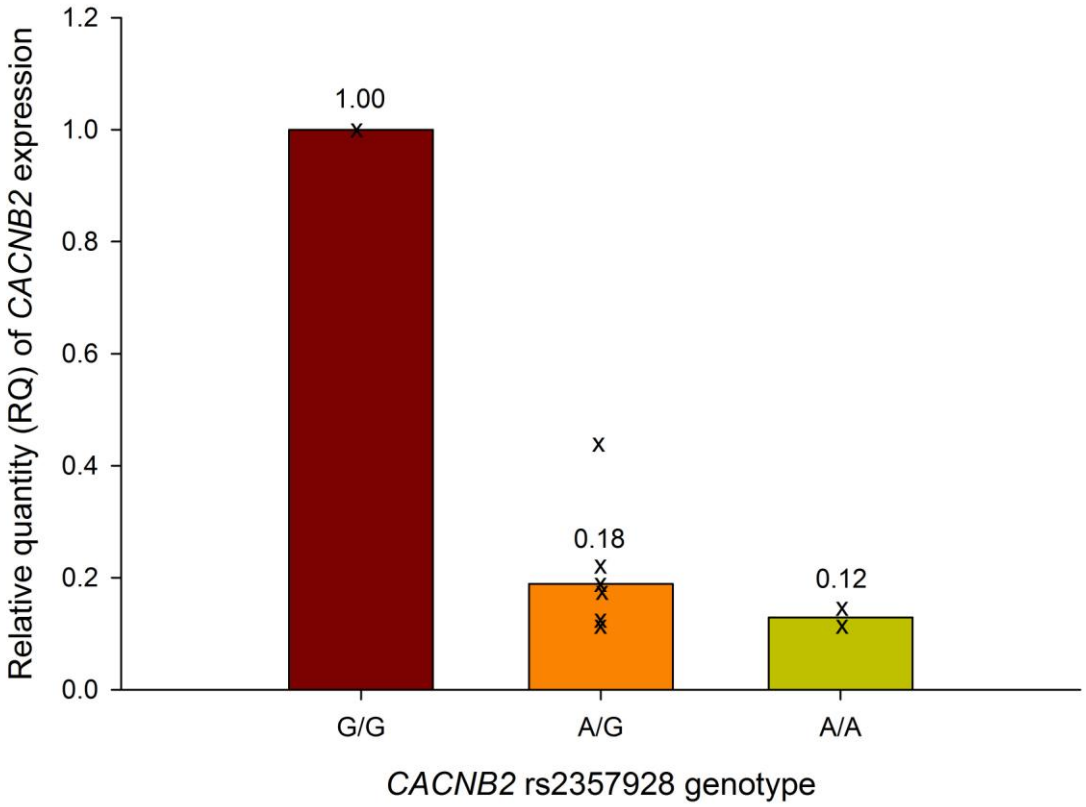

Figure 4-1. Relative *CACNB2* mRNA expression in human arterial tissue, G/G reference genotype, individual RQ values plotted.

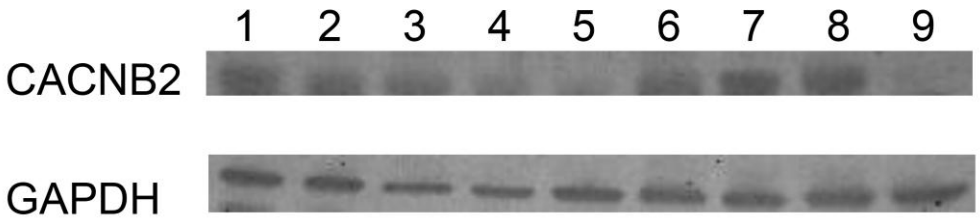

Figure 4-2. Western blot analysis of CACNB2 protein expression relative to GAPDH. Lanes 1-2 rs2357928 A/A genotype; lanes 3-7 rs2357928 A/G genotype, lanes 8-9 rs2357928 G/G genotype

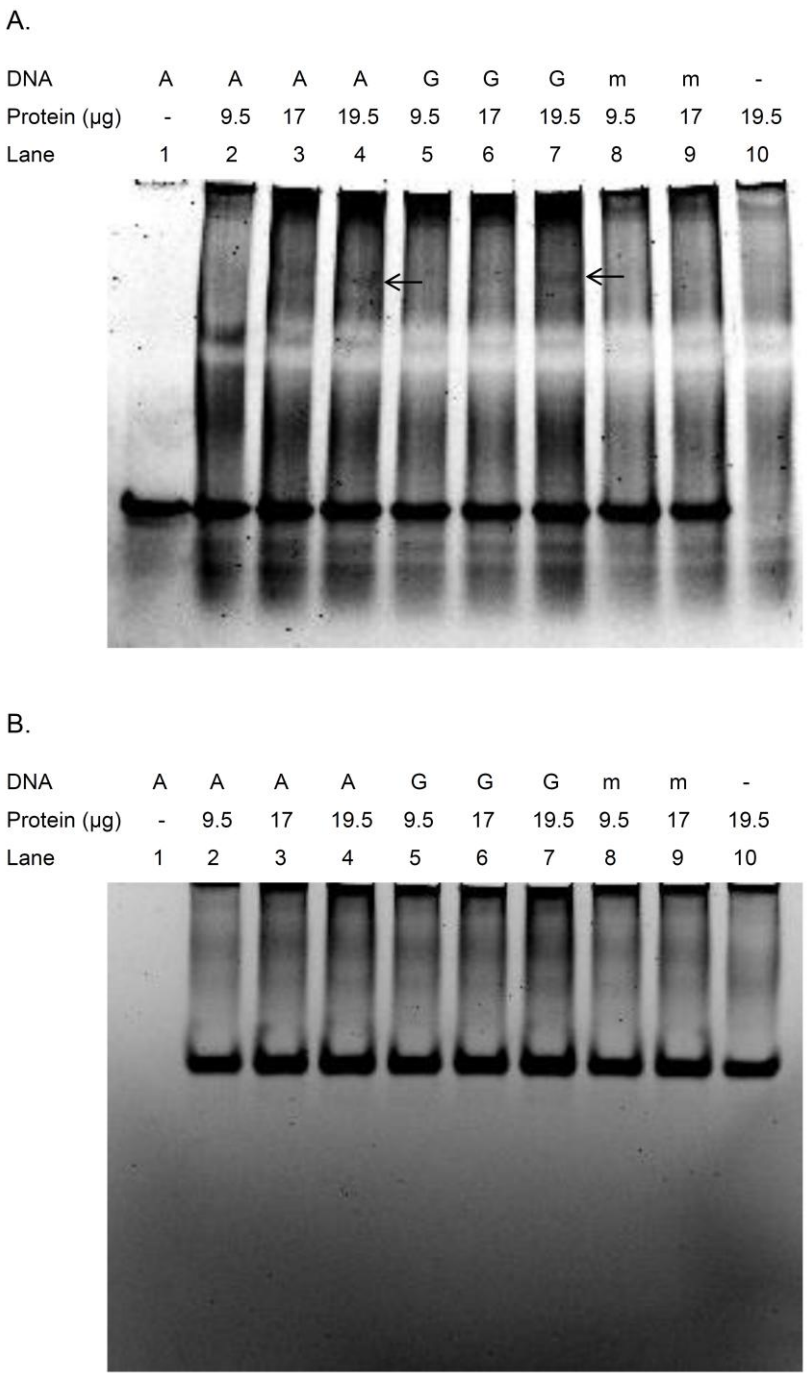

Figure 4-3. EMSA characterization of DNA and protein interactions. 30 ng of DNA sequences corresponding to *CACNB2* rs2357928 A allele (A), G allele (G), and mutant (m) were added to nuclear cell extract as described in Methods. (A.) SYBR® Green nucleic stain of DNA interacting with protein. Bands highlighted in lanes 4 and 6 show interactions that are not seen with the mutant oligonucleotide or protein alone. (B.) SYPRO® Ruby protein stain.

CHAPTER 5
SUMMARY AND CONCLUSIONS

A number of medications commonly utilized in the management of hypertension are known to intercede in the pathway of Ca^{2+} signaling to exert their antihypertensive effects. Perhaps not surprisingly, an increasing amount of evidence demonstrating a relationship between genetic variation within the Ca^{2+} signaling pathway and varied cardiovascular phenotypes has been highlighted in recent literature.[14,19,20,22,23,29,42,45] The overarching goal of the current research project was to advance our understanding of the role of variability within genes of the Ca^{2+} signaling pathway in the treatment responses observed to commonly used antihypertensives.

We first evaluated associations between variation in genes comprising the activation pathway of Ca^{2+} signaling and BP response to antihypertensive therapy in a population of uncomplicated hypertensives. Several strong associations between *CACNA1C* polymorphisms and BP response to atenolol were observed exclusively among PEAR blacks. Notably, the *CACNA1C* rs2239101 SNP that has previously been associated with systolic BP in a large candidate gene cohort study was also associated with greater BP response to atenolol in a manner directionally consistent with the original finding.[29] Among the novel associations reported here, we also identified a common haplotype including *CACNA1C* rs11831085 and rs12425032 that was significantly associated with BP response to atenolol among blacks, despite the limited LD observed in this gene.

We then evaluated the relationship between genetic variation of the Ca^{2+} signaling pathway and clinical susceptibility to adverse cardiovascular outcomes in a high-risk cohort of hypertensives with coronary artery disease. To this end, several significant

associations with adverse cardiovascular outcomes among INVEST-GENES case-control cohort participants were observed. Interestingly, the *CACNA1C* rs2239101 SNP was identified as a risk allele among INVEST-GENES case-control cohort whites, a finding which is counter to previous associations indicating a phenotype of lower systolic BP. Additionally, the *CACNA1C* rs12425032 polymorphism associated with lower BP response to atenolol among PEAR blacks demonstrated a significant pharmacogenetic interaction with treatment strategy among INVEST-GENES case-control cohort blacks, where randomization to the CCB treatment strategy conferred an exaggerated risk for the primary outcome.

Also of considerable interest, the rs3811003 polymorphism within the calsequestrin 2 gene demonstrated a significant interaction with treatment strategy in the INVEST-GENES case-control cohort, as a considerably decreased risk for adverse cardiovascular outcomes was observed among whites randomized to the CCB treatment strategy. Mutations within the coding sequence of *CASQ2* found to affect the Ca^{2+} binding affinity of the protein have previously demonstrated associations with fatal ventricular arrhythmias.[44] The intronic *CASQ2* rs3811003 was found to be in high LD with SNPs previously associated with sudden cardiac death in a separate cohort of patients with coronary artery disease.

Several novel pharmacogenetic interactions with treatment strategy within the INVEST-GENES case-control cohort were also observed among genes encoding phospholamban and the cardiac ryanodine receptor 2.

Finally, we recruited human subjects to participate in a vascular tissue bank study for the purpose of examining whether quantifiable differences in gene expression might

contribute to a clinically significant genetic association found within a regulatory sequence of *CACNB2*. *CACNB2* mRNA expression levels in arterial tissue were found to be significantly different by rs2357928 genotype. Concordant with data from a previously published reporter gene assay, G/G homozygotes demonstrated greater fold change expression than rs2357928 A allele carriers. Although examination of protein expression did not yield similar results, we accept the present sample size is limited and likely confounded by the complex disease pathology of study participants. Larger sample sizes will be required to fully understand the influence of the *CACNB2* promoter SNP, but these data lend additional support for this being a functional polymorphism.

Overall, we have herein documented several novel genetic associations between genes essential to the activation pathway of Ca^{2+} signaling and the cardiovascular phenotypes of BP response and adverse outcomes. Additionally, significant inroads were established for the future examination of the molecular mechanisms that produce the observed cardiovascular phenotypes of clinically meaningful pharmacogenetic associations.

APPENDIX A
NOMINAL ASSOCIATIONS BETWEEN CALCIUM SIGNALING PATHWAY VARIATION AND BLOOD PRESSURE RESPONSE WITHIN THE PHARMACOGENOMICS OF ANTIHYPERTENSIVE RESPONSE (PEAR) STUDY

Table A-1. Associations between *CACNA1C* rs2299657 genotype and blood pressure response to HCTZ among PEAR whites

Response	MAF	ΔDBP Major Hmz. (mmHg)	ΔDBP Het. (mmHg)	ΔDBP Minor Hmz. (mmHg)	*P*[†]	ΔSBP Major Hmz. (mmHg)	ΔSBP Het. (mmHg)	ΔSBP Minor Hmz. (mmHg)	*P*[†]
HCTZ Monotherapy	0.26	-3.3	-5.7	-4.3	*0.005*	-6.1	-9.9	-7.7	*0.002*
HCTZ Add-On	0.26	-2.5	-2.9	-2.6	*0.45*	-5.9	-5.4	-5.2	*0.98*
HCTZ Monotherapy + Add-On	0.26	-3.0	-4.2	-3.3	*0.016*	-6.1	-7.5	-6.3	*0.036*

[†]Adjusted for age, gender, and baseline BP. Response to monotherapy + add-on was adjusted for BP at baseline in the monotherapy group and BP at the first response assessment for the add-on therapy group as well as controlling for the order of drug initiation. DBP = diastolic BP; SBP = systolic BP; Hmz. = homozygote; Het. = heterozygote.

Table A-2. Associations between *CACNA1C* rs2299657 genotype and blood pressure response to atenolol among PEAR whites

Response	MAF	ΔDBP Major Hmz. (mmHg)	ΔDBP Het. (mmHg)	ΔDBP Minor Hmz. (mmHg)	*P*[†]	ΔSBP Major Hmz. (mmHg)	ΔSBP Het. (mmHg)	ΔSBP Minor Hmz. (mmHg)	*P*[†]
Atenolol Monotherapy	0.26	-9.0	-10.6	-11.1	*0.04*	-9.6	-12.0	-13.0	*0.039*
Atenolol Add-On	0.26	-9.8	-9.1	-11.1	*0.29*	-11.2	-9.4	-11.2	*0.73*
Atenolol Monotherapy + Add-On	0.26	-9.3	-10.0	-11.1	*0.03*	-10.4	-10.9	-12.4	*0.15*

[†]Adjusted for age, gender, and baseline BP. Response to monotherapy + add-on was adjusted for BP at baseline in the monotherapy group and BP at the first response assessment for the add-on therapy group as well as controlling for the order of drug initiation. DBP = diastolic BP; SBP = systolic BP; Hmz. = homozygote; Het. = heterozygote.

Table A-3. Associations between *CACNB2* rs7089228 genotype and blood pressure response to HCTZ among PEAR blacks

Response	MAF	ΔDBP Major Hmz. (mmHg)	ΔDBP Het. (mmHg)	ΔDBP Minor Hmz. (mmHg)	$P^{\dagger}$	ΔSBP Major Hmz. (mmHg)	ΔSBP Het. (mmHg)	ΔSBP Minor Hmz. (mmHg)	$P^{\dagger}$
HCTZ Monotherapy	0.32	-8.3	-6.6	-4.2	*0.009*	-14.0	-11.0	-7.1	*0.01*
HCTZ Add-On	0.32	-7.9	-6.6	-6.1	*0.04*	-13.3	-11.5	-14.9	0.37
HCTZ Monotherapy + Add-On	0.32	-8.1	-6.6	-5.2	$8.1x10^{-4}$	-13.7	-11.3	-11.3	*0.015*

†Adjusted for age, gender, and baseline BP. Response to monotherapy + add-on was adjusted for BP at baseline in the monotherapy group and BP at the first response assessment for the add-on therapy group as well as controlling for the order of drug initiation. DBP = diastolic BP; SBP = systolic BP; Hmz. = homozygote; Het. = heterozygote.

Table A-4. Associations between *RYR2* rs16832052 genotype and blood pressure response to atenolol among PEAR blacks

Response	MAF	ΔDBP Major Hmz. (mmHg)	ΔDBP Het. (mmHg)	ΔDBP Minor Hmz. (mmHg)	$P^{\dagger}$	ΔSBP Major Hmz. (mmHg)	ΔSBP Het. (mmHg)	ΔSBP Minor Hmz. (mmHg)	$P^{\dagger}$
Atenolol Monotherapy	0.28	-3.0	-5.9	-4.2	*0.055*	-1.8	-4.8	-3.6	*0.18*
Atenolol Add-On	0.28	-5.2	-5.6	-10.1	*0.004*	-5.7	-5.9	-11.1	*0.018*
Atenolol Monotherapy + Add-On	0.28	-4.1	-5.7	-7.2	*0.002*	-3.7	-5.3	-7.5	*0.023*

†Adjusted for age, gender, and baseline BP. Response to monotherapy + add-on was adjusted for BP at baseline in the monotherapy group and BP at the first response assessment for the add-on therapy group as well as controlling for the order of drug initiation. DBP = diastolic BP; SBP = systolic BP; Hmz. = homozygote; Het. = heterozygote.

Table A-5. Associations between *CACNA1C* rs2238078, rs11831085, rs12425032, rs2239101 TGCT haplotype and blood pressure response to atenolol among PEAR blacks

Response	Frequency	ΔDBP, 0 copy (mmHg)	ΔDBP, 1 copy (mmHg)	ΔDBP, 2 copies (mmHg)	$P^{\dagger}$	ΔSBP,0 copy (mmHg)	ΔSBP, 1 copy (mmHg)	ΔSBP, 2 copies (mmHg)	$P^{\dagger}$
Atenolol Monotherapy	0.15	-4.9	-2.8	-	*0.059*	-4.9	0.7	-	*0.0016*
Atenolol Add-On	0.15	-5.7	-3.9	-	*0.017*	-6.4	-1.7	-	$1.78x10^{-5}$
Atenolol Monotherapy + Add-On	0.15	-6.6	-5.1	-	*0.048*	-7.9	-4.2	-	*0.001*

†Adjusted for age, gender, and baseline BP. Response to monotherapy + add-on was adjusted for BP at baseline in the monotherapy group and BP at the first response assessment for the add-on therapy group as well as controlling for the order of drug initiation.

Table A-6. Associations between *CACNA1C* rs2238078, rs11831085, rs12425032, rs2239101 ATGT haplotype and blood pressure response to atenolol among PEAR blacks

Response	Frequency	ΔDBP, 0 copy (mmHg)	ΔDBP, 1 copy (mmHg)	ΔDBP, 2 copies (mmHg)	$P^{\dagger}$	ΔSBP,0 copy (mmHg)	ΔSBP, 1 copy (mmHg)	ΔSBP, 2 copies (mmHg)	$P^{\dagger}$
Atenolol Monotherapy	0.10	-3.9	-5.8	-	*0.043*	-2.4	-6.7	-	*0.0098*
Atenolol Add-On	0.10	-4.7	-7.0	-	*0.0045*	-4.3	-7.8	-	*0.002*
Atenolol Monotherapy + Add-On	0.10	-5.6	-8.2	-	*0.045*	-6.3	-8.7	-	*0.07*

†Adjusted for age, gender, and baseline BP. Response to monotherapy + add-on was adjusted for BP at baseline in the monotherapy group and BP at the first response assessment for the add-on therapy group as well as controlling for the order of drug initiation.

APPENDIX B
NOMINAL ASSOCIATIONS BETWEEN CALCIUM SIGNALING PATHWAY VARIATION AND ADVERSE CARDIOVASCULAR OUTCOMES IN THE INTERNATIONAL VERAPAMIL SR TRANDOLAPRIL STUDY – GENETIC SUBSTUDY (INVEST-GENES)

Table B-1. Associations between variation within *CACNA1C* and adverse cardiovascular outcomes in INVEST-GENES[a]

Gene	SNP	Race	MAF	Outcome	Treatment strategy	OR (95% CI)	*p-value*
CACNA1C	*rs2299657	White	0.26	Death	CCB	2.53 (1.56-4.10)	$1.6x10^{-4}$
CACNA1C	**rs1034937ǂ**	**Hisp**	**0.09**	**Death**	**βB**	**8.03 (2.23-28.9)**	***0.0014***
CACNA1C	rs1034937ǂ	Hisp	0.09	PO	βB	3.80 (1.26-11.4)	*0.017*
CACNA1C	rs1034937	Hisp	0.09	Death	βB	4.24 (1.45-12.1)	*0.0068*
CACNA1C	**rs4765680**	**Hisp**	**0.18**	**Death**	**βB**	**5.09 (1.87-13.90)**	***0.0015***
CACNA1C	rs4765680	Hisp	0.18	PO	βB	2.93 (1.29-6.65)	*0.01*
CACNA1C	rs4765680	Hisp	0.18	Death	Combined	1.91 (1.09-3.35)	*0.02*
CACNA1C	rs1009281	Hisp	0.37	PO	βB	3.27 (1.51-7.09)	*0.0027*
CACNA1C	**rs2239077**	**Hisp**	**0.39**	**Death**	**βB**	**2.97 (1.22-7.23)**	***0.016***
CACNA1C	rs2239077	White	0.47	Death	CCB	1.71 (1.07-2.72)	*0.024*
CACNA1C	rs2239077	White	0.47	Death	βB	0.55 (0.30-0.98)	*0.04*
CACNA1C	rs1548649	Black	0.27	PO	CCB	0.008 (<0.001-0.416)	*0.016*
CACNA1C	rs3794292ǂ	Hisp	0.08	Death	βB	5.19 (1.35-19.9)	*0.016*
CACNA1C	rs11608641	White	0.08	Death	βB	2.67 (1.18-6.02)	*0.018*
CACNA1C	*rs12425032	Black	0.22	PO	CCB	9.97 (1.33-74.3)	*0.025*

[a]Adjusted for age, sex, history of MI, heart failure, diabetes, ancestry; strongest single associations shown in bold. ǂDominant model;
*Previous association with BP response to atenolol or HCTZ in PEAR; PO = primary outcome

Table B-2. INVEST-GENES *CACNA1C* SNP-treatment interactions[a]

Gene	SNP	Race	MAF	Outcome	OR (95% CI) CCB	OR (95% CI) βB	*p-value*
CACNA1C	**rs1034937**	**Hisp**	**0.09**	**Death**	**0.44 (0.11-1.75)**	**8.03 (2.23-28.9)**	***0.0074***
CACNA1C	rs1034937	Hisp	0.09	PO	0.29 (0.06-1.24)	3.80 (1.27-11.4)	*0.0097*
CACNA1C	rs1009281	Hisp	0.37	PO	0.72 (0.32-1.62)	7.31 (1.78-29.9)	*0.009*
CACNA1C	**rs2370596**	**Hisp**	**0.35**	**PO**	**1.34 (0.59-3.04)**	**0.24 (0.08-0.69)**	***0.0095***
CACNA1C	rs2370596	White	0.27	PO	0.82 (0.49-1.39)	1.78 (1.00-3.16)	*0.04*
CACNA1C	rs4765680	Hisp	0.18	PO	0.54 (0.22-1.35)	2.89 (1.10-7.57)	*0.0096*
CACNA1C	rs1548649	Black	0.27	PO	0.006 (<0.001-0.30)	2.89 (0.66-12.6)	*0.012*
CACNA1C	rs3794292	Hisp	0.08	Death	0.59 (0.18-1.94)	5.19 (1.35-19.9)	*0.014*
CACNA1C	rs1016388	Black	0.26	Death	0.007 (<0.001-1.05)	7.08 (1.11-45.3)	*0.02*
CACNA1C	rs7305879	Black	0.13	PO	8.83 (1.18-66.3)	0.73 (0.14-3.77)	*0.027*
CACNA1C	rs994901	White	0.50	Death	0.50 (0.26-0.99)	2.19 (0.72-6.64)	*0.03*

[a]Shows the dominant model adjusted for age, sex, history of MI, heart failure, diabetes, ancestry; PO = primary outcome; strongest single associations shown in bold

Table B-3. Associations between variation within *CASQ2* and adverse cardiovascular outcomes in INVEST-GENES[a]

Gene	SNP	Race	MAF	Outcome	Treatment strategy	OR (95% CI)	*p-value*
CASQ2	**rs3811003ǂ**	**White**	**0.31**	**Death**	**CCB**	**0.28 (0.14-0.60)**	***9.8x10^{-4}***
CASQ2	rs3811003ǂ	White	0.31	PO	CCB	0.44 (0.26-0.76)	*0.003*
CASQ2	**rs7355132ǂ**	**Black**	**0.19**	**PO**	**Combined**	**4.15 (1.5-11.43)**	***0.0059***
CASQ2	rs7355132ǂ	White	0.25	Death	CCB	0.35 (0.16-0.75)	*0.006*

[a]Adjusted for age, sex, history of MI, heart failure, diabetes, ancestry; ǂDominant model; PO = primary outcome; strongest single associations shown in bold

Table B-4. INVEST-GENES *CASQ2* SNP-treatment interactions[a]

Gene	SNP	Race	MAF	Outcome	OR (95% CI) CCB	OR (95% CI) βB	*p-value*
CASQ2	**rs12036369[b]**	**White**	**0.23**	**Death**	**0.40 (0.19-0.87)**	**1.61 (0.73-3.55)**	***0.01***
CASQ2	rs12036369	White	0.23	PO	0.47 (0.26-0.85)	1.17 (0.67-2.06)	*0.02*
CASQ2	rs2997742[c]	White	0.44	Death	2.54 (1.08-5.96)	0.65 (0.29-1.46)	*0.017*
CASQ2	rs3010396[c]	White	0.45	PO	2.19 (1.04-4.65)	0.68 (0.29-1.58)	*0.037*

[a]Shows the dominant model adjusted for age, sex, history of MI, heart failure, diabetes, ancestry; strongest single association shown in bold. [b]LD, r^2 between rs7355132 (Chapter 3) and rs12036369 among whites = 0.81. [c]LD, r^2 between rs2997742 and rs3010396 among whites = 0.91. PO = primary outcome

Table B-5. Associations between variation within *KCNMB1* and adverse cardiovascular outcomes in INVEST-GENES[a]

Gene	SNP	Race	MAF	Outcome	Treatment Strategy	OR (95% CI)	*p-value*
KCNMB1	rs11739136	Hisp	0.14	Death	βB	3.65 (1.38-9.67)	*0.009*
KCNMB1	rs2301149	Hisp	0.11	PO	βB	3.45 (1.20-9.96)	*0.02*
KCNMB1	rs827778ǂ	Hisp	0.13	Death	CCB	0.26 (0.07-0.95)	*0.04*
KCNMB1	rs827778ǂ	White	0.12	Death	CCB	0.34 (0.12-0.98)	*0.04*
KCNMB1	rs2075612	Hisp	0.38	PO	CCB	0.49 (0.25-0.99)	*0.049*

[a]Adjusted for age, sex, history of MI, heart failure, diabetes, ancestry. ǂDominant model.

Table B-6. INVEST-GENES *KCNMB1* SNP-treatment interactions[a]

Gene	SNP	Race	MAF	Outcome	OR (95% CI) CCB	OR (95% CI) βB	*p-value*
KCNMB1	rs827778	Hisp	0.13	PO	0.43 (0.14-1.28)	2.62 (0.91-7.58)	*0.02*
KCNMB1	rs11742820	White	0.20	PO	1.20 (0.70-2.06)	0.46 (0.24-0.89)	*0.03*
KCNMB1	rs2075612	Hisp	0.39	PO	0.43 (0.18-1.009)	1.94 (0.67-5.60)	*0.04*

[a]Shows the dominant model adjusted for age, sex, history of MI, heart failure, diabetes, ancestry; PO = primary outcome.

Table B-7. Associations between variation within *RYR2* and adverse cardiovascular outcomes in INVEST-GENES[a]

Gene	SNP	Race	MAF	Outcome	Treatment strategy	OR (95% CI)	*p-value*
RYR2	**rs12126370**	**White**	**0.43**	**Death**	**βB**	**2.74 (1.49-5.04)**	***0.001***
RYR2	rs12126370	White	0.43	Death	Combined	1.76 (1.21-2.53)	*0.002*
RYR2	rs2184014	Hisp	0.49	Death	CCB	0.33 (0.16-0.66)	*0.0016*
RYR2	rs3766884[b]	Hisp	0.21	Death	CCB	0.18 (0.06-0.57)	*0.003*
RYR2	rs10158497ǂ	White	0.25	Death	βB	3.62 (1.51-8.66)	*0.003*
RYR2	rs961121	White	0.05	Death	CCB	3.14 (1.45-6.77)	*0.003*
RYR2	rs961121	White	0.05	Death	βB	3.45 (1.52-7.86)	*0.003*
RYR2	rs790882	Hisp	0.22	Death	CCB	0.22 (0.08-0.62)	*0.004*
RYR2	rs1008956ǂ	White	0.46	Death	CCB	0.38 (0.19-0.75)	*0.005*
RYR2	rs2253244ǂ[b]	Hisp	0.30	Death	CCB	0.30 (0.13-0.71)	*0.006*
RYR2	rs2485584ǂ	White	0.47	Death	CCB	0.40 (0.20-0.79)	*0.007*

[a]Adjusted for age, sex, history of MI, heart failure, diabetes, ancestry; strongest single associations shown in bold. ǂDominant model; [b]LD – r^2 between rs3766884 and rs2253244 = 0.58.

Table B-8. INVEST-GENES *RYR2* SNP-treatment interactions[a]

Gene	SNP	Race	MAF	Outcome	OR (95% CI) CCB	OR (95% CI) βB	*p-value*
RYR2	rs1008956[b]	White	0.46	Death	0.38 (0.19-0.75)	1.81 (0.70-4.70)	*0.007*
RYR2	rs790882	Hisp	0.22	Death	0.21 (0.07-0.61)	1.67 (0.53-5.25)	*0.01*
RYR2	rs2184014[c]	Hisp	0.49	Death	0.30 (0.13-0.72)	4.94 (0.59-41.3)	*0.01*
RYR2	**rs12129023**	**Hisp**	**0.14**	**Death**	**0.54 (0.19-1.54)**	**4.13 (1.28-13.3)**	***0.01***
RYR2	rs12129023	Hisp	0.14	PO	0.37 (0.13-1.09)	1.99 (0.71-5.55)	*0.029*
RYR2	rs2253244	Hisp	0.30	Death	0.27 (0.11-0.69)	1.67 (0.54-5.13)	*0.01*
RYR2	rs2490373[b]	White	0.30	Death	0.51 (0.26-1.00)	1.71 (0.75-3.92)	*0.01*
RYR2	**rs12038715**	**Hisp**	**0.35**	**PO**	**0.46 (0.19-1.07)**	**2.53 (0.87-7.29)**	***0.018***
RYR2	rs12038715	Hisp	0.35	Death	0.73 (0.32-1.71)	5.16 (1.07-24.8)	*0.035*
RYR2	**rs3766875[c]**	**Hisp**	**0.56**	**PO**	**0.47 (0.19-1.20)**	**8.51 (0.95-75.8)**	***0.02***
RYR2	rs3766875[c]	Hisp	0.56	Death	0.33 (0.14-0.88)	3.52 (0.41-29.8)	*0.04*
RYR2	**rs1759119**	**White**	**0.47**	**Death**	**3.38 (1.22-9.38)**	**0.67 (0.28-1.57)**	***0.024***
RYR2	rs1759119	Hisp	0.65	Death	2.46 (0.90-6.73)	0.45 (0.13-1.51)	*0.045*
RYR2	rs12128519	White	0.30	Death	0.67 (0.35-1.29)	2.42 (1.01-5.76)	*0.02*
RYR2	rs10158497	White	0.25	PO	0.87 (0.51-1.47)	1.79 (1.01-3.18)	*0.04*

[a]Shows the dominant model adjusted for age, sex, history of MI, heart failure, diabetes, ancestry; strongest single associations shown in bold; [b]LD – r^2 between rs1008956 and rs2490373 = 0.33; [c]LD – r^2 between rs2184014 and rs3766875 = 0.65; PO = primary outcome.

Table B-9. Associations between variation within *PLN* and adverse cardiovascular outcomes in INVEST-GENES[a]

Gene	SNP	Race	MAF	Outcome	Treatment strategy	OR (95% CI)	*p-value*
PLN	rs9489438	Hisp	0.40	Death	CCB	0.46 (0.23-0.92)	*0.027*

[a]Adjusted for age, sex, history of MI, heart failure, diabetes, ancestry.

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