Published in final edited form as:
Ann Thorac Surg. 2014 July ; 98(1): 104–111. doi:10.1016/j.athoracsur.2014.03.017.

Patient Genotypes Impact Survival After Surgery For Isolated Congenital Heart Disease

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Abstract

Background—Survival after cardiac surgery in infancy requires adaptive responses from oxidative stress management and vascular regulation pathways. We tested the hypothesis that genetic variation in these pathways influences post-operative survival in non-syndromic congenital heart disease (CHD) children.

Methods—This is an analysis of a cohort of non-syndromic CHD patients who underwent cardiac surgery with cardiopulmonary bypass before 6 months of age (n=422). Six single

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Meeting Presentation: J. Maxwell Chamberlain Memorial Paper for Congenital Heart Surgery: *Vascular Endothelial Growth Factor and Superoxide Dismutase Gene Variants Have an Additive Adverse Effect on Covariate-Adjusted Transplant Free Survival after Surgery for Isolated Congenital Heart Disease.* Society of Thoracic Surgeons 50th Annual Meeting, Orlando, FL, January 27th, 2014

nucleotide polymorphisms (SNPs) in 6 genes involved in oxidative stress and vascular response pathways, identified through *a priori* literature search, were tested for effects on transplant-free survival. Survival curves, adjusting for confounding covariates, were calculated using the Cox Proportional Hazard Models.

Results—Long-term survival was strongly associated with *VEGFA* SNP rs833069 ($p=7.03\times10^{-4}$) and *SOD2* SNP rs2758331 (p=0.019). To test for joint effects of the 2 SNPs on transplant-free survival, the genotypes were grouped to form a risk score reflecting the cumulative number of risk alleles (0–4 alleles/patient). A higher risk score based on the *VEGFA* and *SOD2* SNP genotypes was associated with worse transplant-free survival ($p=3.02\times10^{-4}$) after confounder adjustment. The total burden of risk alleles was additive; individuals with the highest risk score of 4 (n=59 subjects, 14.2% of the cohort) had a total covariate-adjusted HR=15.64 for worse transplant-free survival.

Conclusions—After cardiac surgery, infants who are homozygous for the high-risk alleles for both the *VEGFA* and *SOD2* SNPs have an approximate 16-fold increased risk of death or heart transplant; suggesting that genetic variants are important modifiers of survival after surgery for CHD.

Keywords

Congenital heart disease, CHD; Ischemia/reperfusion injury (myocardial); Genetics, genomics; Genes/polymorphisms/microarrays; Myocardial remodeling (reshaping, constraining, ventriculectomy); Outcomes (including mortality, morbidity, survival, etc.); Statistics, survival analysis

Introduction

Congenital heart defects (CHDs) are the most common human birth defect. Approximately one-third of CHD cases require surgical intervention, with a majority involving cardiopulmonary bypass (CPB). Long-term mortality in the post-operative stages remains considerable, especially for the more severe heart defects(1).

Oxidative stress is considered to be a major factor following cardiac surgery with CPB due to post-operative organ dysfunction(2). The importance of oxidative stress in postoperative outcomes has been demonstrated through the finding that allopurinol, which blocks free radical formation and its resulting oxidative stress, is associated with decreased cardiac event rate after surgery with CPB in in high-risk infants with hypoplastic left heart syndrome (HLHS)(3).

Studies of postsurgical outcomes in pediatric patients have successfully identified several genetic variants involved in vascular response pathways that affect long-term outcomes. First, endothelin-1 missense variant (*EDN1* G5665T) has been associated with transplant-free survival in patients with functional single ventricle CHD, with the greatest effects in children with the most severe phenotype, HLHS(4). More recently, a randomized clinical trial reported that missense mutations that up-regulated the Renin-Angiotensin-Aldosterone system (RAAS) were associated with impaired ventricular remodeling, renal function, and

somatic growth in infants with functional single ventricle post-cardiac surgery, highlighting the role of vascular response genes on a wide spectrum of postsurgical outcomes(5).

Taken together, these studies suggest that oxidative stress and vascular response play important roles in injury repair and long-term survival in the pediatric CHD population. We sought to examine the effects of specific genetic variants implicated in oxidative stress management and organ recovery on long-term survival in a cohort of children with non-syndromic CHD. Secondarily, we performed an analysis using a genetic risk score, reflecting the number of deleterious alleles each patient has, to determine if the observed genotype effects were independent and additive.

Patients and Methods

Study Design

This is an analysis of a previously described prospective cohort(6–8) of 550 subjects collected to study neurodevelopmental dysfunction following CHD palliation. This specific study sought to identify gene regions related to oxidative stress and vascular response potentially affecting survival in infants after cardiac surgery with non-syndromic CHD. We note that no genome-wide association analyses have been attempted on the phenotype of long-term survival; this is solely a candidate gene study.

Of the 550 original subjects, 56 were removed due to likely genetic syndrome and an additional 72 were removed due to lack of high-quality genotype data, leaving a total of 422 subjects available for analyses. Additional information on data collection (including inclusion/exclusion criteria), operative management, genotyping, and analyses not presented in the main manuscript are found in the Online-Only Appendix Materials.

SNP Selection

To preserve statistical power, we selected 6 candidate SNPs at 6 different genes involved broadly in oxidative and ischemic stress response (see Table 1) *a priori* based on a systematic literature review of published evidence from other investigators, reporting that variants in these genes have a functional impact potentially relevant to the outcomes (see Appendix Table S1). Four of the 6 genes (*EPHX1*, *SOD2*, *CYP2E1*, and *CAT*) are involved in oxidative stress. *VEGFA* and *EDN1* are involved in the vascular response to low output and ischemic states associated with CPB; additionally, *VEGFA* may help promote vascular adaptation to hemodynamic alterations, while the *EDN1* missense SNP that has been associated with transplant-free survival in single-ventricle children(4). All studied SNPs were in Hardy-Weinberg equilibrium in controls.

Analysis

Genotypes were coded using an additive model and all regression models included adjustment for confounders based on genetic ancestry and pertinent clinical covariates. Genetic ancestry was determined using previously described methods(9). Due to the mixed ancestry of the cohort (see Table 2 for demographic information, including genetic ancestry), the first 3 principal component eigenvectors from principal components analysis

(PCA) were used as covariates to adjust for potential population stratification(10). We adjusted for multiple contrasts based on the 6 SNPs by applying a Bonferroni correction of $\alpha = 0.05/6 = 8.3\times10^{-3}$.

Survival analyses and graphics were performed in R (http://www.r-project.org/). Time to long-term mortality was calculated from date of initial surgery to date of death; these data include all deaths, including operative deaths. A Cox Proportional Hazards (CPH) model was used to evaluate the joint effect of the studied SNP and potential covariates. Output from the CPH model was used for plotting of survival curves. An additional analysis included cardiac transplant as an endpoint in addition to any death using the aforementioned methods. Survival analyses were adjusted for the previously reported confounding variables: the first 3 principal component eigenvectors for race, gender, gestational age, gestational weight, diagnostic class(11), preoperative intubation, preoperative LOS, age at first operation, weight at first operation, total CPB time, use of deep hypothermic circulatory arrest (DHCA), total DHCA time, and hematocrit at first operation(12). A European ancestry (EA) only sensitivity analysis for all outcomes was performed to ensure the direction of SNP effects was consistent within the largest genetic ancestry subgroup. A separate, diagnostic-class stratified analysis was performed for transplant free survival to ensure consistency of SNP effects across the physiologic range of heart defects.

Results

Demographic and clinical variables are presented in Table 2. Of the 422 non-syndromic children studied, 16 died operatively and 31 additional children died during the follow-up period, with an average time to mortality of 9.32 years, while an additional 4 patients underwent cardiac transplant. Number of transplants or death by diagnostic class was 8 (3.9%) for class 1, 2 (4.9%) for class 2, 7 (15.2%) for class 3, and 34 (25.9%) for class 4. Median follow-up time for all subjects was 10.18 years.

Survival analyses demonstrated that SNPs at two different loci were associated with long-term survival: *VEGFA* intronic SNP rs833069 (HR=0.37, $p=7.03\times10^{-4}$) and *SOD2* intronic SNP rs2758331 (HR=0.52, p=0.019; Table 3). For both SNPs, each copy of the minor allele was associated with an increase in long-term survival. The *VEGFA* effect was significant after Bonferroni correction, while the *SOD2* effect was not. In the case of *VEGFA* SNP rs833069, each “G” allele led to a dose-dependent increase in survival probability (Figure 1A). A similar effect was noted for *SOD2* SNP rs2758331 (Figure 1B). We note that when testing for transplant-free survival, both *VEGFA* and *SOD2* remained significant (see Appendix Table S2).

To test for joint effects of the 2 SNPs in *VEGFA* and *SOD2* on long-term transplant-free survival, the genotypes were grouped to form a risk score reflecting the cumulative number of risk alleles (0–4 alleles/patient). For both *VEGFA* SNP rs833069 and *SOD2* SNP rs2758331, the minor allele was low-risk, while the more common variant was the high-risk allele for survival (see Table 3 and Figure 1). A higher risk score based on the *VEGFA* and *SOD2* SNP genotypes was associated with worse transplant-free survival ($p=3.02\times10^{-4}$), adjusting for confounders. The total burden of risk alleles was additive; individuals with the

highest risk score of 4 (n=59 subjects, 14.2% of the cohort) had a total covariate-adjusted HR=15.64 for worse transplant-free survival (see Figure 2).

To rule out effects of residual population stratification, we performed a separate sensitivity analysis on the majority European Ancestry (EA) subset of the cohort (n=273 subjects) for the significant SNPs (Table 3). These EA only analyses show consistency of the effect size and directions with the complete cohort. Thus, our results are unlikely to be secondary to population stratification.

To examine the potential for genotype confounding, whereby our identified *VEGFA* and *SOD2* SNPs are associated with less severe CHD, therefore leading to increased survival, we performed separate Cochran-Armitrage tests for both genotypes and diagnostic class. From these analyses, we found that the *VEGFA* SNP rs833069 minor allele was associated with higher-risk diagnostic class subgroups ($p=9.12\times10^{-8}$, see Appendix Table S3). Further analysis of specific diagnoses found that the *VEGFA* minor allele was in excess in HLHS (all diagnostic class 4), while the *VEGFA* major allele was positively associated with both Tetralogy of Fallot (TOF) and Transposition of the Great Arteries (TGA)(see Appendix Tables S4–S6). The vast majority of both the TOF (67/69) and TGA (49/52) subgroups were categorized into diagnostic class 1. *SOD2* genotype was not associated with diagnostic class.

We conducted a sensitivity analysis, stratifying the primary analysis by diagnostic class, to evaluate potential confounding (see Appendix Table S7) by severity of CHD. From these generally underpowered analyses, we noted that *VEGFA* and *SOD2* genotypes remained marginally significant (*VEGFA* p=0.037 and *SOD2* p=0.066) while the genetic risk score remained highly significant (p=0.0067) for class 4 subjects. Genetic risk score was also significant in class 3 subjects (p=0.017). The other effects were not significant, though coefficients trended in the correct direction for the remainder of diagnostic classes. Given this and that class was a covariate in our original regression model, the association of *VEGFA* genotype with CHD class did not account for the association of risk score with survival.

Comment

We have performed a candidate SNP analysis for genes involved in oxidative stress and injury repair pathways in the prediction of long-term survival in children with non-syndromic CHD. SNPs at two genes, vascular endothelial growth factor A, *VEGFA*, and superoxide dismutase 2, *SOD2*, were associated with long-term survival. Moreover, we have shown through a genetic risk score that the effects of the *VEGFA* and *SOD2* SNPs are cumulative – the more copies of the deleterious alleles of either SNP a patient has, the lower their probability of survival is. Through exclusion of subjects with chromosomal or genetic anomalies, we have obtained results that are more accurate representations of the pediatric population with non-syndromic CHD, as genetic anomalies are frequently associated with poorer outcomes in children with CHD(8).

In our analyses, we have identified minor alleles at *VEGFA* and *SOD2* as both being significantly protective against long-term mortality (*VEGFA* $p=7.03\times10^{-4}$ and *SOD2* p=0.019). We also have demonstrated that the effects of both SNPs are independent and

additive when considered together as a genetic risk score [$p=3.02\times10^{-4}$, HR=15.64 in the highest risk group (n=59) compared to the group with no risk alleles (n=11)]. The *VEGFA* SNP studied, rs833069, is in strong linkage disequilibrium (LD; $r^2=0.97$) with one 5′UTR SNP, rs2010963, whose minor allele has been associated with higher *VEGFA* expression(13); thus the SNP studied is associated with higher *VEGFA* expression. Prior experiments injecting exogenous vascular endothelial growth factor (VEGF) into rats found an increase in cardiac vascular permeability and cellular damage, which is hypothesized to be a mechanism through which VEGF causes injury post myocardial infarction (MI)(14). However, other studies have found that lower endogenous VEGF levels are associated with an increased risk of adverse cardiovascular events following MI(15) and that exogenous VEGF can rescue cardiac function after MI(16). Recent animal evidence suggests that while long-term *VEGFA* expression (e.g., from a plasmid) is detrimental to heart remodeling after MI due to the side-effects of hypotension and edema, short-term, pulse-like *VEGFA* expression that more closely matches *in vivo* VEGF dynamics improves survival at 1-year follow-up while avoiding the previous VEGF-associated side-effects(17). Taken together, we hypothesize that our findings reflect a spatiotemporally restricted increased level of *VEGFA* gene expression in patients with the protective allele, which, when endogenously expressed and regulated, improves vascular response to ischemic conditions and increases myocardial function after surgery, leading to improved long-term outcomes. Within this context, our findings could represent a potential therapeutic target using the aforementioned short-term, pulse-like VEGF administration for clinical follow-up after surgical palliation of non-syndromic CHD. The *SOD2* intronic variant analyzed, rs2758331 was studied due to strong LD ($r^2=0.93$) with a well-studied *SOD2* Val16Ala missense SNP, rs4880, which has been reported to increase enzyme activity approximately 33%(18), consistent with a protective improvement in the oxidative stress response.

Post-hoc literature review found a prior report of the *VEGFA* major allele (associated with decreased *VEGFA* expression(13), and decreased survival probability in our data) being associated with TOF(19). Our data replicates this finding, as the *VEGFA* major allele was associated with both TOF and TGA, which represented relatively minor (low morbidity/low mortality) heart defects in our cohort (vast majority diagnostic class 1). Additionally, the *VEGFA* minor allele, which is associated with increased *VEGFA* expression(13) and increased survival in our data, was associated with the most severe heart defect, HLHS (all 130 subjects with HLHS were in the highest risk diagnostic class 4). Had the *VEGFA* minor allele been associated with both lower class, less severe CHD and improved survival, the class association might have led to a false positive survival association. However, we find that the *VEGFA* minor allele is associated with the most severe CHD, and independently (as demonstrated by both the adjustment for diagnostic class in the original analyses and the significant and protective effects of *VEGFA* in the stratified sensitivity analysis of class 4 subjects) is protective against death or heart transplant in these highest-risk children. Thus, we conclude that there are not confounding effects of *VEGFA* (or *SOD2*, which was not associated with diagnostic class) on survival, as the protective effects of *VEGFA* were strongest in children with the most severe CHD. Further research into the mechanisms through which *VEGFA* expression influences the type and severity of CHD is now warranted.

Some limitations of this study must be considered. First, power was limited due to the size of the cohort and the lack of comparable cohorts with which to consider pooling data. We addressed this by limiting the number of hypotheses, including not attempting genome-wide analyses. As noted, all SNPs tested had or tagged known functional effects, improving the probability of true associations. However, true positives with smaller effect sizes may have been missed. Second, although the cohort is largely of European ancestry, we used data from subsets of all genetic ancestries and adjusted for this through usage of a standard statistical method for adjusting for population stratification(10) and performed sensitivity analyses in the majority EA subset of the cohort. These analyses suggest that the observed associations are not due to population stratification. Finally, our literature-identified and tested SNPs do not represent all biologically plausible candidates. We could only test variants of interest that were represented or in high LD with SNPs on our genotype chip, limiting hypotheses tested.

More work must be done to establish these findings before they can be implemented into the clinical setting. First, these results should be replicated in an independent cohort; unfortunately, no comparable genetic study of non-syndromic CHD patients is available at this time. Moreover, it must be noted that any replication of our work would require an inception cohort with DNA obtained prior to the first surgery. Failure to obtain DNA prior to the first surgery will likely lead to a survivor bias, as those with the deleterious alleles identified in our work will die at a rate disproportionate to the rest of the cohort.

In conclusion, the results presented offer evidence that long-term survival in children with non-syndromic CHD is likely affected by variation in genes involved in oxidative stress and vascular response mechanisms. Given the high incidence of CHD and the frequent need for surgical palliation, further molecular follow-up of these genes is imperative. Identification of these candidate genes and their differential susceptibility to oxidative and ischemic stress provides a potential window into novel pathways that can aid in the development of therapies and preventative strategies to aid in decreasing the morbidity and mortality of necessary cardiac surgery in infants with non-syndromic CHD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank all subjects and families for their participation. Genotyping was performed by the Center for Applied Genomics at the Children’s Hospital of Philadelphia.

This work was supported by a grant from the Fannie E. Rippel Foundation, an American Heart Association National Grant-in-Aid (9950480N), HL071834 from the National Institutes of Health, and a Washington State Life Sciences Discovery Award to the Northwest Institute for Genetic Medicine. DSK was supported by 1F31MH101905-01.

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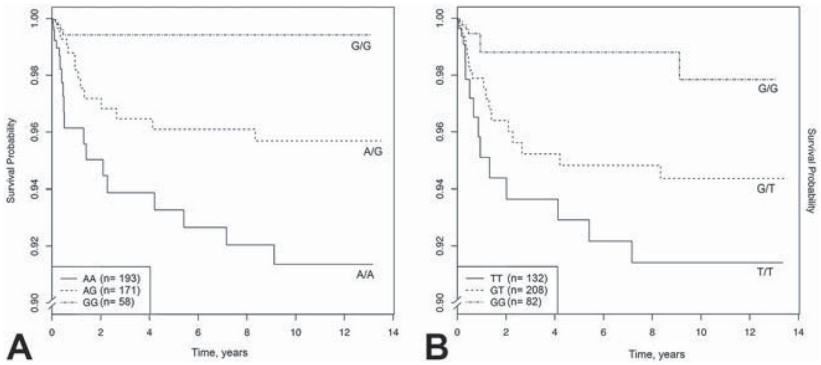

Figure 1. Vascular response related *VEGFA* SNP rs833069 (a) and oxidative stress related *SOD2* SNP rs2758331 (b) genotypes predict long-term survival

The survival curves show a dose dependent effect of each SNP. Note the range of the y-axis, "Survival Probability". *VEGFA* = vascular endothelial growth factor A; *SOD2* = superoxide dismutase 2.

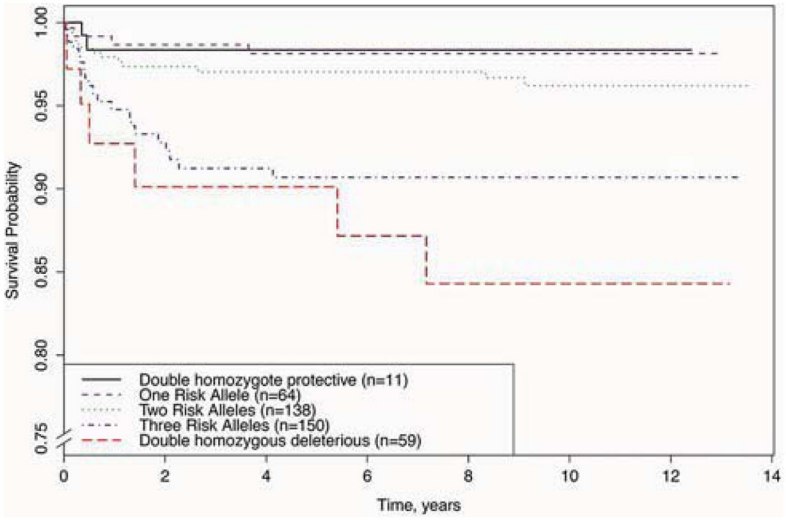

Figure 2. Genetic risk score reflecting number of *VEGFA* and *SOD2* risk alleles carried by each patient is predictive of long-term survival

Note the range of the y-axis, “Survival Probability”.

Table 1

Description of SNPs studied.

SNP	Gene[a]	Variant Type	Chr:Position[b]	Major/Minor Allele	Minor Allele Frequency	*Gene Name* and Description
rs1051740	*EPHX1*	Tyr113His	1:224,086,256	A/G	0.291	*Epoxide hydrolase 1*. Converts epoxides to non-toxic forms
rs5370	*EDN1*	Lys198Asn	6:12,404,241	T/G	0.187	*Endothelin 1*. Pro-peptide of endothelin 1, a potent vasoconstrictor
rs833069	*VEGFA*	Intronic[c]	6:43,850,557	A/G	0.346	*Vascular endothelial growth factor A*. Growth factor mediates vascular permeability and endothelium growth/apoptosis inhibition
rs2758331	*SOD2*	Intronic[d]	6:160,025,060	T/G	0.436	*Superoxide dismutase 2*. Mitochondrial protein; converts superoxide byproducts to hydrogen peroxide
rs10776686	*(CYP2E1)*	Intergenic	10:135,182,921	A/G	0.051	*Cytochrome P450 2E1*. Endoplasmic reticulum-associated enzyme, involved in varied processes
rs1001179	*CAT*	5′UTR	11:34,416,807	T/C	0.159	*Catalase*. Key antioxidant heme enzyme present in peroxisome

3′UTR = 3 prime un-translated region of a gene. 5′UTR = 5 prime un-translated region of a gene. Chr = chromosome. LD = linkage disequilibrium. SNP = single nucleotide polymorphism.

[a] Intergenic SNPs are represented in parentheses naming the nearest gene, e.g. *(CYP2E1)*.

[b] Position information and annotation from reference assembly 36.3.

[c] Intronic SNP, rs833069, is in strong LD (r^2 = 0.97) with *VEGFA* 5′UTR SNP rs2010963, which has been reported to increase expression levels of *VEGFA*.

[d] Intronic SNP, rs2758331, is in strong LD (r2 = 0.93) with *SOD2* Val16Ala missense SNP rs4880.

Table 2

Baseline and operative characteristics of the cohort

Baseline Characteristics	Cohort Subset (n=422)
Gender, n (%)	
Female	176 (41.7%)
Male	246 (58.3%)
Ethnicity, n (%)	
Asian/Pacific Islander, Hispanic, or other ancestry	51 (12.1%)
African ancestry, not Hispanic	98 (23.2%)
European ancestry, not Hispanic	273 (64.7%)
Gestational Age, weeks, mean ± SD	38.5 ± 2.05
Gestational Weight, kg, mean ± SD	3.15 ± 0.62
Diagnostic Class, n (%)	
I: 2 ventricles, no arch obstruction	204 (48.3%)
II: 2 ventricles, arch obstruction	41 (9.7%)
III: 1 ventricle, no arch obstruction	46 (10.9%)
IV: 1 ventricle, arch obstruction	131 (31.1%)
Specific CHD Diagnoses, n (%)	
Hypoplastic Left Heart Syndrome	130 (30.8%)
Tetralogy of Fallot	64 (15.2%)
Transposition of the Great Arteries	34 (8.1%)
Ventricle Septal Defect	40 (9.5%)
Ventricle Septal Defect, Coarctation of Aorta	19 (4.5%)
Single Ventricle	30 (7.1%)
Other Distinct CHD Diagnoses	105 (24.9%)
Preoperative intubation, n (%)	119 (28.2%)
Preoperative length of stay, days, mean ± SD	2.14 ± 2.61
Age at first operation, days, mean ± SD	42.1 ± 54.7
Weight at first operation, kg, mean ± SD	3.82 ± 1.25
Total cardiopulmonary bypass time in first operation, min, mean ± SD	67.1 ± 40.3
Use of DHCA, n (%)	259 (61.4%)
Total DHCA time in first operation, min, mean ± SD	41.4 ± 18.3

Baseline Characteristics	Cohort Subset (n=422)
Hematocrit level after hemodilution in first operation, %, mean ± SD	27.8 ± 4.1
Genetic risk score category[a]	
0 risk alleles	11 (2.6%)
1 risk allele	64 (15.2%)
2 risk alleles	138 (32.7%)
3 risk alleles	150 (35.5%)
4 risk alleles	59 (14.0%)
Long-term mortality, n (%)	47 (11.1%)
Time to mortality, years, mean ± SD	9.32 ± 3.48
Heart Transplants, n (%)	4 (1.0%)

CHD = congenital heart defect. DHCA = deep hypothermic circulatory arrest.

[a]Number of *VEGFA* SNP rs833069 or *SOD2* SNP rs2758331 major alleles, which are both associated with worse long-term survival.

Table 3

SNP results ($p \leq 0.05$) for long-term mortality.

SNP	Gene	EA-only HR (95% CI)[a]	Full Cohort HR (95% CI)[a]	EA-only *P*[b]	Full Cohort *P*
rs833069	*VEGFA*	0.25 (0.105–0.609)	0.37 (0.211–0.659)	0.0021	7.03×10^{-4}
rs2758331	*SOD2*	0.29 (0.133–0.636)	0.52 (0.304–0.900)	0.0019	0.019

CI = Confidence Interval. EA = European Ancestry, non-Hispanic. HR = Hazard Ratio from survival analyses.

[a] HR and 95% CI were calculated using Cox proportional hazards methods for the outcome of long-term survival, adjusting for the covariates listed in the methods.

[b] Analyses performed on the majority genetic ancestry group (EA) of the cohort (n=273 subjects), using analysis methods outlined in a.