APPENDIX

Patient Genotypes Impact Survival After Surgery For Isolated Congenital Heart Disease

Running title: *VEGFA* / *SOD2* and Long-Term Survival

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Supplemental Methods:

Operative Management

Details about the operative management of patients in this cohort have been previously reported(1,2). In brief, alpha-stat blood gas management and modified ultrafiltration were used in all patients. DHCA was used at the surgeon's discretion, and if used, patients first underwent core and topical hypothermia to a nasopharyngeal temperature of 18^{o}C.

Data Collection

Subjects who were ≤ 6 months of age and undergoing major surgical treatment of CHDs with CPB or DHCA were eligible for preoperative enrollment. Exclusion criteria for the original cohort included: (1) multiple congenital anomalies, (2) medical geneticist determination of likely genetic or phenotypic syndrome other than chromosome 22q-, and (3) language other than English spoken in the home.

Data on preoperative factors that might affect postsurgical outcomes, including gestational age, birth head circumference, and birth weight, were obtained from hospital records. Weight and age at surgery were recorded for the initial operation and for subsequent procedures with CPB. Operative variables were recorded, including the durations of CPB and DHCA, lowest nasopharyngeal temperature, and hematocrit level after hemodilution. Hospital length of stay (LOS) was recorded for the initial hospitalization. The postoperative LOS outcome is a measure of postoperative morbidity, and may reflect dysfunction in any of a multitude of organ systems.

Genotyping

Whole blood or buccal swab samples were obtained before surgery and were stored at 4^{o}C. Genomic DNA was isolated from WBCs and genotyping was performed using the Illumina HumanHap 550k BeadChip at the University of Pennsylvania Center for Applied Genomics. Copy number variation was not included in these analyses. High quality SNP data was retained by eliminating patients with call rates < 97% and genetic variants with call rates <99%. In addition, all genetic variants with a minor allele frequency (MAF) of < 0.5% (for power considerations) and SNPs that were out of Hardy-Weinberg equilibrium (HWE) ($p < 10^{-6}$) were removed from analyses. After these filters, 514,139 SNPs remained with a genotyping rate of 99.772%. Of the 494 non-syndromic subjects, 422 had genotype data that passed quality control and were considered in these analyses.

Analysis of Immediate Morbidity and Mortality Outcomes

Logistic/linear regression analyses were performed in PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/), with survival analyses and graphics produced by R (http://www.r-project.org/). We employed separate techniques to test association with our outcome measures. Linear regression was used for the continuous post-operative LOS data, which were Winsorized (extreme values set to 3 standard deviations from the mean, or 40.43 days) to obtain an approximate normal distribution. Post-operative LOS was first adjusted through a separate regression model for the following potentially confounding variables: the first 3 principal component eigenvectors for race, gender, gestational age, gestational weight, diagnostic class, preoperative intubation, preoperative LOS, age at first operation, weight at first operation, total CPB time, use of DHCA, total DHCA time, and hematocrit at first operation.

These surgical covariates had previously been determined to influence outcomes. After covariate adjustments the residuals became the predicted variables for testing genotype effects utilizing an additive model and linear regression. The same analyses were performed for post-operative mortality using logistic regression methods.

Supplemental Results on Outcomes Not Presented in the Primary Manuscript

Post-operative LOS and long-term survival were correlated with each other, with a pairwise correlation coefficient r = 0.47. The overall mean post-operative LOS was 11.4 ± 9.7 days, but 21.4 days for those who suffered operative deaths and 11.0 days for those who did not (see **Table 2**).

Post-operative LOS was associated with two SNPs: *CYP2E1* intergenic SNP rs10776686 (Beta Coefficient = 6.71, p = 0.016; **Table 3**) and *EPHX1* Tyr113His missense rs1051740 (Beta Coefficient = -4.10, $p = 5.04x10^{-3}$). We note that with removal of the 16 subjects who suffered early mortality following surgery, only the *EPHX1* SNP rs1051740 was significantly associated with post-operative LOS (p = 0.049). The carriers of the *CYP2E1* minor allele had a mean covariate-adjusted LOS of 6.7 days longer than those with only the major alleles. In contrast, the covariate-adjusted mean post-operative LOS was 4.1 days shorter for each copy of the *EPHX1* Tyr113His missense SNP.

Though underpowered with only 16 cases, we sought to determine if genetic variants predicted initial post-operative mortality, which was correlated with post-operative LOS (pairwise correlation coefficient r = 0.53). Using logistic regression methods adjusting for the same confounding variables as in our other analyses, we found that the same two SNPs that were associated with post-operative LOS were also significant for initial post-operative mortality: *CYP2E1* intergenic SNP rs10776686 (OR = 216.1, $p = 3.03x10^{-3}$) and *EPHX1* Tyr113His missense rs1051740 (OR = 0.091, p = 0.044). Taken in conjunction, both the *EPHX1* SNP

rs1051740 and *CYP2E1* intergenic SNP rs10776686 were significant at the Bonferroni-adjusted level ($p \leq 8.3x10^{-3}$) for one of these outcomes, respectively, though with the caveat that the analyses were underpowered. All test results are summarized in **Appendix Table S2**.

Supplemental Discussion on *CYP2E1* and *EPHX1* Mechanisms of Oxidative Stress

As expected due to the correlated outcomes, the *CYP2E1* and *EPHX1* SNP effect directions were consistent for both initial operative mortality and post-operative LOS. The minor allele at *CYP2E1* intergenic SNP rs10776686 was associated with both increased operative mortality and a longer stay. The functional variant for our *CYP2E1* findings is likely rs2031920, a 5'UTR variant that is in LD ($r^2 = 1.0$) with our tagging intergenic SNP. Rs2031920, originally identified as a restriction fragment-length polymorphism (when exposed to *Rsa1*), has been reported to cause approximately ten-fold increase in gene expression of *CYP2E1*(3). Increased *CYP2E1* expression may result in poorer postsurgical outcomes through a variety of mechanisms. First, the CYP2E1 enzyme has been reported to generate reactive oxygen species even in the absence of substrate(4); thus, in the context of increased gene expression, CYP2E1 enzymatic activity may increase oxidative injury in a range of tissues including the myocardium. This hypothesis is supported by the finding of increased *CYP2E1* expression in end-stage dilated cardiomyopathy and ischemic heart disease, compared to healthy myocardium(5).

The *EPHX1* rs1051740 Tyr113His allele was found to be protective and was associated with decreased LOS and mortality. Epoxide hydrolase 1 is an important enzyme for chemical detoxification; the studied genetic variant, *EPHX1* Tyr113His, decreases enzyme activity, and has been associated with elevated measures of oxidative stress(6). However, the mechanisms of oxidative stress regulation are not well understood and microsomal epoxide hydrolase (EPHX1) may have differential effects on oxidative stress management and tissue injury depending on the setting and other factors. It has been well established that inhibition of the soluble epoxide

hydrolase (EPHX2) is cardioprotective(7). Therefore, while EPHX1 is clearly involved in oxidative stress management, how it affects oxidative stress burden in this setting or whether oxidative stress regulation is the foundation for our observed genetic effect on outcomes remains to be determined.

Appendix Table S1. Summary of literature of studied SNPs.

SNP	Gene[a]	Variant Type	Oxidative Stress Category[b]	Summary of Gene Function and Literature Findings
rs1051740	*EPHX1*	Tyr113His	Detoxification	Enzyme converts epoxides to non-toxic forms. Inhibition of soluble epoxide hydrolase is cardioprotective(7). Missense mutation associated with: lung and other tobacco-related cancers(8), ovarian cancer(9), and numerous pharmacogenetic interactions with phenytoin(10), among other drugs.
rs2758331	*SOD2*	Intronic	Detoxification	Enzyme detoxifies superoxide byproducts of mitochondrial respiration. Tags *SOD2* Val16Ala missense SNP, rs4880 (r^2 = 0.93), which increases enzyme activity by approximately 33%(11) and has also been associated with coronary artery disease in female diabetics(12), and a decreased risk of human pancreatic cancers(13).
rs10776686	*(CYP2E1)*	Intergenic	Free radical generation and detoxification	Enzyme involved in metabolic and other detoxifying processes. SNP tags, by linkage disequilibrium, *CYP2E1* 5'UTR SNP rs2031920 (r^2 = 1.0), which increases *CYP2E1* expression ~ten-fold(3,14). CYP2E1 activity can generate reactive oxygen species in the absence of substrate(4) and has increased expression levels in end-stage dilated cardiomyopathy and ischemic heart disease(5). CYP2E1 also metabolizes halothane and related volatile anesthetics(15), alcohol(16), tobacco(17), and other carcinogens(18); the 5'UTR variant is associated with many cancers(17,19,20).
rs1001179	*CAT*	5'UTR	Detoxification	Enzyme is key peroxisome-associated antioxidant protein. SNP associated with increased transcriptional activity of the *CAT* gene(21) and increased risk of postmenopausal breast cancer in conjunction with hormone replacement therapy(22).

rs833069	*VEGFA*	Intronic	Vascular response and cell survival	Protein is involved in vascular permeability during inflammation and growth/apoptosis-inhibition. SNP tags 5'UTR *VEGFA* SNP, rs2010963 (r^2 = 0.97), which increases *VEGFA* expression(23). *VEGFA* is protective from risk of adverse cardiovascular events after myocardial infarction(24), and exogenous vascular endothelial growth factor can rescue cardiac function in late-stage ischemic heart disease(25) and after myocardial infarction(26). *VEGFA* also has roles in inflammation and cell survival(27): the 5'UTR SNP is positively associated with the disease time-course and severity of proliferative diabetic retinopathy(28,29), yet protective for psoriatic arthritis(30), and amyotropic lateral sclerosis(31), among other diseases.
rs5370	*EDN1*	Lys198Asn	Replication of Kirshbom(32) findings; vascular response	Protein is a pro-peptide of endothelin-1, a potent vasoconstrictor. Missense mutation associated with poorer transplant-free survival in children with single-ventricle CHD(32), asthma and atopic asthma(33), delayed onset of type 2 diabetes and retinopathy(34), decreased high-density lipoprotein cholesterol(35), and blood pressure levels (36).

[a] = Intergenic SNP naming the nearest gene.

Appendix Table S2. Results of association tests for 6 SNPs for initial operative mortality, postoperative length of stay, and long-term mortality.

SNP	Gene[a]	OR/Beta/HR (95% CI)	*P*
Initial Operative Mortality[b]			
rs1051740	***EPHX1***	**0.09 (0.0089-0.94)**	**0.044**
rs5370	*EDN1*	0.67 (0.18-2.48)	0.552
rs833069	*VEGFA*	0.55 (0.15-1.98)	0.360
rs2758331	*SOD2*	0.93 (0.29-3.01)	0.901
rs10776686	***(CYP2E1)***	**216.1 (6.18-7553)**	**3.03×10^{-3}**
rs1001179	*CAT*	3.01 (0.75-12.07)	0.12
Postoperative Length of Stay[c]			
rs1051740	***EPHX1***	**-4.10**	**5.04×10^{-3}**
rs5370	*EDN1*	-2.73	0.067
rs833069	*VEGFA*	-0.95	0.470
rs2758331	*SOD2*	-0.41	0.740
rs10776686	***(CYP2E1)***	**6.71**	**0.016**
rs1001179	*CAT*	1.11	0.510
Long-Term Survival[d]			
rs1051740	*EPHX1*	1.06 (0.59-1.88)	0.850
rs5370	*EDN1*	0.88 (0.51-1.53)	0.662
rs833069	***VEGFA***	**0.37 (0.21-0.66)**	**7.03×10^{-4}**
rs2758331	***SOD2***	**0.52 (0.30-0.90)**	**0.019**
rs10776686	*(CYP2E1)*	0.91 (0.25-3.31)	0.881
rs1001179	*CAT*	0.73 (0.39-1.37)	0.331
Transplant-Free Survival[d, e]			
rs1051740	*EPHX1*	0.90 (0.53-1.55)	0.713
rs5370	*EDN1*	0.68 (0.41-1.16)	0.162
rs833069	***VEGFA***	**0.47 (0.29-0.78)**	**3.35×10^{-3}**

rs2758331	***SOD2***	**0.62 (0.39-0.98)**	**0.040**
rs10776686	*(CYP2E1)*	2.11 (0.77-5.75)	0.142
rs1001179	*CAT*	0.95 (0.53-1.69)	0.858

CI = Confidence Interval. HR = Hazard Ratio from survival analyses. Beta = Linear regression coefficient. OR = Odds Ratio from logistic regression analyses; OR > 1 indicates increased rate of death. SNP = single nucleotide polymorphism.

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

[b] OR and 95% CI were calculated using logistic regression for the outcome of initial operative mortality, adjusting for the covariates listed in the methods.

[c] Beta coefficients were calculated through linear regression for the outcome of postoperative length of stay, adjusting for the covariates listed in the methods.

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

[e] Transplant-free survival included cardiac transplantation as an endpoint (in addition to death) for survival analyses, using the methods outlined in [d].

Appendix Table S3. *VEGFA* SNP rs833069 minor allele is associated with worse diagnostic class.

	VEGFA SNP rs833069 genotype			
Diagnostic Class		Major	Heterozygous	Minor
	Class 1	114	74	15
	Class 2	15	16	10
	Class 3	22	17	6
	Class 4	33	66	28

Cochran-Armitrage Test of Trend Statistics (two-sided alternative hypothesis):
Z statistic = 5.34
$P = 9.12 \times 10^{-8}$

Appendix Table S4. *VEGFA* SNP rs833069 minor allele is associated with Hypoplastic Left Heart Syndrome (HLHS).

	VEGFA SNP rs833069 genotype		
	Major	Heterozygous	Minor
No HLHS	152	107	31
HLHS	32	66	28

Cochran-Armitrage Test of Trend Statistics (two-sided alternative hypothesis):
Z statistic = 5.14
$P = 2.82 \times 10^{-7}$
All 131 HLHS subjects categorized into class 4.

Appendix Table S5. *VEGFA* SNP rs833069 major allele is associated with Tetralogy of Fallot (TOF).

	VEGFA SNP rs833069 genotype		
	Major	Heterozygous	Minor
No TOF	144	148	55
TOF	40	25	4

Cochran-Armitrage Test of Trend Statistics (two-sided alternative hypothesis):
Z statistic = -2.86
P = 0.0042
67/69 TOF subjects categorized in class 1; remainder in class 3.

Appendix Table S6. *VEGFA* SNP rs833069 major allele is associated with Transposition of the Great Arteries (TGA).

	VEGFA SNP rs833069 genotype		
	Major	Heterozygous	Minor
No TGA	152	156	56
TGA	32	17	3

Cochran-Armitrage Test of Trend Statistics (two-sided alternative hypothesis):
Z statistic = -2.82
P = 0.0048
49/52 TGA subjects categorized in class 1; remainder in class 2.

Appendix Table S7. Associations of *VEGFA*, *SOD2*, and genetic risk score with transplant-free survival stratified by diagnostic class.

	Total *N*	*N* Events	Association Tested	Hazard Ratio (HR)	P-Value
Class 1	204	8			
			VEGFA	0.28	0.26
			SOD2	0.27	0.25
			Genetic Risk Score	0.88	0.96
Class 2	41	2			
			VEGFA	0.95	0.99
			SOD2	0.082	0.98
			Genetic Risk Score	21.77	0.98
Class 3	46	7			
			VEGFA	4.77×10^{-7}	0.32
			SOD2	0.00031	0.17
			Genetic Risk Score	53.1	0.017
Class 4	131	34			
			VEGFA	0.51	0.037
			SOD2	0.56	0.066
			Genetic Risk Score	17.49	0.0067

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