Burden of potentially pathologic copy number variants is higher in children with isolated congenital heart disease and significantly impairs covariate-adjusted transplant-free survival

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ABSTRACT

Objectives: Copy number variants (CNVs) are duplications or deletions of genomic regions. Large CNVs are potentially pathogenic and are overrepresented in children with congenital heart disease (CHD). We sought to determine the frequency of large CNVs in children with isolated CHD, and to evaluate the relationship of these potentially pathogenic CNVs with transplant-free survival.

Methods: These cases are derived from a prospective cohort of patients with nonsyndromic CHD (n = 422) identified before first surgery. Healthy pediatric controls (n = 500) were obtained from the electronic Medical Records and Genetic Epidemiology Network, and CNV frequency was contrasted for CHD cases and controls. CNVs were determined algorithmically; subsequently screened for >95% overlap between 2 methods, size (>300 kb), quality score, overlap with a gene, and novelty (absent from databases of known, benign CNVs); and separately validated by quantitative polymerase chain reaction. Survival likelihoods for cases were calculated using Cox proportional hazards modeling to evaluate the joint effect of CNV burden and known confounders on transplant-free survival.

Results: Children with nonsyndromic CHD had a higher burden of potentially pathogenic CNVs compared with pediatric controls (12.1% vs 5.0%; $P = .00016$). Presence of a CNV was associated with significantly decreased transplant-free survival after surgery (hazard ratio, 3.42; 95% confidence interval, 1.66-7.09; $P = .00090$) with confounder adjustment.

Conclusions: We confirm that children with isolated CHD have a greater burden of rare/large CNVs. We report a novel finding that these CNVs are associated with an adjusted 2.55-fold increased risk of death or transplant. These data suggest that CNV burden is an important modifier of survival after surgery for CHD. (*J Thorac Cardiovasc Surg* 2016;151:1147-51)

Covariate-adjusted transplant-free survival by potentially pathogenic CNV in CHD cases.

Central Message

Isolated CHD cases have greater CNV burden. We also report a novel finding that CNVs are associated with an increased risk of death.

Perspective

Large genomic copy number variants (CNVs) are rare and potentially pathogenic. We found that children with isolated congenital CHD have a greater burden of large/rare CNVs, and that these CNVs are associated with an increased risk of death. These data suggest that CNV burden is an important modifier of survival after surgery for CHD and has the potential for use in risk stratification.

See Editorial Commentary page 1152.

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Abbreviations and Acronyms

CHD	= congenital heart disease
CHOP	= The Children's Hospital of Philadelphia
CNV	= copy number variant
CPB	= cardiopulmonary bypass
DHCA	= deep hypothermic circulatory arrest
ECMO	= extracorporeal membrane oxygenation
eMERGE	= electronic Medical Records and Genetic Epidemiology
LOS	= length of stay

Supplemental material is available online.

Congenital heart disease (CHD) represents the most common human birth defect, often requiring surgical intervention with cardiopulmonary bypass (CPB) or circulatory arrest soon after birth. Survival after surgery has improved, but long-term mortality remains considerable, particularly for more severe CHDs, including single-ventricle lesions.¹

Genetic factors, particularly those that are rare and alter proteins, are hypothesized to be major contributors to human disease.² Copy number variants (CNVs), duplicated or deleted regions of the genome, have been reported as potential causes of sporadic CHDs.^{3,4} CNVs are reportedly more frequent in children with CHD compared with controls.⁵ In addition, CNVs larger than 300 kb and overlapping a gene have been reported to be more frequent in CHD cases and associated with poorer growth and cognitive outcomes.⁶ Of note, previous studies reporting the prevalence of CNVs in children with CHD have included syndromic patients (eg, those with DiGeorge 22q11.2 microdeletions).

We previously used data from this cohort of children with isolated CHD to demonstrate the strong protective effects of *VEGFA* and *SOD2* genetic variants on transplant-free survival.⁷ Given the protein-disrupting potential of these large, gene-overlapping CNVs, we hypothesize that such CNVs also likely affect survival. Thus, in the present study we sought to determine the frequency of these potentially pathogenic CNVs among children with CHD compared with healthy pediatric controls, and to evaluate whether these large CNVs affect transplant-free survival in the first 3 years of follow-up after surgical correction of CHD.

METHODS**Ethics Statement**

Between October 1998 and April 2003, subjects were enrolled at The Children's Hospital of Philadelphia (CHOP) on a protocol approved by the Institutional Review Boards of CHOP and the University of

Washington. Informed written consent was obtained from a parent or guardian of each subject.

CHD Case Population

In this analysis of a previously described prospective cohort of 550 participants enrolled in a prospective study at CHOP to study neurodevelopmental dysfunction after surgical correction for CHD (hereinafter referred to as the "CHD cases"),⁸⁻¹⁰ patients aged 6 months or younger who underwent CPB with or without deep hypothermic circulatory arrest (DHCA) for repair of CHD were eligible for enrollment. Exclusion criteria included (1) multiple distinct congenital anomalies, (2) a recognizable genetic or phenotypic syndrome, and (3) a language other than English spoken in the home. Recognition of dysmorphic features can be difficult in neonates, however, and in some patients genetic syndromes were identified at subsequent evaluation, and these subjects were removed from the dataset before analysis.

This study examined a subset of the cohort with genetic data (n = 422) to establish the prevalence of large, gene-overlapping CNVs compared with that in healthy pediatric controls, and determine whether these potentially pathogenic CNVs were associated with differential transplant-free survival. We note that no genome-wide association analyses have been attempted using these CNV data; this is solely a study of the global burden of large, gene-overlapping CNVs and how they affect transplant-free survival in the first 3 years after surgical correction of CHD.

Of the original 550 CHD cases, 73 were excluded owing to the lack of high-quality genotype data, leaving a total of 477 subjects for analysis. An additional 55 subjects were excluded owing to the presence of DiGeorge syndrome or other chromosomal/genetic abnormalities, which would be expected to bias both the estimation of CNV prevalence and the evaluation of the effects of CNVs on survival for CHD, given that patients with genetic syndromes generally have worse survival.¹¹ A total of 422 patients were considered after these exclusions. Details of data collection (including further information on inclusion/exclusion criteria), operative management, and genotyping have been reported previously.⁷

Control Population

Healthy control subjects (hereinafter referred to as "controls") from the same site (CHOP) for comparisons of CNV prevalence were obtained from the Electronic Medical Records and Genetic Epidemiology (eMERGE) consortium.¹² A total of 500 healthy controls without CHD or other conditions associated with increased CNV prevalence (eg, autism, schizophrenia) were analyzed for the presence of large, gene-overlapping CNVs.

Genetic Evaluation to Exclude Subjects With Syndromic CHD

CHD cases were evaluated by a genetic dysmorphologist at the 1-year and/or 4-year examinations. Patients were classified as having no indication of genetic syndrome or chromosomal abnormality (normal, isolated CHD), suspected genetic syndrome (suspect), or a definite genetic syndrome or chromosomal abnormality (genetic). Following this classification, each CHD case's genetic record was reviewed by a second senior board-certified medical geneticist, blinded to the genetic data, to determine whether to include or exclude the subject from the current analysis, which focuses on nonsyndromic subjects. Based on this review, 55 CHD cases with a known or suspected genetic syndrome were excluded from our analysis owing to the potential for genetic confounding effects on CNV prevalence and the resulting effect on transplant-free survival within the first 3 years of follow-up after surgery.

Genotyping

Whole blood or buccal swab samples were obtained before surgery in CHD case subjects and at study enrollment in controls, and stored at

4°C. Genomic DNA was isolated from white blood cells and genotyping was performed at CHOP's Center for Applied Genomics.

CNV Determination and Validation

CNVs were determined algorithmically with Illumina HumanHap 550k BeadChip data for the CHD cases and Illumina 610k-Quad BeadChip data for the controls using the programs PennCNV¹³ and GWASTools.¹⁴ In brief, CNVs were considered potentially pathogenic after filtering for size (>300 kb), PennCNV quality score >100, overlap with genes, and novelty, as reported previously.⁶ Novelty was determined by comparing the called CNVs to the Database of Genomic Variants (<http://projects.tcag.ca/variation/>). CNVs were filtered from the data if >50% of the CNV overlapped with another nonpathogenic CNV already cataloged in the Database of Genomic Variants. CNVs identified as pathogenic and present in the Database of Genomic Variants were not filtered from the data. To prevent false-positive CNV calls from algorithmic methods, we filtered all CNVs for a minimum of 95% overlap in calls from the PennCNV and GWASTools programs. The foregoing methods were applied separately for the CHD cases and controls. A complete list of the CNVs determined algorithmically for both the CHD cases and controls is provided in Table E1.

CNVs were validated by quantitative polymerase chain reaction (qPCR) at CHOP's Center for Applied Genomics. Of the 38 tested CNVs from the CHD cases with available DNA, in 2 samples CNVs failed to validate (ie, showed the reference copy number) and 1 sample failed to undergo DNA amplification (likely owing to poor DNA quality) and thus could not be tested for CNV. Excluding the DNA quality-related qPCR failure, 35 of 37 predicted CNVs were successfully validated, for a validation rate of 94.6% (Table E1). Information on qPCR primers information for the 38 tested CNVs is provided in Table E2.

Statistical Analyses

All analyses and graphics were performed in R (<http://www.r-project.org/>) using standard regression packages. The χ^2 test was used to test the significance of the difference in frequency of large, gene-overlapping CNVs between the CHD cases and controls.

Genetic ancestry was determined as described previously.¹⁵ Owing to the mixed ancestry of the cohort, the first 3 principal component eigenvectors were used as covariates in Cox proportional hazards regression models, to adjust for potential population stratification.¹⁶

In CHD cases, time to long-term mortality was calculated from the date of initial surgery to the date of death. A Cox proportional hazards model was used to evaluate the joint effect of the global burden of potentially pathogenic CNVs and covariates affecting survival. Output from this model was used in plotting survival curves. Survival analyses were adjusted for the previously reported confounding variables: the first 3 principal component eigenvectors for race, gestational age, birth weight, diagnostic class (coded as a dummy variable with diagnostic class 1 as the reference group),¹⁷ total surgical support time (ie, total minutes on either CPB or DHCA), and extracorporeal membrane oxygenation (ECMO) use. Diagnosis class was assigned based on preoperative diagnosis according to a previously proposed scheme¹⁷: class I, 2-ventricle heart without arch obstruction; class II, 2-ventricle heart with arch obstruction; class III, single-ventricle heart without arch obstruction; and class IV, single-ventricle heart with arch obstruction. Confidence intervals (CIs) and 2-sided *P* values were calculated based on an asymptotic normal distribution of the estimated hazard ratio (HR) using Wald statistics.

RESULTS

Baseline characteristics of the studied subset of the CHD cases stratified by presence or absence of a potentially pathogenic CNV are presented in Table 1. Compared with

TABLE 1. Baseline characteristics of the CHD cases, stratified by the presence or absence of potentially pathogenic CNV

Characteristic	No CNV (n = 371)	CNV (n = 51)	Combined (n = 422)	<i>P</i> value
Male sex, n (%)	208 (56)	37 (73)	245 (58)	.040*
Ethnicity, n (%)				.49*
Caucasian	240 (65)	33 (65)	273 (65)	
African	85 (23)	14 (27)	99 (23)	
Hispanic	18 (5)	1 (2)	19 (5)	
Asian	12 (3)	0 (0)	12 (3)	
Native American	7 (2)	2 (4)	9 (2)	
Other	9 (2)	1 (2)	10 (2)	
Gestational age, wk, mean $\pm$ SD	38.5 $\pm$ 2.0	38.0 $\pm$ 2.1	38.5 $\pm$ 2.1	.062†
Birth weight, kg, mean $\pm$ SD	3.16 $\pm$ 0.60	3.03 $\pm$ 0.72	3.15 $\pm$ 0.62	.13†
Diagnostic class, n (%)				.88*
Class 1	179 (48)	25 (49)	204 (48)	
Class 2	36 (10)	5 (10)	41 (10)	
Class 3	39 (11)	7 (14)	46 (11)	
Class 4	117 (32)	14 (27)	131 (31)	
Preoperative LOS, d, mean $\pm$ SD	2.2 $\pm$ 2.6	2.1 $\pm$ 2.7	2.1 $\pm$ 2.6	.57†
Intubation, n (%)	104 (28)	15 (29)	119 (28)	.97*
Total surgical support time, min, mean $\pm$ SD	30 $\pm$ 50	20 $\pm$ 35	29 $\pm$ 48	.22†
Delayed sternal closure, n (%)	56 (15)	5 (10)	61 (14)	.48*
ECMO use, n (%)	22 (6)	1 (2)	23 (5)	.40*
Postoperative LOS, d, mean $\pm$ SD	16 $\pm$ 23	18 $\pm$ 29	16 $\pm$ 24	.62†
Mortality, n (%)	34 (9)	13 (25)	47 (11)	—

CNV, Copy number variant; SD, standard deviation; LOS, length of stay; ECMO, extracorporeal membrane oxygenation. * χ^2 test. †Wilcoxon rank-sum test.

CHD cases without a potentially pathogenic CNV, those with a potentially pathogenic CNV included a significantly higher proportion of males (73% vs 56%; *P* = .040) and had a nominally lower gestational age (mean, 38.0 vs 38.5 weeks; *P* = .062). Birth weight was not significantly different (mean, 3.03 kg in CHD cases with a qualifying CNV vs 3.16 kg in those without; *P* = .13). No significant differences between the 2 subgroups were seen in terms of distribution of race/ethnicity (with Europeans composing the majority of the cases, followed by individuals of African ancestry), diagnostic class, preoperative length of stay (LOS), incidence of preoperative intubation, total surgical support time, incidence of delayed sternal closure, ECMO use, and postoperative LOS.

Fifty-one of the 422 CHD cases evaluated (12.1%) had a CNV that exceeded 300 kb, overlapped with a gene, and was novel (ie, potentially pathogenic). In comparison, 25 such potentially pathogenic CNVs were identified in 500 (5.0%) of the healthy control cohort (see Table E1 for a list of identified CNVs). Applying the χ^2 test revealed a significant difference in the proportion of potentially

TABLE 2. Association of potentially pathogenic CNV on transplant-free survival in the CHD cases, adjusting for confounders with Cox proportional hazards regression (n = 422, with 47 deaths or heart transplants observed)

Covariate	HR (95% CI)	P value
Gestational age	0.90 (0.80-1.02)	.93
Birth weight	0.99 (0.99-1.02)	.19
Male sex	0.84 (0.44-1.58)	.64
Diagnostic class 2	1.19 (0.25-5.71)	.82
Diagnostic class 3	2.77 (0.91-8.41)	.073
Diagnostic class 4	9.70 (3.89-24.22)	1.13×10^{-6}
Total surgical support time	1.01 (1.00-1.02)	.037
ECMO use	14.44 (6.73-31.00)	7.42×10^{-12}
Genetic ancestry PC1	—	.40
Genetic ancestry PC2	—	.24
Genetic ancestry PC3	—	.0086
Potentially pathogenic CNV*	3.43 (1.66-7.09)	.0009

HR, Hazard ratio; CI, confidence interval; ECMO, extracorporeal membrane oxygenation; PC, principal component eigenvector (for genetic ancestry adjustment); CNV, copy number variant. *Presence of a potentially pathogenic CNV is associated with a 2.1% increased risk of transplant or death.

pathogenic CNVs between the CHD cases and the controls (12.1% vs 5.0%; $P = .00016$).

To examine the possible association between the presence of a large, gene-overlapping, novel CNV and transplant-free survival, we applied a Cox proportional hazards model. Survival analyses demonstrated a strong increased risk of death associated with potentially

pathogenic CNVs in a model jointly adjusting for the following covariates: the first 3 principal component eigenvectors for race, gestational age, birth weight, sex, diagnostic class, total surgical support time, and ECMO use (HR, 3.43; 95% CI, 1.66-7.09; $P = .0009$) (Table 2 and Figure 1). A Cox proportional hazard model R^2 comparison estimated the effect of CNV burden on transplant-free survival as 2.1%. Sensitivity analyses of the effect of covariate-adjusted CNV burden on transplant-free survival across diagnostic classes are presented in Table E3.

DISCUSSION

Here we confirm previous published reports^{5,6} that large, gene-overlapping, novel CNVs are present at higher frequency in children with isolated, nonsyndromic CHD compared with controls (12.1% vs 5.0%). CNVs present in a catalog of nonpathogenic CNVs were excluded, because they were less likely to contribute to survival given their known benign status. Note that we computed CNV frequency specifically for participants with isolated CHD not attributed to any known genetic or chromosomal anomaly that could have biased our results. In addition, we report for the first time that large, gene-overlapping, novel CNVs are associated with an estimated 3.43-fold increased risk of death compared with patients without such CNVs (95% CI, 1.66-7.09; $P = .00009$). Overall,

FIGURE 1. Covariate-adjusted long-term survival by the presence of potentially pathogenic CNV in CHD cases (n = 422, with 47 observed events). Note the discontinuous y-axis, which begins at an adjusted survival probability of 0.8. The 95% CIs for covariate-adjusted survival probability are 0.960 to 0.990, 0.947 to 0.985, 0.941 to 0.982, 0.939 to 0.981, and 0.931 to 0.978 for the no CNV group and 0.901 to 0.997, 0.798 to 0.959, 0.762 to 0.943, 0.762 to 0.943, and 0.762 to 0.943 for the CNV group at 0.5, 1, 1.5, 2, and 2.5 years of follow-up, respectively. CNV, Copy number variant.

this novel association of large, gene-overlapping CNVs and survival further emphasizes the importance of genetic factors in explaining complex phenotype variation and outcomes.

CNVs, which can result in the duplication or deletion of entire genes (ie, a change in the copy number of a particular gene), are one of several types of mutations currently thought to account for the “missing heritability” of complex genetic traits not yet adequately explained by common genetic variants studied in genome-wide association scans.² In children, CNVs have been implicated in the pathogenesis of numerous diseases, including schizophrenia¹⁸ and autism.¹⁹ CNVs also have been identified as potential causes of specific CHD diagnoses.^{3,4} In the present study, we have expanded on previous work and herein present evidence that the global burden of large, gene-overlapping CNVs is likely pathogenic, with a significantly increased risk of heart transplant or death after surgery seen in CNV carriers. Of note, analyses in adult cohorts have also identified an association between large CNVs and mortality.²⁰

Some limitations of this study should be considered. First, statistical power was limited owing to the size of the CHD case cohort and lack of comparable cohorts. We addressed the rarity of each individual CNV by focusing on the global burden of these large, gene-overlapping, and novel CNVs; however, given this pooling approach to analysis, we were unable to determine whether CNVs in a given region were more responsible than others for the effect on survival. In addition, because of this analytic method, we cannot specifically identify the reason for the impact of CNVs on survival. As noted in Table E1, multiple chromosomal regions are affected, and it is unlikely that a single common pathway is acting to affect survival in these patients. Finally, because we lack genotype data on parents of the affected CHD cases, we are unable to infer whether these rare CNV events are de novo or inherited. De novo variants in affected children of unaffected parents are considered more likely to be pathogenic.

In conclusion, our results confirm that large, gene-overlapping, novel CNVs are enriched in children with isolated CHD compared with healthy children, and provide new evidence that these CNVs are associated with poorer survival. Further follow-up of the pathogenic effects of these potentially pathogenic CNVs in a similar prospective cohort lacking survivor bias is imperative. Given the approximate 3.5-fold enrichment of these pathogenic CNVs in children with isolated CHD, validation of these results could lead to the development of new preventive and risk assessment strategies aimed at decreasing the morbidity and mortality of CHD.

Conflict of Interest Statement

Authors have nothing to disclose with regard to commercial support.

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Key Words: copy number variation, survival, congenital heart disease, genetics

TABLE E1. qPCR validation of 38 CNVs identified in 38 unique CHD participants with available DNA

Group	CNV region	Size	CNV type	Validate
CHD	chr22:17257787-19792353	2,534,566	Deletion, 1 copy	1
CHD	chr22:17257787-19792353	2,534,566	Deletion, 1 copy	1
CHD	chr7:4102511-4867717	765,206	Duplication, 1 copy	1
CHD	chr18:13417800-15112502	1,694,702	Duplication, 1 copy	1
CHD	chr22:17257787-19792353	2,534,566	Deletion, 1 copy	1
CHD	chr22:17257787-19792353	2,534,566	Deletion, 1 copy	1
CHD	chr22:17257787-19792353	2,534,566	Deletion, 1 copy	1
CHD	chr22:17257787-19792353	2,534,566	Deletion, 1 copy	1
CHD	chr15:20306549-20685685	379,136	Deletion, 1 copy	1
CHD	chr22:21122400-21554058	431,658	Deletion, 1 copy	1
CHD	chr22:17257787-19792353	2,534,566	Deletion, 1 copy	1
CHD	chr15:20306549-20685685	379,136	Deletion, 1 copy	1
CHD	chr22:21011312-21554058	542,746	Deletion, 1 copy	1
CHD	chr7:143056311-143505123	448,812	Duplication, 1 copy	1
CHD	chr16:15032942-16197033	1,164,091	Duplication, 1 copy	1
CHD	chr22:17257787-19792353	2,534,566	Deletion, 1 copy	1
CHD	chr15:20306549-20685685	379,136	Deletion, 1 copy	1
CHD	chr15:20306549-20685685	379,136	Deletion, 1 copy	1
CHD	chr10:133136594-133608348	471,754	Duplication, 1 copy	1
CHD	chr10:46410734-47173619	762,885	Duplication, 1 copy	1
CHD	chr22:17257787-19726528	2,468,741	Deletion, 1 copy	1
CHD	chr22:21024486-21441861	417,375	Deletion, 1 copy	1
CHD	chr22:17257787-19792353	2,534,566	Deletion, 1 copy	1
CHD	chr3:95067163-95989113	921,950	Duplication, 1 copy	1
CHD	chr10:2682656-3123648	440,992	Duplication, 1 copy	1
CHD	chr1:239320770-240023059	702,289	Duplication, 1 copy	1
CHD	chr12:33415349-34669982	1,254,633	Duplication, 1 copy	1
CHD	chr22:21024486-21554058	529,572	Deletion, 1 copy	1
CHD	chr13:102725899-103348161	622,262	Duplication, 1 copy	1
CHD	chr22:17257787-19792353	2,534,566	Deletion, 1 copy	1
CHD	chr22:17257787-19790220	2,532,433	Deletion, 1 copy	1
CHD	chr22:17257787-19792353	2,534,566	Deletion, 1 copy	1
CHD	chr15:20306549-20778963	472,414	Deletion, 1 copy	1
CHD	chr22:20885078-21401228	516,150	Deletion, 1 copy	1
CHD	chr22:17257787-18686993	1,429,206	Deletion, 1 copy	1
CHD	chr8:11620338-11935618	315,280	Duplication, 1 copy	0
CHD	chr2:111303487-111617069	313,582	Duplication, 1 copy	0
CHD	chr9:194201-589666	395,465	Duplication, 1 copy	NA*
CHD	chr22:21100917-21554058	453,141	Deletion, 1 copy	—
CHD	chr15:20306549-20685685	379,136	Deletion, 1 copy	—
CHD	chr6:65228140-66383666	1,155,526	Deletion, 1 copy	—
CHD	chr16:15032942-16197033	1,164,091	Deletion, 1 copy	—
CHD	chr7:158059922-158621330	561,408	Duplication, 1 copy	—
CHD	chr7:57212608-57593745	381,137	Duplication, 1 copy	—
CHD	chr16:21892987-22331199	438,212	Duplication, 1 copy	—
CHD	chr15:20306549-20685685	379,136	Deletion, 1 copy	—
CHD	chr22:17257787-19792353	2,534,566	Deletion, 1 copy	—
CHD	chr22:17257787-19792353	2,534,566	Deletion, 1 copy	—
CHD	chr15:20306549-20685684	329,335	Deletion, 1 copy	—
CHD	chr22:17257787-19729278	2,471,491	Deletion, 1 copy	—
CHD	chr22:21024486-21554058	529,572	Deletion, 1 copy	—
CHD	chr22:17257787-19792353	2,534,566	Deletion, 1 copy	—
CHD	chr22:20784580-21330892	546,312	Deletion, 1 copy	—
CHD	chr22:21100917-21554058	453,141	Deletion, 1 copy	—
CHD	chr22:17257787-19792353	2,534,566	Duplication, 1 copy	—

(Continued)

TABLE E1. Continued

Group	CNV region	Size	CNV type	Validate
CHD	chr1:244544759-244871447	326,688	Duplication, 1 copy	—
CHD	chr3:11622173-11958591	336,418	Duplication, 1 copy	—
CHD	chr22:21328337-21979242	650,905	Deletion, 1 copy	—
CHD	chr22:17257787-19792353	2,534,566	Deletion, 1 copy	—
CHD	chr22:17257787-19792353	2,534,566	Deletion, 1 copy	—
CHD	chr22:21046568-21401228	354,660	Deletion, 1 copy	—
CHD	chr6:162521711-162900893	379,182	Duplication, 1 copy	—
CHD	chr12:24803300-25155526	352,226	Duplication, 1 copy	—
CHD	chr15:20306549-20685684	329,335	Duplication, 1 copy	—
CHD	chr22:21328337-21979242	650,905	Deletion, 1 copy	—
CHD	chr22:17257787-19792353	2,534,566	Duplication, 1 copy	—
CHD	chr22:19066315-19792353	726,038	Duplication, 1 copy	—
CHD	chr3:1394026-1975594	581,568	Duplication, 1 copy	—
CHD	chr12:33415349-34565140	1,149,791	Duplication, 1 copy	—
CHD	chr22:17257787-19792353	2,534,566	Deletion, 1 copy	—
CHD	chr9:4117491-4537288	419,797	Duplication, 1 copy	—
CHD	chr22:17257787-19792353	2,534,566	Deletion, 1 copy	—
CHD	chr3:832325-1403635	571,310	Duplication, 1 copy	—
CHD	chr22:17257787-19792353	2,534,566	Deletion, 1 copy	—
CHD	chr9:36587-360439	323,852	Deletion, 1 copy	—
CHD	chr3:133579573-133940085	360,512	Deletion, 1 copy	—
Control	chr22:19420148-19792353	372,205	Duplication, 1 copy	—
Control	chr18:1070791-1767013	696,222	Duplication, 1 copy	—
Control	chr4:117695261-118972510	1,277,249	Deletion, 1 copy	—
Control	chr3:2721456-3035501	314,045	Duplication, 1 copy	—
Control	chr6:93770526-94218919	448,393	Duplication, 1 copy	—
Control	chr8:13592327-14702986	1,110,659	Duplication, 2 copies	—
Control	chr4:189370201-189766567	396,366	Deletion, 1 copy	—
Control	chr21:46289633-46909417	619,784	Duplication, 1 copy	—
Control	chr21:46192812-46909417	716,605	Duplication, 1 copy	—
Control	chr21:46391256-46909417	518,161	Duplication, 1 copy	—
Control	chr3:832325-1403635	571,310	Duplication, 1 copy	—
Control	chr15:20314760-20635884	321,124	Duplication, 1 copy	—
Control	chr21:46086792-46909417	822,625	Duplication, 1 copy	—
Control	chr1:246136535-246563486	426,951	Duplication, 1 copy	—
Control	chr7:71532565-71921501	388,936	Deletion, 1 copy	—
Control	chr15:20321135-20778963	457,828	Deletion, 1 copy	—
Control	chr19:47975960-48387680	411,720	Duplication, 1 copy	—
Control	chr15:20314760-20635884	321,124	Duplication, 1 copy	—
Control	chr2:78591176-79811160	1,219,984	Deletion, 1 copy	—
Control	chr3:540961-1325458	784,497	Duplication, 1 copy	—
Control	chr21:46449972-46909417	459,445	Duplication, 1 copy	—
Control	chr3:832325-1415351	583,026	Duplication, 1 copy	—
Control	chr12:1142624-1461019	318,395	Duplication, 1 copy	—
Control	chr15:29807358-30302218	494,860	Duplication, 1 copy	—
Control	chr21:20828763-21672012	843,249	Duplication, 1 copy	—

Total CNVs validated by qPCR: 35 of 37 (94.6%) (excluding 1 sample failure)*

Positions based on the hg18 genome build. *CNV*, Copy number variant; *CHD*, congenital heart defect; *qPCR*, quantitative polymerase chain reaction. *Sample failure did not amplify tested CNV regions or control CNV regions, indicating poor DNA quality; denoted by “NA.”

TABLE E2. Primer design for qPCR validation of CNVs in the CHD cohort

No.	Targeted region(s)	Amplicon position (hg18)	Amplicon sequence	UPL probe	Left primer sequence	Right primer sequence
1	chr1:239320770-240023059	chr1:239824508-239824631	tcacaatgggtctgatctgcattcacttc cagctgctggttagctcttttagcaggc ctctggcaccctcagcagtcggaggc acagaagctgcaaaaggatcttcg aaactgggcagagaaaa	11	tcacaatgggtctgatctgc	ttttctctgccagtttcg
1	chr2:111303487-111617069	chr2:111461205-111461304	aaccactctttggcctgctccaaactg ggctgaatctgtggtgggacaagct gagaaccaggtacgtattactcag gccatcatctgctccttcagtgg	26	aaccactctttggcctgct	ccactgaaggcagatgatga
1	chr3:95067163-95989113	chr3:95285939-95285998	gctcctggagggaataatagctct gctgcagtgctgcttccaggtag tgtgctttcc	84	gctcctggagggaataat	ggaaagcacactacctggaca
1	chr7:4102511-4867717	chr7:4214233-4214299	ccaacctcttgcgttctcaacagatt tactactggggagcagacagc cagaacgaaacggagaaaa	16	ccaacctcttgcgttctca	ttttccggttctgttctgg
1	chr7:143056311-143505123	chr7:143297051-143297125	caaacttctctgttctactcttgtgtcc cagcagtcagagggaaccttta aaataaactcagcttggaggc	3	caaacttctctgttctactctt	gcctccaaagctgagttatatt
1	chr8:11620338-11935618	chr8:11748292-11748409	cgacaggggatgaaaggaggcct gctccggcattggccaacacca gcaggcagcagaggaggccc agagctgccacatgttgaagcc ggatcctagatccacctggaga ggaca	27	cgacaggggatgaaagag	tgctcctccagggtggatct
1	chr9:194201-589666	chr9:460625-460697	caagaaagcaccgccactccagtg ggaacggacacgcgtgcacac agccacgagtgaggttgaag aggccc	76	caagaaagcaccgccact	gggcctcttcaaacctcac
1	chr10:2682656-3123648	chr10:2822646-2822705	cccgtctctgatttactgtgaatc ctggctgtgtgcagatgatggc tgagagcaaagg	76	cccgtctctgatttactg	cctttgctctcagccatcat
1	chr10:46410734-47173619	chr10:47128596-47128719	ggtaccatggacctgactggag aatatttcaccgctggccctg ggctccaggagagcccacca ccaccatggcccaggatgga ggcagcactggcgccaggg gcaagcaggtcacagatgcca	58	ggtaccatggacctgactgg	tggcatctgtgacctgctt
1	chr10:133136594-133608348	chr10:133261877-133261938	cttggttaaccgccagacgggat cccatccagccattcagctg ccatcttgggaaaggagac	42	cttggttaaccgccagacg	gtctccctttccaggatg
1	chr12:33415349-34669982	chr12:33644954-33645013	aatccccaggacggtgtttgtact tggggagcagccttagtagca gcaagccactgagtg	32	aatccccaggacggtgtt	cactcagtggttctgcteta
1	chr13:102725899-103348161	chr13:102965076-102965136	tcacctatccctggtgcattagggc ttttaactctgccttcaggttg attgggaattgcat	13	tcacctatccctggtgcatt	atgcaattcccaatcaacct
5	chr15:20306549-20685685	chr15:20415639-20415748	acttgcaagctatagggaatttt tcttaatggaaggaggagat accatgtatgacttctacacg tcaattttgataaaataagag aaaaggaaacatggcagaatgtg	12	acttgcaagctatagggaatttt	cacattctgcatgtttcctt
1	chr16:15032942-16197033	chr16:16045782-16045846	aacggcttcacctcttgtgtcc aggcagccgggtgaagggtg tgtactctccaagatcctgc	27	aacggcttcacctcttgt	gcaggatccttggaggagta

(Continued)

TABLE E2. Continued

No.	Targeted region(s)	Amplicon position (hg18)	Amplicon sequence	UPL probe	Left primer sequence	Right primer sequence
1	chr18:13417800- 15112502	chr18:13731942- 13732020	caggcacagagatgaacca gctgcatgagagtata cctactccagcttaatttt attggtgtgactgttgatg	11	caggcacagagatgaaccag	gataccaacagtcacaccaa
14	chr22:17257787- 19792353, chr22:17257787- 19726528, chr22:17257787- 19790220, chr22:17257787- 18686993	chr22:17729380- 17729493	ttctcatcgctgtcactgctgc tctccaggaggtctcggg gcctcagctctttcacaaag gttctgctcttttaacctgc acaaaaacattacatcac acttccttcagaaacc	79	ttctcatcgctgtcactgct	ggtttctgaagggaagtgtga
5	chr22:20885078- 21401228, chr22:21011312- 21554058, chr22:21024486- 21441861, chr22:21024486- 21554058, chr22:21122400- 21554058	chr22:21222566- 21222627	cgagcaccttactggaatgaaggc tgctctgcctgtgctcaaacctct acttttcgc	13	cgagcaccttactggaatga	gcgaaaagtagatggtttgagc
	SNCA positive control	chr4:90962489- 90962560	gctgagaagaccaaagag caagtgacaaatgttgga ggagcagtggtgacggg tgtgacagcagtagccag	68	gctgagaagaccaaagagcaa	ctgggtactgctgtcacac
	GAPDH positive control	chr12:6515824- 6515886	gctgcattcgccctctaatgg ggaggtggcctagggtg ctcacatattctggaggagcctc	10	gctgcattcgccctctta	gaggctcctcagaatatgtga

UPL, Universal probe library (from Roche Life Sciences); SNCA, synuclein alpha gene (positive control); GAPDH, glyceraldehyde-3-Phosphate Dehydrogenase gene (positive control).

TABLE E3. Sensitivity analyses from Cox proportional hazards regression by diagnostic class for the outcome of covariate-adjusted transplant-free survival

Diagnostic class	Total	Events	CNV burden, HR (95% CI)	P value
1	204	9	16.54 (1.71-159.80)	.015
2	41	2	60.47 (0.15-2.45 × 10 ⁹)	.646
3	46	5	6.64 (0.51-86.33)	.148
4	131	31	2.47 (9.67-6.31)	.059

Cox proportional hazards regression analyses reported adjusted for all variables reported in the Statistical Analyses section with the exception of diagnostic class (which was singular in each sensitivity analysis). CNV, Copy number variant; HR, hazard ratio; CI, confidence interval.