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Association of Congenital Cardiovascular Malformations With 33 Single Nucleotide Polymorphisms of Selected Cardiovascular Disease-Related Genes

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Abstract

Rationale—Clark (1996) proposed that abnormal blood flow is related to some to congenital cardiovascular malformations (CCVM), particularly CCVM with obstruction to blood flow. Our hypothesis is that CCVM may relate to genes that affect blood coagulation or flow. We studied whether polymorphisms of such genes are related to CCVM; previously association of these SNPs conotruncal CCVM is described (Shaw et. al. 2005)

Methods—We assessed risk of pulmonary stenosis (PS, N=120), atrial septal defect (ASD, N=108), aortic stenosis (AS, N=36), and coarctation of the aorta (CoAo, N=64), associated with 33 candidate genes, selected for their relationship to blood flow affected by homocysteine metabolism, coagulation, cell–cell interaction, inflammation, or blood pressure regulation.

Results—Effects were specific to cardiac phenotype and race. CoAo was associated with *MTHFR* (–667) C>T (odds ratio [OR] for TT 3.5, 95% confidence limits [CI] 1.4–8.6). AS was associated with a polymorphism of *SERPINE1*, G5>G4, OR = 5.6 for the homozygote with 95% CI 1.4 –22.9. Unique polymorphisms were associated with increased risk of ASD and PS: *NPPA* 664G>A with ASD (OR of 2.4, 95%CI 1.3 – 4.4) and *NOS3* (–690) C>T with PS (OR 6.1; 95%CI 1.6 – 22.6 in the African American population only). For ASD, the *NPPA* (–664) G>A SNP there was increased risk from the variant genotype only in maternal smokers (OR 2.6; 95%CI 1.0–7.2).

Conclusions—Genes affecting vascular function and coagulation appear to be promising candidates for the etiology of cardiac malformations and warrant further study.

INTRODUCTION

Congenital cardiovascular malformations (CCVM) are thought to have a genetic component due to the familial tendency of some phenotypes and the specific heritable nature of some rare cardiac defects, e.g. Holt Oram syndrome (Isphording et. al. 2004; Heinritz et. al. 2005; McDermott et. al. 2005; Borozdin et. al. 2006). A multitude of studies have been published in the last two decades attempting to define genes associated with cardiac development often using mouse or zebrafish models, and to identify genes associated with specific CCVM. In

most studies of humans with a common CCVM phenotype, specific genes associated with nonsyndromic CCVM are present in perhaps 15 % of cases. In the last two decades a parallel group of observations have demonstrated the association of environmental exposures with CCVM, e.g. exposure to solvents, retin A, and high homocysteine levels. (Jenkins et. al. 2007) The relationship of these two fields of study of pathogenesis of CCVM to one another is often unclear.

In this study we explored gene-only and gene-environment effects of 33 single nucleotide polymorphisms (SNPs) in candidate genes from specific pathways, with respect to the risk of pulmonary stenosis, atrial septal defect, aortic stenosis, or coarctation of the aorta. These last two types of CCVM have strong risk associated with family history of CCVM, and surveillance of relatives of people with these CCVM shows frequent CCVM in family members (Loffredo, et. al. 2004). Congenital cardiovascular malformations are specific in their pathogenesis and risk factors for occurrence (Ferencz, 1985). Thus our analyses are carried out for each defect separately. It is our hypothesis that CCVM may occur due to abnormal blood flow patterns in the embryo, and thus be related to genes whose activity may affect blood coagulation and/or blood flow. We studied whether or not polymorphisms affecting activity of genes that are related to coagulation or vascular regulation are related to CCVM. We thus selected genes for this study that regulate blood pressure, alter coagulation, promote an inflammatory response, or affect homocysteine metabolism. Genes that affect cell cell interaction were included as genes that may affect the endothelium in development. Previous studies of this panel of genes studied here showed association of some polymorphisms with conotruncal cardiac malformations. (Shaw, et. al. 2005) The homocysteine metabolism group includes *MTHFR* (methylene tetrahydrofolate reductase), and *CBS* (cystathione beta synthase). Homozygosity of the *MTHFR* 677C>T variant is associated with elevated plasma homocysteine among subjects with lower folate intakes (Bathum, et. al. 2007; Ulvik, et. al. 2007). Elevated maternal homocysteine levels are associated with the occurrence of congenital cardiac malformations (Hobbs, et. al. 2006; Hobbs, et. al. 2006; Verkleij-Hagoort, et. al. 2006; Verkleij-Hagoort, et. al. 2007). Verkleij-Hagoort et al. later reported that various polymorphisms of genes affecting folate metabolism did not bear risk for congenital heart malformations, but their meta-analysis pooled multiple forms of cardiovascular malformations as an analytic group. The authors did confirm again in this study the risk of hyperhomocysteinemia for CCVM (Verkleij-Hagoort, et. al. 2007).

MATERIALS AND METHODS

Study population

Case and control infants were all participants enrolled in the Baltimore Washington Infant Study (BWIS) (Ferencz, et. al. 1993). The BWIS is a population based prospective case control study of risk factors for congenital cardiovascular malformations (CCVM) in which cases and controls were entered into the study from 1981 to 1991. Demographic and historical exposure data were available for all infants and obtained by face to face interviews of parents in the Baltimore Washington Infant Study (Ewing, Loffredo, Beaty, 1997). Family history of CCVM in a first degree relative was obtained from the parents at the time of the interview. Cases and controls are recorded as white or black or other because that was the language used to query the mother and father as to how they identified themselves and their infant. The phrasing has now been changed to Caucasian, African American, or Other respectively in this paper. Controls were identified on a population basis of live births in the regions. The controls thus included live born infants without CCVM but with other noncardiac malformations. These control infants did not undergo formal surveillance for CCVM after birth, but the BWIS included all cases of CCVM described before one year of age in the population of study including all regional pediatric cardiology practices and the

Medical Examiner's office. Thus CCVM would be unlikely to have been missed in the control infants (Ferencz, et. al. 1993). 1.3% of control infants were affected by non-cardiac birth defects in contrast to 24% of case infants overall in the entire BWIS population (Ferencz, et. al. 1997).

Cases included 120 infants with valvar pulmonic stenosis (PS), 108 infants with secundum ASD (ASD), 36 infants with aortic stenosis (AS), and 64 infants with coarctation of the aorta (CoAo) compared to 477 live born control infants (Co) who were matched by year and hospital of birth. Phenotypes were established by echocardiography, cardiac catheterization, surgical inspection, and/or autopsy for cases by cardiologists participating in the BWIS DNA samples:

Our analyses were of infant newborn screening blood specimens (filter paper) obtained after birth; this blood was the source of the DNA for genotyping. Of the 488 control infants whose mothers were interviewed, a blood specimen was obtained and genotyped for 477; these 477 infants are the control group for this study. Blood samples adequate for genotyping could not be obtained in the other 11 infants. All interviews and DNA samples were obtained with approval from The Institutional Review Board of the University Of Maryland School Of Medicine.

Genotyping assays

Genomic DNAs were extracted from blood spots as previously described. (Iovannisci 2000). We employed a multilocus allele-specific hybridization assay (Cheng, et. al. 1999) for genotyping. Briefly, the first step was a multiplex PCR amplification using a primer blend containing a biotinylated primer pair amplification of each polymorphic site. Next, the biotin-tagged amplification products were hybridized to a linear array of immobilized oligonucleotide probes specific for each allele of the polymorphic sites. Following a stringent wash, chromogenic reagents were used to visualize the biotin-tagged amplicons that remained hybridized. After color development arrays were scanned for archiving, and genotypes were interpreted by two observers. All genotyping was performed blinded to subjects' case or control status and independently scored by two individuals. A no-template negative control was included with each batch of samples genotyped. 10% of the samples were randomly selected and genotyping repeated to ensure assay reproducibility. Assay specificity was confirmed by the inclusion of samples whose genotypes had previously been established by DNA sequencing.

Statistical analysis

We estimated the magnitude of association of cardiac defects with each SNP comparing heterozygous to homozygous wild type, and homozygous variant allele to homozygous wild type using odds ratios (ORs) and 95% confidence limits. Nonparametric testing was carried out using the Mann Whitney U test. Gene-environment interactions were also explored for SNPs that showed initial positive results using historically reported occupational and leisure time exposures reported in the Baltimore Washington Infant Study, specifically maternal or paternal smoking, maternal exposure to solvents, maternal alcohol use, and maternal vitamin use. Maternal vitamin use was not recorded for all cases and controls in the study; the date was collected only in the latter years of the study. Analyses were performed to explore whether effect modification existed between *NOS3* genotypes and maternal smoking (defined as any cigarette smoking in the period 1 month before through 2 months after conception) or between reported vitamin use in pregnancy and *MTHFR* genotype on the risk of offspring for CoAo. Associations were estimated by odds ratios (ORs) and their 95% confidence intervals (CI) from logistic regression models using SPSS software (version 8.0). Frequencies of covariates are displayed in Table I. Gene frequencies were analyzed by

reported ethnicity of the infant. Comparison of gene frequencies in Caucasian and African American control populations were made using chi square analyses. Hardy Weinberg equilibrium was assayed in controls and in the four genes found associated with CCVM using a p value criterion of 0.05. All SNPs studied were in Hardy Weinberg equilibrium. We did not assay gene deletions or repeat polymorphisms (Edwards 2000).

Logistic regression analyses were performed separately for each cardiac malformation, including in models those variables found to be significant by non parametric testing. These included family history of cardiac malformations, tobacco use by mothers or fathers, and those leisure and occupational exposures known to be associated with the malformation, e.g. solvent exposure.

Selection of SNPs for study

Rather than using tagged SNPs, we chose candidate genes for study which are involved in one of five pathways that are relevant to cardiovascular function and homeostasis: homocysteine metabolism, coagulation, cell-cell interaction, inflammatory response, and blood pressure regulation.

MTHFR polymorphisms that result, in combination with a low folate intake, in elevated homocysteine levels are associated with CCVM in other studies.(Verkleij-Hagoort, et. al. 2006; Verkleij-Hagoort 2006; Ulvik, et. al. 2007). SNPs selected for their function in blood pressure regulation include: *AGTR1* (angiotensin receptor 1) (Bonnardeaux, et. al. 1994), two variants of *NPPA* (atrial natriuretic peptide precursor) (John, et. al. 1995); *AGT* (angiotensin) (Markovic, et. al. 2005); and *ADD1* (adducin 1) (Lanzani, et. al. 2005) as well as *SCNN1A* (sodium channel, nonvoltage-gated 1 alpha), a gene encoding sodium channels with effect on blood pressure (Rossier 1997; Huang, et. al. 2005) and *GNB3* (guanine nucleotide binding protein 3) (Siffert, et. al. 1998).

Hypotheses about flow related effects on cardiac development have been proposed for many years (Ferencz, et. al. 1984; Ferencz, 1985; Clark, 1986; Clark, 1996; Ferencz, 2000; Opitz and Clark, 2000; Ferencz, 2002); thus genes that affect vascular tone and blood pressure may be relevant to pathogenesis of cardiac malformations.

Polymorphisms of the gene *ADRB2* (adrenergic beta receptor 2) are widely studied and associated with hypertension, heart failure, asthma and obesity among other clinical conditions (Park, Kim and Lee 2005; Dallongville, et. al. 2003; Corbalan, et. al. 2002). The *NOS3* gene (nitric oxide synthase 3) is associated with blood pressure regulation and with endothelial function (Fulton, et. al. 1999). Genes selected for relevance to inflammation include *MMP3* (matrix metalloproteinase 3) (Fang, et. al. 2005; Samnegard, et. al. 2005), *SELE* (selectin E = endothelial leukocyte adhesion molecule 1) (Kamata, et. al. 2005), *ICAM1* (intercellular adhesion molecule 1) (Sligh, et. al. 1993), *TNF* (tumor necrosis factor) (Kamata, et. al. 2005) and *LTA* (lymphotoxin alpha) (Ozaki, et. al. 2002), another member of the *TNF* family. Eight genes affect coagulation status and/or endothelial function including: *F2* (clotting factor 2) (Poort, et. al. 1996), *F5* (clotting factor 5) (Simioni, et. al. 1997), *F7* (clotting factor 7) (Girelli, et. al. 2000), *SERPINE1* (serpin peptidase inhibitor, clade E, member 1 = plasminogen activator inhibitor type 1) (Nilsson, et. al. 1985), *FGB* (fibrinogen b beta polypeptide) (Tybjaerg-Hansen, 1997), and *ITGA2* (integrin alpha 2) (Kunicki, et. al. 1997) and *ITGB3*, (integrin beta 3) (Weiss, et. al. 1996). Altered endothelial function has been proposed as a unifying mechanism for pathogenesis of congenital cardiovascular malformations based on the extensive data of the Baltimore Washington Infant Study (Ferencz, 1985; Ferencz, et. al. 1984). Hematologic disorders with thrombus formation show epidemiologic associations with congenital cardiovascular malformations in the same studies (Ferencz, 2000; Ferencz, 2002).

RESULTS

Demographics

The frequency of each diagnosis and distribution of race and sex are shown in Table I. As others have noted, AS was more common in male infants compared to control populations with an OR of 2.9 (95% CI 1.4–6.3). Risk of AS was much higher among Caucasian infants compared to African American infants. (OR 8.7, 95% CI 2.3–33.2). CoAo risk was also associated with Caucasian race (OR= 4.9, 95% CI 2.1–22.4). ASD was more common in female infants (OR=2.3, 95% CI 1.5–3.6).

Impact of race on study of SNP alleles

When analyses are restricted to the population based control population of infants, significant differences in allele frequency are found in 26 of the 33 SNPs tested comparing Caucasian to African American infants, the two major groups in this study. (Table II). Thus comparison of the case population to the control population needed to be stratified on race to allow for valid inference. There are insufficient numbers of CoAo or AS occurring in African American infants to allow this, so analyses of these two defects were restricted to Caucasian cases and controls. Analyses of ASD cases and PS cases, which occurred in approximate proportion to the racial distribution of the control population, were, analyzed for cases and controls totally, and separately, for the Caucasian and African American subsets. Overall, the frequencies of each genotype in the control population are comparable to those reported for Caucasian and African or African American populations in reference populations. (<http://www.ncbi.nlm.nih.gov/projects/SNP/>).

Coarctation of the Aorta

(Caucasian cases and controls only)

As shown in Table III, *MTHFR* 677C>T is associated with increased OR of CoAo. Risk of CoAo with *MTHFR* 677C>T is increased in the homozygous compared to the heterozygous variant. For the heterozygous variant OR is 2.8 (95% CI 1.4–5.5); for the homozygous variant OR is 3.5 (95% CI 1.4–8.6). Maternal vitamin use as a binary variable did not alter the risk of the *MTHFR* polymorphism for CoAo. However data on maternal vitamin use was only collected in the last three years of the BWIS.

Aortic Stenosis

The polymorphic variant *SERPINE1* G5/G4, (OR for heterozygote vs. wild type 3.7, 95% CI 0.9 to 14.7; OR for homozygote vs. WT 5.6, 95 % CI 1.4 to 22.9) was associated with increased risk of AS. Analyses were restricted to Caucasian cases and controls because of the paucity of African American AS cases. Known environmental exposures associated with AS in this dataset included maternal exposure to solvents and maternal exposure to laboratory chemicals. Only one instance of maternal environmental exposure (to solvent) occurred in an infant heterozygous for *SERPINE1* G5/G4 and none in infants homozygous for G4. No association of family history of cardiac malformations, smoking, or mother's age were found for AS in this dataset.

Family History

Family history of CCVM was obtained from interview of mother, and, if available, both parents, (17) as described in the BWIS. A family history of CCVM was present in five infants from the pool of infants described here with AS or CoAo. Comparing the Caucasian infants with AS or CoAo and family history of CCVM to the Caucasian infants with AS or CoAo without such a family history, a family history of congenital CCVM in a first degree

relative was associated with Arg to Gly shift in *ICAM1* (intercellular adhesion molecule 1) at position 241 with odds 12.3 and 95% CI 2.1 to 70.7. No association of any of the SNPs studied with family history of CCVM was found in the control population or among infants with PS or ASD.

Atrial Septal Defect

An association of the *NPPA* 664G allele with ASD was observed with an OR of 2.4 (95% CI 1.3 to 4.3). There was evidence of effect modification of this genotype with maternal smoking. In the absence of smoking, the *NPPA* 664G allele had an OR of 0.8 (95% CI 0.3 to 2.8) compared to smoking mothers for whom the OR was 2.6 with 95% CI 1.0 to 7.2. (Table V) No race-specific effects were observed and the distribution of Caucasian and African American infants with ASD was not different than the distribution of controls.

Pulmonary stenosis

PS occurred in both African American and Caucasian infants in proportion to the control population. Analysis stratified by race demonstrated association of *NOS3* (−690) T with PS in African American but not in Caucasian infants. (Table VI). No evidence of gene environment interaction was observed, either for all PS in the study or for Caucasian or African American infants separately.

DISCUSSION

Phenotypic specificity

Of the four genes associated with cardiac malformations in this study, all are, in a broad sense, associated with vascular regulation and coagulation. This is consistent with the hypothesis that some cardiac malformations are the consequence of altered flow in development and also consistent with the known progression during fetal development of two types of CCVM, aortic stenosis and pulmonary stenosis.

Previous work by Shaw et al. (Shaw, et. al. 2005) surveyed this panel of genetic polymorphisms in infants with conotruncal anomalies, including d-transposition of the great arteries and Tetralogy of Fallot. (OMIM #18750). In that study, four gene variants were associated with increased risk of conotruncal anomalies: *NPPA* 2238T>C, *F7* (−323) del/ins, *F2* 20210G>A, and *ITGB3* Leu33Pro genes. Our findings, combined with these previous results, show associations that are specific to the cardiac phenotype. In three of four malformations, race of the infant is an additional important variable in the statistical models of risk. In two malformations, AS and CoAo, the proportion of Caucasians is much greater than in the population-based controls. For the instance of PS, only African American infants show an association with *NOS3* SNP. Unlike infants with conotruncal anomalies, CoAo or AS are each strongly associated with family history of CCVM, yet these two phenotypes showed associations with different gene variants tested in this study. The variable of family history of CCVM was associated with a different polymorphism, of *ICAM1*, in the AS and CoAo population. Intercellular adhesion molecule 1 (*ICAM1*) is a ligand for lymphocyte function antigens and is also homologous to a neural cell adhesion molecule. We did not find reports of *ICAM1* studied in cardiac development. *NCAM*, (neural cell adhesion molecule) but not *ICAM1*, is known to be important in forming groups of early embryonic cells and their boundaries (Knudsen, McElwee, and Myers 1990).

Homocysteine metabolism

Mothers of infants with CCVM have higher homocysteine levels, compared to mothers whose infants were born without heart defects (Hobbs, et. al. 2006; Hobbs, et. al. 2006a) In another study, maternal genotypes 677CT and 677TT were associated with increased risk of

conotruncal cardiac defects when no periconceptional folate was given (van Beynum, et. al. 2006). Others have reported no association of *MTHFR* polymorphisms and combined phenotypes of congenital heart defects (Stortri, et. al. 2003; Verkleij-Hagoort, et. al. 2007). In our study *MTHFR* polymorphism is strongly associated with coarctation of the aorta with increased odds for the homozygous allele state compared to the heterozygote. Using the limited numbers of cases with reported vitamin use in our study, vitamin use before 8 weeks pregnancy is associated with no risk of CoAo for the *MTHFR* polymorphism (OR 1.1, 95%CI 0.2 to 5.4). When vitamin use begins at eight or more weeks of pregnancy, the OR for the heterozygote vs. wild type of *MTHFR* 677C>T is 8.7 with 95%CI 1.2 to 61.4 and for the homozygote vs. wild type with late vitamin use is 10.7 with 95%CI 1.0 to 109. Obviously the very wide confidence intervals, due to the small numbers of cases in each group, limit the reliability of the finding but the interaction with vitamin use is in the expected direction.

In contrast to our results, one previous study limited to CoAo and AS failed to demonstrate a relationship to the *MTHFR* 677C>T polymorphism (McBride, et. al. 2005). The lack of association of *MTHFR* polymorphisms with coarctation of the aorta may reflect different vitamin supplementation between their groups and ours, or may reflect different design. Our study is population based compared to population based controls; the McBride study is a family based association design. Another study of *MTHFR* genotypes found associations with a variety of CCVM including CoAo, hypoplastic left heart, pulmonary stenosis, subaortic stenosis, and aortic valvar stenosis (Junker, et. al. 2001). In our study, pulmonary stenosis and valvar aortic stenosis are not associated with *MTHFR* genotypes.

Coagulation, endothelium, and blood flow

It has been hypothesized based on epidemiologic studies that coagulation abnormalities and/or abnormal or reduced red cells are related to the pathogenesis of CCVM (Ferencz, et. al. 1984; Ferencz, 1985; Correa-Villasenor, 1991; Ferencz, 2002;). The hypothesis was based on association of heritable blood disorders including hemophilia and von Willebrand's disease with CCVM (Ferencz, et. al. 1993). Three gene variants affecting coagulation associated with increased risk of conotruncal anomalies have been described: *F2* 20210A>G, *F7* (-323) del/ins, and *ITGB3* Leu33Pro (Shaw, et. al. 2005).

Endogenous nitric oxide is an important factor in regulating vascular tone and endothelial function and may affect flow and thrombus formation in the course of cardiac development. *NOS3* is expressed in the murine embryo at 9.5 days gestation, a time point which coincides with the development of a unidirectional circulatory system. (Teichert, et. al. 2008). Mice with genetic deficiency of eNOS have septal defects, increased frequency of bicuspid aortic valve, and heart failure, with decreased myocardial angiogenesis. (Zhao, Lu and Feng 2002).

The *NOS3* polymorphism is associated with PS only in the population of African American cases, suggesting an additional gene-gene or gene-environment interaction. The African American population in this study was found in two geographic clusters consistent with an environmental exposure that might be found predominantly in this population. A previous report (Shaw, et. al. 2005) identified increased risk of conotruncal anomalies associated with interaction of maternal smoking with any of three *NOS3* polymorphisms that result in decreased enzymatic activity.

A polymorphism of *SERPINE1*, a gene controlling plasminogen activator inhibitor activity is associated with valvar AS. The 5G polymorphism is less transcriptionally active than the 4G allele, leading to less fibrinolysis with the 4G polymorphism. (Rossaak, et. al. 2000). No data were identified by these authors about the expression of *SERPINE1* in embryonic tissues.

We also observed an interaction in this study between the variant *NPPA* 664G allele and maternal cigarette smoking during early pregnancy such that infants with the G allele whose mothers reported smoking had an increased risk of ASD. Zeller et al (Zeller, et. al. 1987) described localized expression of genes for atrial natriuretic factor in mouse cardiac embryos through development. *NPPA* is expressed in specific regions of developing mouse atrium in the embryo at 9.5 days. At this time the basic structure of the heart is established. (Soufan, et. al. 2004) The atrial natriuretic peptide is known to have a vasodilatory effect through a nitric oxide dependent mechanism.

Limitations of this study

The cases in this study represent the spectrum of specific CCVM phenotypes observed over a decade in a large population based study with complete ascertainment, yet each phenotype is relatively infrequent. Thus the study is limited by small numbers of each diagnosis. The phenotypes are highly homogeneous and established by careful clinical evaluation.

A large number of gene polymorphisms were investigated, and so one might anticipate that random associations would be found. Using the Bonferroni adjustment for multiple testing, one would predict that a p value of 0.001515 would in this data as tested correspond to a true probability, adjusted for multiple testing of 0.05. The measured p values in for association of *MTHFR* SNP with coarctation of the aorta are near but not less than the Bonferroni adjusted value, p0.0056 for the homozygote and p 0.0030 for the heterozygote. For the association of *NOS3* and pulmonary stenosis in African American infants p is 0.0026; and for AS homozygotes with *SERPINE1* p 0.0079. Thus while none of our values for significance meet the Bonferroni adjusted criterion, the values are highly suggestive of associations as noted. For the association of *SERPINE1* with AS and the association of *MTHFR* with CoAo, the OR of the homozygous SNP was higher than the OR for the association of the heterozygous SNP. The comparison of the homozygous to heterozygous allele in the case of either CoAo or AS lacks significance by direct testing; however the numbers of cases in each cell is small.

MTHFR and *NPPA* polymorphisms have been reported by others as associated with CCVM. *NOS3* polymorphisms in association with smoking have also been reported by Shaw as associated with increased risk of CCVM. (Shaw, et. al. 2005) The gene variants that we find associated with CCVM are ones whose activity may affect blood coagulation and/or blood flow and are supported by established hypotheses about alteration in blood flow and the etiology of some CCVM, particularly CCVM in which obstruction to blood flow is the major manifestation. Limited information is available regarding the expression of these genes during human embryological development, especially during the first six weeks when the cardiovascular system develops. Such research including animal models would provide important evidence for the possible mechanisms underlying the associations we have reported.

Implications for future research

The associations of specific SNPs with cardiac phenotype were highly specific in this study, i.e. the genes associated with different phenotypes were distinct. This finding argues strongly for analysis by specific, well characterized, phenotypic categories to identify associations with genetic variation. The limitation of this approach is in the limited numbers of well characterized cases with the same phenotype even when based, as this study is, on a very large population based data collection.

26 of the 33 SNPs assessed in this study had variant alleles which show significantly different frequencies in African American compared to Caucasian control infants in this

study. This finding is consistent with known variance in prevalence of those SNPs in populations of varying ethnicity. However, it mandates that data analysis be made with matched ethnicity of cases and controls which further reduces the sample sizes available. Small sample size is a limitation of this study. AS and CoAo are extremely uncommon in, e.g., African American populations, so that studies were carried out only in Caucasian cases and controls.

Using a case control study design, genetic associations with CCVM have been identified that are phenotype specific. Gene variants associated with higher levels of plasma homocysteine were associated with increased risk of CoAo. Genes that may affect vascular properties through endothelial function and coagulation were associated with CoAo, PS, and AS. Interaction of an environmental factor, maternal smoking, with a *NPPA* gene variant, is associated with atrial septal defect in this model as well.

The genes identified in this study as associated with AS, CoAo, PS and ASD are known to affect coagulation or vascular regulation. Further studies of these genes in the etiology of CCVM is warranted

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Table 1

Demographic Characteristics of Cases and Controls

Race	Controls	%	AS	%	CoAo	%	PS	%	ASD	%
Caucasian	307	64	33	92	56	85	72	60	62	57
African American	162	34	2	5	6	9	45	37	40	37
Other	7	1	1	3	2	3	3	3	6	6
Significance Race			0.0004		0.0000		0.43		0.37	
Sex										
Male	240	50	27	75	36	56	50	42	33	31
Female	236	50	9	25	28	44	70	58	75	69
Significance Sex			0.004		0.36		0.09		0.001	

Co controls; AS aortic stenosis; CoAo coarctation of the aorta; PS pulmonary stenosis; ASD secundum atrial septal defect

Table II

Allele Frequencies in White and Black Control Infants

Gene		White	Black	p values
MTHFR 677C>T rs1801133 NM_005957.3:c.849C>T	Wild type	134	113	0.001
	Heterozygote	124	47	
	Homozygote	32	2	
NOS3 (-922)A>G rs1800779 D26607:g.498A>G	Wild type	118	112	0.001
	Heterozygote	141	48	
	Homozygote	41	2	
NOS3 (-690) C>T rs3918226 D26607:g.730C>T	Wild type	253	158	0.001
	Heterozygote	43	4	
	Homozygote	4	0	
NOS3 Glu298Asp rs1799983 D26607:g.G7002G>T	Wild type	131	123	0.001
	Heterozygote	135	36	
	Homozygote	34	3	
AGTR1 1166A>C rs5186 Z11162.1:c.1629A>C	Wild type	145	126	0.001
	Heterozygote	131	33	
	Homozygote	24	3	
AGT Met235Thr rs699 NM_000029.2:c.916T>C	Wild type	95	6	0.001
	Heterozygote	156	52	
	Homozygote	49	104	
NPPA 664G>A rs5063 NM_006172.1:c.193G>A	Wild type	279	147	0.141
	Heterozygote	21	13	
	Homozygote	0	2	
NPPA 2238T768>C rs5065 NM_006172.1:c.553T>C	Wild type	206	51	0.001
	Heterozygote	80	78	
	Homozygote	12	32	
ADD1 Gly460Trp rs4961 AH003627.1:c.1378G>T	Wild type	187	120	0.008
	Heterozygote	99	41	
	Homozygote	14	1	
SCNN1A Trp493Arg rs5742912 NM_001038.4:c.1576T>C	Wild type	275	159	0.008
	Heterozygote	23	2	
	Homozygote	2	0	
SCNN1A Ala663Thr rs2228576 NM_001038.4:c.2096G>A	Wild type	144	119	0.000
	Heterozygote	123	40	
	Homozygote	31	3	
GNB3 825C>T rs5443 U47924.1:g.57721C>T	Wild type	136	13	0.001
	Heterozygote	137	73	
	Homozygote	27	76	

Gene		White	Black	p values
ADRB2 Arg16Gly rs1042713 M15169.1:c.1633A>G	Wild type	37	38	0.001
	Heterozygote	143	85	
	Homozygote	120	39	
ADRB2 Gln27Glu rs1042714 M15169.1:c.1666C>G	Wild type	88	92	0.001
	Heterozygote	163	63	
	Homozygote	49	7	
MMP3 (-1171)A5>A6 rs3025058 J04732.1:g.138insdelA	Wild type	69	6	0.001
	Heterozygote	178	64	
	Homozygote	52	91	
F2 20210G>A rs1799963 M17262:g.26784G>A	Wild type	289	158	0.576
	Heterozygote	6	3	
	Homozygote	2	0	
F5 Arg506Gln rs6025 NM_000130.3:c.1746G>A	Wild type	282	161	0.021
	Heterozygote	16	1	
	Homozygote	2	0	
F7 (-323)10-bp del>ins rs5742910 J02933:g.198_199delinsCCTATATCCT	Wild type	223	87	0.001
	Heterozygote	71	58	
	Homozygote	6	17	
F7 Arg353Glu rs6046 NM_000131.2:c.1289G>A	Wild type	231	125	0.821
	Heterozygote	64	33	
	Homozygote	5	4	
SERPINE1 (-675)G5>G4 rs1799768 J03764.1:g.2491dupG	Wild type	66	70	0.001
	Heterozygote	152	84	
	Homozygote	82	8	
SERPINE1 11053G>T rs7242 J03764.1:g.14216G>T	Wild type	53	42	0.080
	Heterozygote	153	73	
	Homozygote	94	46	
FGB (-455)G>A rs1800790 X05018.1:g.1045G>A	Wild type	191	141	0.001
	Heterozygote	88	18	
	Homozygote	19	0	
ITGA2 873G>A rs1062535 NM_002203.2:c.867G>A	Wild type	105	78	0.009
	Heterozygote	150	71	
	Homozygote	45	13	
ITGB3 Leu33Pro rs5918 NM_000212.2:c.196T>C	Wild type	207	127	0.097
	Heterozygote	87	33	
	Homozygote	6	2	
SELE Ser128Arg rs5361 NM_000450.1:c.561A>C	Wild type	246	150	0.008
	Heterozygote	50	12	
	Homozygote	3	0	

Gene		White	Black	p values
SELE Leu554Phe rs5355 NM_000450.1:c.1839C>T	Wild type	272	154	0.143
	Heterozygote	27	7	
	Homozygote	1	0	
ICAM1 Gly241Arg rs1799969 NM_000201.1:c.778G>A	Wild type	242	150	0.002
	Heterozygote	56	11	
	Homozygote	2	1	
TNF (-376)A>G rs1800750 X02910.1:g.240G>A	Wild type	291	158	0.742
	Heterozygote	9	4	
	Homozygote			
TNF (-308)A>G rs1800629 X02910.1:g.308G>A	Wild type	211	123	0.381
	Heterozygote	82	37	
	Homozygote	7	3	
TNF (-244)G>A rs673 M16441:c.3851G>A	Wild type	299	138	0.001
	Heterozygote	1	22	
	Homozygote	0	2	
TNF (-238)G>A r361525 X02910.1:g.378G>A	Wild type	264	153	0.018
	Heterozygote	35	7	
	Homozygote	1	2	
LTA Thr26Asn rs1041981 X01393.1:c.258C>A	Wild type	132	39	0.001
	Heterozygote	143	85	
	Homozygote	25	38	
CBS 844ins68bp	Wild type	241	92	0.001
	Heterozygote	59	70	
	Homozygote			

The frequencies of each allele are shown for Caucasian and African American infants in the control population. The significances refer to the difference in allele frequencies between the two populations as determined by chi square analysis

Table III

Genes Associated with Coarctation of the Aorta in White Cases and Controls

Genotype	MTHFR 677C>T	
	Control	Case
Wild type	134	12
Heterozygous	134	33
Homozygous	32	10
Heterozygote vs. wild type	2.8	
95% CI	1.4–5.5	
Significance	0.004	
Homozygote vs. wild type	3.5	
95% CI	1.4–8.6	
Significance	0.006	

The odds are given for the comparison between the gene wild type and the heterozygotic form as well as comparison between the gene wild type and the homozygotic form. 95% CI is the 95% confidence intervals of the OR in the line above.

Table IV

Gene Associated With Increased Odds of Aortic Stenosis

Genotype	SERPINE1 G5>G4	
	Control	Cases
Wild type	66	2
Heterozygote	152	17
Homozygote	82	14
Heterozygote vs. wild type	3.7	
95% CI	0.9–14.7	
Significance	0.05	
Homozygote vs. wild type	5.6	
95% CI	1.4–22.9	
Significance	0.01	

The odds are given for the comparison between the gene wild type and the heterozygotic form; as well as comparison between the gene wild type and the homozygotic form. 95% CI is the 95% confidence intervals (CI) of the OR in the line above.

Table V

NPAA 664 G>A Associated With Atrial Septal Defect

Genotype	NPAA 664G>A		NPAA 664G>A		NPAA 664G>A	
	Control	Case	NonSmokers		Smokers	
Wild Type	430	87	Control	Case	Control	Case
			176	35	111	27
Heterozygote	37	18	18	3	11	7
Homozygote	2	0				
Heterozygote vs. wild type	2.4		0.8		2.6	
95% confidence interval	1.3 to 4.4		0.2 to 2.8		1.0 to 7.2	
Significance	0.04		0.79		0.06	

The odds are given for the comparison between the gene wild type and the heterozygotic form. 95% CI is the 95% confidence interval of the OR in the line above.

Table VI

NOS3 (−690) C>T Is Associated with Pulmonary Stenosis Only in Black Infants

	OR for PS			
	NOS3 (−690)C>T		NOS3 (−690)C>T	
Race	White		Black	
Case or Control	Control	Case	Control	Case
Wild type	253	60	158	39
Heterozygote	43	9	4	6
Homozygote	4	0	0	0
Heterozygote vs. Wild type	0.9 (0.4 to 1.9)		6.1 (1.6 to 22.6)	
95% confidence Interval	0.4 to 1.9		1.6 to 22.6	
Significance	0.75		0.003	

The odds are given for the comparison between the gene wild type and the heterozygotic form. 95% CI is the 95% confidence intervals of the odds ratio in the line above.