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Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Coronary artery calcification (CAC) is an important indicator of future coronary artery disease events. Since elevated blood pressure (BP) is an important predictor of CAC, genetic polymorphisms in the renin-angiotensin system and their interaction may play a role in explaining CAC quantity variation.

Material/Methods: As part of the Epidemiology of Coronary Artery Calcification Study, 166 asymptomatic women and 166 asymptomatic men were genotyped for the insertion/deletion polymorphism in the *angiotensin-converting enzyme* (*ACE*) gene and the –6 promoter polymorphism of the *angiotensinogen* (*AGT*) gene. We used a novel method to detect gene-gene interaction and compared it to the standard two-way analysis of variance (ANOVA) method.

Results: Based on a two-way ANOVA model, there was no evidence for epistasis for either systolic BP or CAC in either men or women. However, using a novel method, we found evidence of significant gene-gene interaction in systolic BP in men and gene-gene interaction in both systolic BP levels and CAC quantity in women.

Conclusions: Our study demonstrates that new methods of assessing epistasis maybe important in understanding the complex genetics of systolic blood pressure as well as subclinical coronary atherosclerosis.

key words: epistasis • blood pressure • gene-gene interactions • coronary artery calcification • statistical

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BACKGROUND

Although there have been many studies of candidate genes for hypertension and coronary artery disease (CAD), the vast majority of studies have focused on risk factors or clinical end points and have only investigated single-gene effects. As we move into a more genomic era of studying the genetic architecture of complex diseases like CAD, its subclinical phenotypes, and its risk factors, it is likely that the interactions between genes will play a greater role than the contribution of any single gene [1]. One limitation to progress in this area arises from the limitations of our methods of detecting gene-gene interactions and their impact on disease risk. For example, traditional statistical models of interaction (e.g., a two-way analysis of variance with interaction) use the phenotypic variance explained by the interaction (mean square due to interaction) as the measure of the contribution of interaction to the trait of interest. This traditional approach for detecting interaction has been challenged by Cheverud and colleagues [2,3] because it does not test the main underlying null hypothesis of epistasis – that is, the distance between the genotypic means estimated for one locus does not change across the genotypic background marked by a second locus [4]. This view of epistasis has been called a "physiological epistasis" model because it focuses on the average phenotypic value of individuals carrying a particular multilocus genotype and does not depend on the population frequency of the genotype class. In traditional statistical genetics, definitions of interaction rely on the estimation of the mean square value due to interaction after frequency dependent estimation of the mean square value due to the main effects of each genetic locus [5,6].

The term epistasis has been used for more than 100 years. In the classical sense, epistasis refers to the masking of the phenotypic effects of one gene by the effects of a second gene and a gene is epistatic when its presence suppresses the effect of the other gene. This original definition of epistasis is biological, rather than statistical (Moore, 2005) [7]. Today, we recognize that gene-gene interactions can act directly to change transcription or translation levels or indirectly, by way of their protein products, to alter phenotypes in ways that are separate from their independent effects (Thorton-Wells et al., 2004) [8]. What we are referring to here is not classical epistasis; but rather, the idea that multiple polymorphisms can affect phenotypes in complex ways not necessarily captured by standard analytical approaches to interactions (Thorton-Wells et al., 2004) [8].

In the study presented here we illustrate the differences between the traditional statistical genetics approach and "physiological epistasis" approach by testing for epistatic effects of the *angiotensin-converting enzyme* (*ACE*) insertion (I)/deletion (D) polymorphism and in the *anginotensiogen* (*AGT*) promoter polymorphism at position –6 on interindividual variation in systolic blood pressure (SBP) and coronary artery calcification (CAC) quantity levels.

CAC is an active component of atherosclerosis, the primary cause of CAD [9,10]. CAC can be accurately and non-invasively measured using electron beam computed tomography (EBCT) [11,12]. A direct relationship exists between CAC and both histological and *in vivo* intravascular ultrasound measures of atherosclerotic plaque [13–17]. EBCT measure-

ment of CAC is a highly sensitive and specific method for detecting obstructive CAD [18]. Measures of the presence and quantity of CAC predict future CAD end points in both symptomatic and asymptomatic individuals [19–22].

Little is known about the genetic basis of CAC. Before adjusting for any risk factors, 43.5% of the variation in CAC quantity was attributable to genetic factors [23]. After adjusting for risk factors, including SBP, 41.8% of the residual variation in CAC quantity was attributable to genetic factors [23]. There have been few candidate gene studies in CAC measured by EBCT [24,25] and only one genetic linkage study has been published [26]. Lipoprotein(a), which has a strong genetic component, is associated with both the presence and quantity of CAC [27].

Important predictors of CAC include measures of high blood pressure [28–30] and pulse pressure which are under both environmental and genetic control. The renin-angiotensin system (RAS) plays a cardinal role in salt and water homeostatsis and vascular tone regulation, and is important in the pathobiology of hypertension and vascular disease [31,32]. Given the high prevalence of prehypertension and hypertension in the United States [33], many studies have focused on genes for blood pressure regulation and the organ damage resulting from uncontrolled high blood pressure. Two well-studied genes that are part of RAS are *AGT* on chromosome 1 and *ACE* on chromosome 17. A common variant in the *AGT* gene is an A→G substitution 6 base pairs upstream from the site of transcription initiation in the proximal promoter region. Inoue et al. [34] found that the *AGT*-6 polymorphism is in very tight linkage disequilibrium with the threonine to methionine polymorphism at residue 235 (M235T), an *AGT* polymorphism that numerous studies have focused on as a candidate gene for hypertension and CAD [31,35–40]. The most widely studied polymorphism in the *ACE* gene is an insertion(I)/deletion(D) that has been reported to be associated with increased levels of ACE activity [41] and increased risk of myocardial infarction [41–45].

MATERIAL AND METHODS

Sample

The sample for this analysis originated from the Epidemiology of Coronary Artery Calcification (ECAC) study [46]. Participants in this study were recruited from the Rochester Family Heart Study, a community-based cross-sectional study of the genetic epidemiology of CAD and essential hypertension in Rochester, Minnesota [47,48]. Individuals were recruited into the ECAC Study independent of risk factor status and were at least age 20 at the time of recruitment, not pregnant or lactating, and never had coronary or non-coronary heart surgery. Each of the 1,240 ECAC participants underwent a physical examination, donation of a blood sample, review of medical records and an EBCT examination during a visit to the Mayo Clinic. The study protocols were approved by the Mayo Clinic and University of Michigan Institutional Review Boards. All individuals provided their written informed consent.

During an interview, participants reported current medication use and history of physician-diagnosed hyperten-

sion. Standard enzymatic methods were used to measure total cholesterol [48]. Body mass index (BMI) was calculated (weight/height²; (kg)/(m²)). SBP and diastolic blood pressure (DBP) levels were measured in the right arm with a random-zero sphygmomanometer (Hawksley and Sons). Three measures at least two minutes apart were taken and the average of the second and third measurements was used. A total of 222 females and 239 males had genotype data for both the *ACE* and *AGT* polymorphisms. Thirty women and 40 men less than 30 years old were excluded from this analysis because of the low prevalence of detectable CAC in younger individuals [49]. Another 3 men had a history of myocardial infarction or stroke and were excluded from analyses. Since some participants were members of the same family and all analyses were sex-specific, one woman and one man from each family were randomly selected for the present analyses. The final sample included 166 unrelated women and 166 unrelated men who were asymptomatic for CAD.

Genotype measurement

Genotyping of the candidate gene polymorphisms was carried out by subjecting 20 ng aliquots of genomic DNA for each individual in the study to PCR amplification and subsequent restriction-endonuclease digestion. DNA was extracted from blood drawn at the time of the physical exam. The oligonucleotide primers used for the *AGT*-6 polymorphism were [5'-GTGTCGCTTCTGGCATCTGTCCTTCTG G-3'], [5'-TACCCAGAACAACGGCAGCTTCTTCCACT-3'], and [5'-CCGGTTACCTTCTGCTGTACAGCCCAGAACAA CGGCAGGCTTCTTCCATC-3'] (Inoue et al., 1997). The primers used for the *ACE* D/I polymorphism in intron 16 were [5'-CTGGAGACCACTCCCATCCTTTCT-3'] and [5'-GATGTGGCCATCACATTCGTCAGAT-3'] [50]. The insertion corresponds to an *alu* repetitive sequence and is 287 bp long [50]. The fragments were then visualized by the use of ethidium-bromide staining after electrophoresis on 10% vertical acrylamide gels. Samples initially scored as heterozygotes were subjected to successive enzyme digestions and rescored to minimize the possibility of mistypings due to incomplete digestions. Genotypes were scored separately by two trained laboratory workers and any discrepant typings were subjected to examination by a third party. All laboratory personnel were blind to the CAC and risk factor status of individuals being genotyped. All genotyping was performed under the supervision of Dr. Eric Boerwinkle at the University of Texas, Houston.

Coronary artery calcification measurement

Quantity of CAC was measured using an Imatron C-100 or C-150 EBCT scanner (Imatron Inc., South San Francisco, California). A scan run consisted of 40 contiguous 3-mm thick tomograms initiating at the root of the aorta and proceeding caudally to the apex of the heart. Each tomogram took 100 msec of scan time. All images were triggered at end-diastole using electrocardiographic gating and were acquired during two to four breath holds.

Tomograms were initially scored by a radiologic technologist using an automated scoring system [51]. CAC was defined as a hyperattenuating focus of ≥4 contiguous pixels in size, within 5 mm of the arterial midline, and with a computed tomography (CT) number above 130 Hounsfield units (HU) throughout the focus. An experienced radiologist then reviewed the technical quality and scoring accuracy of each tomogram before making interpretations regarding the findings. Quantity of CAC was defined as the area of CAC in mm squared totaled over all foci in four epicardial arteries (left main, left anterior descending, left circumflex, and right coronary arteries).

Statistical analysis

We identified a group of unrelated men and women. In this group of men and women, we tested the null hypothesis of Hardy-Weinberg equilibrium for the *AGT* and *ACE* polymorphisms using chi-square tests. All subsequent analyses were sex-specific. All statistical analyses were performed using SAS statistical software (Version 8.02, SAS Institute, Cary, NC).

The logarithm of the quantity of CAC plus the value one (log [CAC area +1]) was taken in order to reduce skewness in the distribution of CAC quantity. Since blood pressure is such a significant predictor of CAC [28] we investigated the epistatic effects of *ACE* and *AGT* polymorphism on log (CAC area +1) adjusting for SBP, as well as age, age², hypertension status and use of blood pressure lowering medications and on SBP adjusted for age, age², hypertension status and use of blood pressure lowering medications.

One-way analysis of variance (ANOVA) was used to test for effects of each polymorphism alone on the covariate adjusted SBP levels and the covariate adjusted CAC levels. Two-way ANOVA was used to test for interaction effects between the *AGT* and *ACE* polymorphisms on the covariate adjusted SBP levels and the covariate adjusted CAC levels by coding *AGT* and *ACE* genotypes as class variables, with 2-degrees of freedom for each main effect test and 4-degees of freedom for the test of interaction effects.

As an alternative to the traditional statistical genetics methods of detection of interaction, the method developed by Cheverud and Routman [2] was used to estimate and test for the sum of the individual epistatic effects between *AGT* and *ACE* on covariate adjusted SBP levels and covariate adjusted CAC levels. This method allows the partition of each of the nine two-locus genotypic values (i.e., the two-way cell means) into nonepistatic and epistatic values that are not weighted by allele frequencies. Using the Cheverud and Routman notation, let G_{ijkl} be the average phenotypic value for individuals with genotypes *ij* at the *AGT* locus and *kl* at the *ACE* locus.

Then the marginal genotypic values, or single-locus values (SLV), for *AGT* ($G_{ij..}$) are calculated as:

$$G_{ij..} = (G_{ij11} + G_{ij12} + G_{ij22})/3,$$

while the marginal genotypic values, or single-locus values (SLV), for *ACE* ($G_{..kl}$) are calculated as:

$$G_{..kl} = (G_{11kl} + G_{12kl} + G_{22kl})/3.$$

Non-epistatic values (ne_{ijkl}) are calculated as:

$$ne_{ijkl} = G_{ij..} + G_{..kl} - G_{....}$$

Table 1. Descriptive characteristics of the sample.

	Men (n=166)		Women (n=166)	
	Mean ±SD	Range	Mean ±SD	Range
Age, years	48.7±9.1	30.2–75.9	49.6±9.0	30.1–82.0
Weight, kg	89.5±14.0	56.4–151.5	72.8±15.2	46.8–132.5
Height, cm	179.0±5.9	163.1–195.6	164.0±5.5	148.9–180.1
BMI, kg/m^2	27.9±4.1	20.6–47.6	27.1±5.6	18.4–50.1
SBP, mmHg	118.5±14.4	92.5–175.0	116.7±15.0	85.0–179.0
DBP, mmHg	78.2±9.5	54.0–105.0	74.6±8.5	56.0–104.0
Cholesterol, mg/dl	192.9±34.6	115.0–309.0	188.0±38.7	68.0–352.0
CAC Area	23.7±64.6	0.0–510.0	7.2±32.1	0.0–329.8
Log(Area +1)	1.5±1.7	0.0–6.2	0.5±1.2	0.0–5.8
Hypertension,%	17.5%		10.9%	
Blood pressure lowering medications,%	9.0%		7.2%	
Prevalence of detectable CAC,%	53.0%		21.1%	

where $G_{....}$ is defined as the unweighted average of the nine genotypic values. The epistatic value (e_{ijkl}) for each two-locus genotypic is simply the difference between the genotypic value and the non-epistatic value for that particular genotype *ij* at the first locus and genotype *kl* at the second locus:

$$e_{ijkl} = G_{ijkl} - ne_{ijkl}.$$

The error variance for a particular e_{ijkl}, say e_{1111}, is calculated as follows:

$$V(e_{1111}) = [16V(G_{1111}) + 4[V(G_{1112}) + V(G_{1122}) + V(G_{1211}) + V(G_{2211})]$$

$$+ V(G_{1212}) + V(G_{1222}) + V(G_{2212}) + V(G_{2222})]/81.$$

Note that this formula can be used to derive the remaining eight variances. In general, 16 is the coefficient for the genotypic value variance of the two-locus genotype of interest, 4 is the coefficient for the genotype value variances with the same genotype as the genotype of interest at one locus but not the other, and 1 is the coefficient for the other 4 genotypic value variances. A measure of the overall level of epistasis (E^2) is the sum of the squares of the individual epistatic values:

$$E^2 = (e^2_{1111} + e^2_{1112} + e^2_{1122} + e^2_{1211} + e^2_{1212} + e^2_{1222} + e^2_{2211} + e^2_{2212} + e^2_{2222})$$

The significance of the overall level of epistasis can be tested using an F-test with 4, N-9 degrees of freedom (N = sample size) that compares the epistasis mean-square error (EMS) and residual mean-square error (RMS)

where EMS = $[(N_{1111}e^2_{1111} + N_{1112}e^2_{1112} + N_{1122}e^2_{1122} + N_{1211}e^2_{1211} + N_{1212}e^2_{1212} + N_{1222}e^2_{1222} + N_{2211}e^2_{2211} + N_{2212}e^2_{2212} + N_{2222}e^2_{2222})$ $- (\Sigma N_{ijkl}e_{ijkl})^2/N)]/4$ and RMS is the pooled within-genotype variance. The RMS can be obtained from a two-way ANOVA with the main effects and interaction effects of the two loci. Individual tests of epistatic values can be conduct-

Table 2. Two locus genotype frequencies for men and women.

Men	GG	GA	AA	Total
DD	17	23	11	51
DI	30	43	14	87
II	12	10	6	28
Total	59	76	31	
Women	**GG**	**GA**	**AA**	**Total**
DD	22	20	2	44
DI	27	50	14	91
II	10	18	3	31
Total	59	88	19	

ed to identify the particular set of genotypes that are interacting using the T-statistic, $T = e_{ijkl}/V(e_{ijkl})$.

RESULTS

In this sample, the 166 asymptomatic unrelated men ranged in age from 30 to 75 years with a mean of 49 years. The 166 asymptomatic unrelated women ranged in age from 30 to 82 years with a mean of 50 years (Table 1). The descriptive statistics for height, weight, BMI, SBP, DBP, fasting plasma-cholesterol levels, CAC, hypertension status, and use of blood pressure lowering medications are also presented in Table 1. Overall, the frequency of the *AGT* G allele was 0.602 and the frequency of the *ACE* I allele was 0.446 in the sex-combined unrelated sample. The chi-square tests for the null hypotheses of Hardy-Weinberg equilibrium were not rejected for either the *AGT* or the *ACE* polymorphisms (P=0.569
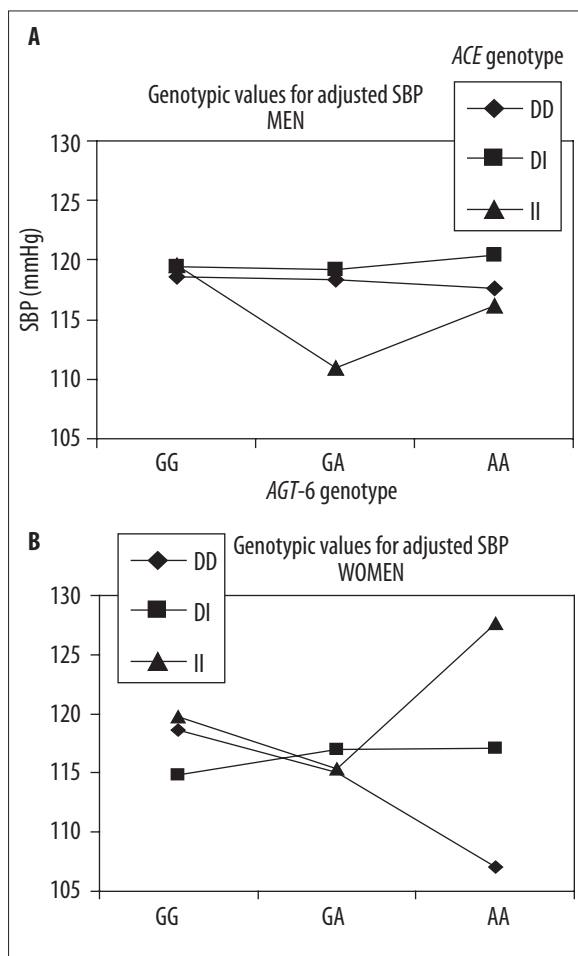
Figure 1AB. Genotypic values for adjusted SBP for men and women.

Figure 2AB. Genotypic values for adjusted log (CAC Area+1) for men and women.

and P=0.121, respectively). The two-locus allele frequencies for men and women are given in Table 2.

In men, SBP was adjusted for age, age², hypertension status, and use of blood pressure lowering medications which accounted for 51% of the variation in SBP (data not shown). Based on one-way ANOVA there was no association between either polymorphism and adjusted SBP levels in men (P=0.739 for AGT; P=0.246 for ACE). There was no evidence for epistasis based on two-way ANOVA (P=0.541). However, using the physiological epistasis model, we found significant evidence of epistasis in adjusted SBP levels in men involving the GG/II and GA/II genotype (P<0.05) (Table 3A and Figure 1A).

In men, log (CAC area +1) was adjusted for age, age², SBP, hypertension status, and use of blood pressure lowering medications which accounted for 18% of the variation in this trait. There was evidence for a possible association between ACE and adjusted CAC quantity based on one-way ANOVA (P=0.051). The two-locus genotypic values in Figure 2A suggest a positive main effect of the ACE II genotype on CAC quantity regardless of AGT-6 genotype. The AGT-6 polymorphism was not associated with a significant main effect on CAC quantity (P=0.631). There was no evidence of a significant gene-gene interaction between ACE I/D and AGT-6 polymorphisms on adjusted CAC levels in
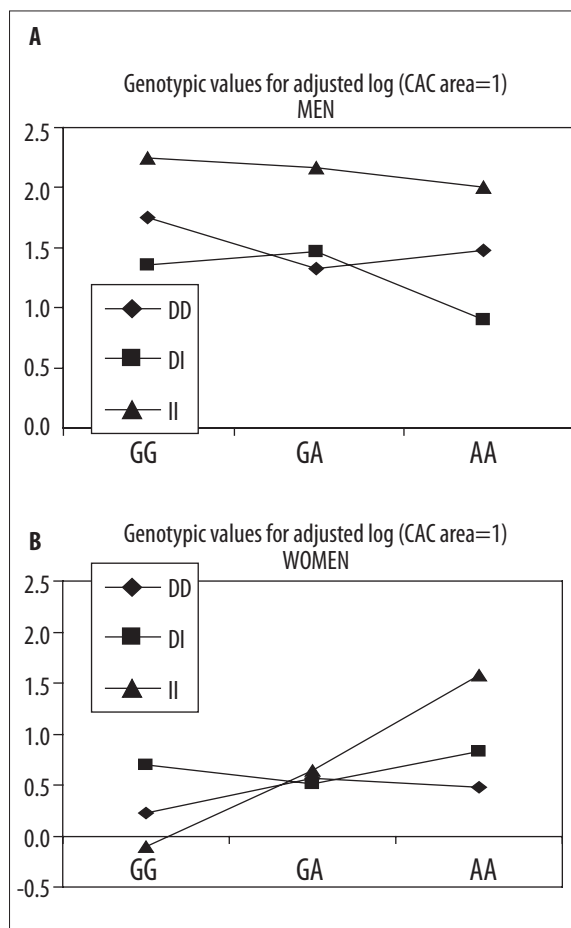
this sample of men based on the physiological epistasis model used here (Table 3B and Figure 2A) or a two-way ANOVA (P=0.426).

In women, the covariates age, age², hypertension status, and use of blood pressure lowering medications explained 40% of the variation in SBP. Based on one-way ANOVA there was no association between either polymorphism and adjusted SBP levels in women (P=0.838 for *AGT*; P=0.799 for *ACE*). There was also no evidence for epistasis using the two-way ANOVA (P=0.563). With respect to the role of epistasis in determining adjusted SBP levels we found that the GG/DD, GA/DD, AA/DD, GG/DI, GA/II, and AA/II were associated with statistically significant epistatic effects using the physiological epistasis method (P=0.033) (Table 3B and Figure 1B). Here the *ACE* genotype only has an effect when the *AGT* genotype is AA.

In women, the covariates age, age², SBP, hypertension status, and use of blood pressure lowering medications accounted for 35% of the variation in log(CAC area+1). There was no association between either polymorphism and adjusted CAC levels based on one-way ANOVA (P=0.166 for *AGT*; P=0.544 for *ACE*). The *AGT*-6 and *ACE* I/D two-locus genotypic values of adjusted log (CAC +1) are given in Table 4B and illustrated Figure 2B. The crossover in the plot is suggestive of additive by additive epistatic effects in women.

Table 3A. Genotypic, nonepistatic and epistatic values for men for systolic blood pressure (in mm Hg) adjusted for age, age^2, hypertension status, and use of blood pressure lowering medications. E^2=24.858; F=1.005; P=0.407.

	Genotypic values			
	GG	**GA**	**AA**	**SLV**
DD	118.58	118.32	117.65	118.18
DI	119.40	119.18	120.38	119.65
II	119.62	110.99	116.15	115.59
SLV	119.20	116.16	118.06	

	Nonepistatic values		
	GG	**GA**	**AA**
DD	119.57	116.54	118.43
DI	121.04	118.01	119.90
II	116.98	113.94	115.84

	Epistatic values ±standard errors		
	GG	**GA**	**AA**
DD	−1.00±1.10	1.78±1.07	−0.78±1.26
DI	−1.65±1.00	1.18±0.95	0.47±1.12
II	2.64±1.17	−2.95±1.40	0.31±1.62

	t-values (ratio of epistasis value to standard error)		
	GG	**GA**	**AA**
DD	−0.91	1.66	−0.62
DI	−1.65	1.24	0.42
II	2.26*	−2.00*	0.19

* Indicates statistically significant at α=0.05;
SLV – denotes the single locus values.

Table 3B. Genotypic, nonepistatic, and epistatic values for men for log(CAC area+1) adjusted for age, age^2, systolic blood pressure, hypertension status, and use of blood pressure lowering medications. E^2=0.143; F=0.366; P=0.833.

	Genotypic values			
	GG	**GA**	**AA**	**SLV**
DD	1.75	1.33	1.48	1.52
DI	1.36	1.47	0.90	1.24
II	2.25	2.17	2.00	2.14
SLV	1.79	1.66	1.46	

	Nonepistatic values		
	GG	**GA**	**AA**
DD	1.67	1.54	1.35
DI	1.40	1.27	1.07
II	2.29	2.16	1.97

	Epistatic values ±standard errors		
	GG	**GA**	**AA**
DD	0.08±0.19	−0.21±0.17	0.13±0.21
DI	−0.03±0.15	0.20±0.15	−0.17±0.19
II	−0.04±0.20	0.01±0.20	0.04±0.23

	t-values (ratio of epistasis value to standard error)		
	GG	**GA**	**AA**
DD	0.41	−1.26	0.64
DI	−0.24	1.38	−0.88
II	−0.22	0.03	0.16

SLV – denotes the single locus values.

The mean adjusted log(CAC area+1) for women with the *ACE* II genotype is lower than or comparable to the other *ACE* genotypes for women with the *AGT* GG or AG genotypes. However, it is higher than the other *ACE* genotypes for women with the *AGT* AA genotype. In this case it is not possible to answer the question, does the *ACE* I allele tend to higher or lower CAC. The answer is higher if a person is *AGT* AA and lower if they are *AGT* GG.

Using a two-way ANOVA model, the F-test for interaction between the *AGT*-6 and *ACE* I/D polymorphisms on CAC was not statistically significant in women (P=0.153). The overall F-test for interaction using the Cheverud and Routman method [2] was marginally statistically significant (P=0.052). The estimates of the nonepistatic and epistatic values along with the standard errors and associated t-values for individual epistatic values for adjusted log(CAC area+ 1) are displayed in Table 4B. The individual epistatic values that were each statistically significant (P<0.05) were GG/DD, GA/DD, AA/DD, GG/DI, GG/II, AA/DI and AA/II indicating that the genotypic values associated with 7 of the 9 two-locus gen-

otype classes were affected by epistatic effects. Interestingly, in women, the significant epistatic effects on SBP demonstrated some differences as compared to CAC.

Discussion

It is well known that common chronic diseases such as CAD, hypertension, and diabetes have multifactoral inheritance patterns. Over the last 15 years there has been a plethora of single gene association studies aimed at understanding the genetic architecture of these common diseases. Many, or perhaps most, of the findings from single gene association studies have not been replicated by other studies. From a single gene, Mendelian perspective of disease, this lack of replicability calls into question the validity of the genetic inferences made from the individual studies. From the perspective of what we know of the genetic architectures of complex traits studied in animals and plants [52,53], this lack of repeatability is expected. Differences in the joint frequency distribution of genetic and environmental factors across samples, coupled with the substantial role of interaction between these factors, makes for a very complex ge-

Table 4A. Genotypic, nonepistatic and epistatic values for women for systolic blood pressure (in mm Hg) adjusted for age, age^2, hypertension status, and use of blood pressure lowering medications. E^2=145.513; F=2.701; P=0.033

Genotypic values				
	GG	**GA**	**AA**	**SLV**
DD	118.67	115.02	107.03	113.57
DI	114.85	117.03	117.08	116.32
II	119.77	115.36	127.65	120.93
SLV	117.76	115.80	117.25	

Nonepistatic values			
	GG	**GA**	**AA**
DD	114.39	112.43	113.89
DI	117.14	115.18	116.63
II	121.75	119.79	121.24

Epistatic values ±standard errors			
	GG	**GA**	**AA**
DD	4.27±1.03	2.59±0.96	−6.85±1.02
DI	−2.29±1.17	1.85±1.02	0.45±1.35
II	−1.98±1.35	−4.43±1.37	6.41±1.98

t-values (ratio of epistasis value to standard error)			
	GG	**GA**	**AA**
DD	4.15*	2.69*	−6.74*
DI	−1.97*	1.81	−0.33
II	−1.47	−3.23*	3.24*

* Indicates statistically significant at α=0.05;
SLV – denotes the single locus values.

Table 4B. Genotypic, nonepistatic and epistatic values for women for log(CAC area+1) adjusted for age, and age^2,systolic blood pressure, hypertension status, and use of blood pressure lowering medications. E^2=0.812; F=2.400; P=0.052.

Genotypic values				
	GG	**GA**	**AA**	**SLV**
DD	0.23	0.57	0.48	0.42
DI	0.70	0.51	0.83	0.68
II	−0.10	0.64	1.58	0.71
SLV	0.28	0.57	0.96	

Nonepistatic values			
	GG	**GA**	**AA**
DD	0.10	0.39	0.79
DI	0.35	0.65	1.04
II	0.38	0.68	1.07

Epistatic values ±standard errors			
	GG	**GA**	**AA**
DD	0.13±0.07	0.17±0.08	−0.31±0.07
DI	0.35±0.09	−0.14±0.09	−0.21±0.11
II	−0.48±0.10	−0.04±0.10	0.51±0.09

t-values (ratio of epistasis value to standard error)			
	GG	**GA**	**AA**
DD	2.01*	2.08*	−4.64*
DI	3.69*	−1.63	−1.98*
II	−4.75*	−0.37	5.82*

* Indicates statistically significant at α=0.05;
SLV – denotes the single locus values.

netic architecture where similar genes will show different results in different populations [52;54–57].

New statistical approaches that incorporate multiple loci in candidate gene analysis are necessary in order to detect small context-specific genetic effects in the coronary atherosclerosis process. In general, the analysis of variance methods are more conservative for testing interaction effects than the physiological epistasis method presented by Cheverud and Routman [2]. Specifically, it has been shown that as allele frequencies deviate from 0.5, the additive and dominant components of variability for a single locus subsume more and more of the variability that is actually due to epistasis [3]. This phenomena results in smaller estimates of the component of variability due to epistasis than is actually observed in the genotypic mean differences and this results in reduced power to detect gene-gene interactions using the analysis of variance methods.

While numerous studies have examined the separate, single-site effects of polymorphisms in the *AGT* and *ACE* genes

and their effect on CAD risk factors and outcomes, few have explored epistatic effects between the two polymorphisms. To our knowledge, the only other study to investigate interaction effects between *AGT* and *ACE* on CAD examined the prevalence of myocardial infarction (MI) in a case-control study based on the population of St. Petersburg, Russia [58]. The cases were 198 MI survivors at least age 55 years. Sex of these survivors was not described. Controls were males between 6–17 years. The researchers reported that the main effects for the M235T and *ACE* I/D polymorphisms were not significant, but they found significant evidence of epistasis (the frequency of double homozygotes TTII was greatly increased in the group of MI patients as compared with those in the control group). Since *AGT*-6 and M235T have been found to be in strong linkage disequilibrium, with the 235T variant occurring more than randomly expected with the *AGT*-6 A allele, our results point to the two-loci genotype of double homozygotes for both *ACE* I and a variant form of the gene for *AGT* as potentially being associated with a higher risk for CAD since the AAII genotypes had high mean CAC levels in both men and women

In addition to the evidence for gene-gene interactions for SBP in both men and women and CAC quantity in women we also found that men and women differ in their level of epistasis between the *ACE* and *AGT* polymorphisms. There are two main reasons why this result might be expected. First, the physiological differences between men and women in their lipid metabolism and other metabolic determinants of CAC quantity are very likely to affect the mapping function between genotype and phenotype. Second, men have much higher levels of CAC quantity than women and it's unlikely that the same main effects or epistatic effects of two common susceptibility gene mutations will persist across the entire phenotypic range of CAC quantity seen in a population.

CONCLUSIONS

In conclusion using a physiological epistasis approach that focuses on phenotype values of people with a specific multilocus genotype and independent of the population frequency of the genotype class, we identified evidence for epistasis not found through the traditional two-way ANOVA approach. Our results support the hypothesis that gene-gene interaction in the RAS system may be a predisposing factor for a subclinical measure of coronary atherosclerosis in women as well as SBP in both men and women. These findings may provide some insight into the wide variation in results from studies that have focused only on the main effects of these genes.

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