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## Contribution of Copy Number Variation to Down Syndrome-associated Atrioventricular Septal Defects

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### Abstract

**Purpose**—The goal of this study was to identify the contribution of large copy number variants (CNV) to Down syndrome (DS) associated atrioventricular septal defects (AVSD), whose risk in the trisomic population is 2000-fold more compared to general disomic population.

**Methods**—Genome-wide CNV analysis was performed on 452 individuals with DS (210 cases with complete AVSD; 242 controls with structurally normal hearts) using Affymetrix SNP 6.0 arrays, making this the largest heart study conducted to date on a trisomic background.

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### SUPPLEMENTARY MATERIAL

Supplementary information is linked to the online version of the paper at <http://www.nature.com/gim>

### DISCLOSURE

The authors declare no conflicts of interest.

**Results**—Large common CNVs with substantial effect sizes ( $OR > 2.0$ ) do not account for the increased risk observed in DS-associated AVSD. In contrast, cases had a greater burden of large rare deletions ( $p < 0.01$ ) and intersected more genes ( $p < 0.007$ ) when compared to controls. We also observed a suggestive enrichment of deletions intersecting ciliome genes in cases compared to controls.

**Conclusion**—Our data provide strong evidence that large rare deletions increase the risk of DS-associated AVSD, while large common CNVs do not appear to increase the risk of DS-associated AVSD. The genetic architecture of AVSD is complex and multifactorial in nature.

### Keywords

Copy Number Variation; Congenital heart defect; Down Syndrome; Atrioventricular septal defects; Ciliome

## INTRODUCTION

Congenital heart defects (CHD) are the most prevalent and serious of all recognized structural birth defects, occurring in 8 to 10 of every 1000 live births in the U.S.<sup>1</sup> Infant death rates due to CHD are high, with 44.0 deaths per 100 000 infants whose parents self-report as having European Ancestry (EA) and 56.2 deaths per 100 000 infants who reported as having African Ancestry (AA). Even with improved treatments, affected infants who survive birth require multiple surgeries with lengthy hospitalizations. Many of these individuals experience lifelong disabilities such as cognitive impairment, depression, anxiety disorder and behavioral problems and reduced quality of life. Affected individuals also have an increased risk of sudden cardiac death in later childhood and adulthood. In total, CHD account for over 2.6 billions of healthcare dollars spent each year in U.S.

CHD encompass a large, heterogeneous group of structural and functional abnormalities that arise during embryogenesis. As with most complex disorders, the underlying etiology reflects the combined influence of both genetic susceptibility and environmental exposures.<sup>2,3</sup> In humans, studies of the genetic architecture of CHD have implicated multiple classes of genetic variants, including both rare<sup>3-5</sup> and common single nucleotide variants<sup>6</sup> and rare structural variants.<sup>7-10</sup> Genes identified as the cause of rare familial forms of CHD have been found to play a role in simplex forms of CHD as well. Recently, using an exome sequencing approach and filtering for *de novo* events, CHD-susceptibility variants have been identified in histone-modifying genes.<sup>11</sup>

One of the most important contributors to CHD is chromosome aneuploidy.<sup>3</sup> In the current study we have focused on trisomy 21, or Down syndrome (DS [MIM 190685])<sup>12</sup>, as it is the most common autosomal aneuploidy condition that survives to term. The prevalence of DS is approximately 1 in 700 live births in the U.S., rendering it the most commonly identified form of intellectual and development disability and a leading cause of birth defects. CHD occurs in 40–50% of individuals with DS, the majority being septal defects. The most striking and severe form of DS-associated CHD is atrioventricular septal defect (AVSD [MIM 606215])<sup>12</sup>, also known as atrioventricular canal defect. While the incidence of non-syndromic AVSD in the general population is very low, 0.83 in 10 000 live births,<sup>13</sup>

approximately 20% of individuals with DS have complete AVSD, corresponding to a 2000-fold increased risk. More than 65% of all AVSD cases occur in children with DS.<sup>14</sup> This condition typically requires surgery in the first year of life. Thus, the costs and burdens of CHD are clearly amplified among those with DS and their families.

While trisomy 21 dramatically increases the risk of CHD and AVSD, nearly 80% of children with DS do not have an AVSD, and 50% of children with DS are born with structurally normal hearts. The increased dosage of genes on chromosome 21 therefore explains only part of the increased risk for CHD in DS and suggests that additional variants throughout the genome may play a role. Thus individuals with DS represent a “sensitized” population in whom genetic studies might reveal novel factors contributing to the risk of developing CHD, and any genetic mechanisms revealed may be relevant to both the trisomic and disomic individuals. Indeed, our studies of the genetic risk factor, *CRELD1* ([MIM 607170])<sup>12</sup>, have shown that the contributing missense mutations are found in both simplex euploid and DS-associated AVSD<sup>15</sup> and that mutation of *CRELD1* increases risk for CHD when expressed on a trisomic mouse background similar to trisomy 21.<sup>16</sup> This approach, i.e., using a carefully phenotyped group of individuals with a known genetic susceptibility factor for CHD, is the human equivalent of a sensitized screen used in model organisms. This strategy can be successful in revealing variation and identifying novel members of pathways affecting developmental or functional processes.<sup>17</sup> Recent findings from genome wide and chromosome-21 specific SNP and CNV association studies on a relatively smaller set of individuals with DS and other heart forms highlight the complex etiology seen in DS associated CHD.<sup>2,4,5,18-20</sup>

Here we report the largest genetic study to date of a carefully phenotyped collection of individuals with DS and AVSD (cases, n=210) compared with individuals who have DS and documented structurally normal hearts (controls, n=242). The cases and controls in this study were selected from the extreme ends of the observed phenotypic distribution of heart development in children with DS. We sought to test two specific hypotheses. First, we hypothesized that common (>0.01 frequency) CNVs of large effect contribute to the dramatically increased risk of AVSD in the DS population. Second, we hypothesized that the genome-wide burden of rare (<0.01 frequency) CNVs increases the risk of AVSD in the DS population, much as they do for other CHD in the disomic population. We found no support for the common variant hypothesis, but did find a significant increase in burden for rare deletions in cases. We also found a suggestive enrichment of genes involved in the cilium pathway, echoing a prior finding from a gene expression study of a similar collection of cases and controls.<sup>21</sup> Taken together, these results suggest the genetic architecture of AVSD is multifactorial and complex, even in the DS population.

## MATERIALS AND METHODS

### Sample Ascertainment

Details regarding the recruitment and enrollment have been documented previously.<sup>18,22</sup> Briefly, participants with a diagnosis of full trisomy 21 were enrolled with the vast majority confirmed by karyotype. Individuals with partial or mosaic trisomy 21 were not enrolled. Study participants were recruited through multiple centers across the United States.

Protocols were approved by institutional review boards at each participating center and informed consent was obtained from a custodial parent for each participant. To minimize phenotypic heterogeneity of cases, we focused on AVSD as the most severe heart phenotype seen associated with DS. A single cardiologist (K.J.D.) reviewed medical records and classified cases as individuals with DS who had a complete, balanced AVSD documented most often by echocardiogram or surgical reports (DS+AVSD). Controls were those with a structurally normal heart, patent foramen ovale or patent ductus arteriosus (DS+NH). Medical records and echocardiography were used to document normal heart status. Only participants whose mother reported as non-Hispanic EA were included in the current study. This information was obtained from a questionnaire administered to the mother of the child with DS by a trained interviewer. The data files are available at GEO repository, accession number GSE60607.

### Genotyping and CNV Detection

Genomic DNA was isolated using the Puregene DNA purification kit according to the manufacturer's instructions from lymphoblastoid cell lines (Qiagen Inc, CA, USA). The quantity and quality of the extracted DNA were determined on the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, DE, USA) and integrity of the genomic DNA was assessed on 0.8% agarose gel stained with ethidium bromide. Genotyping and CNV detection were performed using the Affymetrix Genome-Wide Human SNP Array 6.0 at Emory University as per manufacturer's protocol. Genotype calling was performed using Birdseed algorithm (version 2), as implemented in the Affymetrix Power Tools software (APT 1.12.0). Individual arrays with <86% call rate, <0.4 contrastQC and mismatched gender concordance were excluded from downstream analyses. Additionally, samples were removed if the heterozygosity was greater than 3 standard deviations (SD) above the mean and the total call rate less than 94.5% (outliers with >3 SD below the total call rate) indicating potential sample mixture. Samples with inconsistent family structure based on genotype calls were also excluded from downstream analysis. SNPs with completion rate <95% as well as those that deviated from Hardy-Weinberg equilibrium were also excluded from analysis. A total of 773 359 autosomal SNPs were retained, while 133 301 autosomal SNPs were excluded.

From the total 471 genotyped samples, 9 were excluded due to poor data completeness or inconsistent family structure (130 cases had parental genotype information). Matching of cases and controls was evaluated by principal component analyses (PCA) using Eigenstrat software v4 (Figure S1).<sup>23</sup> Seven outlier samples were excluded based on PCA analysis. We also excluded 3 samples with excess number of CNVs (>3 SD) called from downstream analyses as these likely result from experimental artifacts. The final data set consisted of 452 subjects, of which 210 were cases and 242 were controls.

For CNV analysis, data normalization and log<sub>2</sub> ratios for autosomes were calculated using APT 1.12.0. We used stringent criteria to filter CNVs as previously described.<sup>24</sup> To minimize false positive findings, we first used three algorithms GLAD, GADA and BEAST to identify CNVs.<sup>24,25</sup> CNVs called by only a single algorithm were removed from analysis. When algorithms disagreed on break point determination, the smallest start point and the

largest end point (largest interval) were considered. As a further filter, we required that each CNV include at least 20 SNPs. For deletions, we required that the genotypes be homozygous at 90% of sites. For duplications, we required that the percentage of heterozygotes exceed the genome-wide average heterozygosity of 27% reported for SNPs genotyped on the microarray. Finally, we visually inspected the log<sub>2</sub> ratio plots and excluded 8 large deletions that we judged to be cell line artifacts.

### Statistical Analysis

Prior to conducting association tests, we excluded CNVs that overlapped centromeric regions (Table S1), as these regions are highly repetitive with few SNP markers on commercial arrays, making CNV calls intersecting these regions unreliable. Global burden of CNVs between cases versus controls, was performed using PLINK.<sup>26</sup> Deletions and duplications were analyzed separately. We also performed region-based and position-based association tests for CNVs among the case-control group. Region-based association analyses were conducted to see whether any specific genomic region was associated with case CNVs vs control CNVs. For this, we used the hg19 UCSC gene list as the candidate genomic region. Position-based analyses test for association of individual CNVs and asked if any single genomic position was associated with cases vs controls. We did both the burden and association analyses first on all deletions/duplications and then by filtering deletions/duplications by their frequency and then by size (large>100kb). The frequency-based filtering was based on frequency among cases and controls combined and defined as rare variants (<0.01) and as common variants (>0.01). Empirical *p*- values from 1 000 000 random permutations were reported for each test.

We performed gene set enrichment analyses using the CNV enrichment test implemented in PLINK v1.07.<sup>27</sup> The list of genes we used for enrichment analyses is provided in Table S2 and is primarily based on the candidate gene lists published elsewhere with modifications.<sup>11,21</sup> For the genes in the ciliome pathway, we excluded any mouse-genome based genes for which a corresponding human ortholog (hg19) could not be found using UCSC genome browser. Since our CNV analyses were focused on autosomes, we also excluded candidate genes falling on sex chromosomes. For comparison, we used the same hg19 gene list mentioned above. Enrichment analyses were conducted for all deletions, rare deletions (<0.01), and all duplications. Similar to burden tests, empirical *p* values from 1 000 000 random permutations were reported.

### Validation of CNVs

We attempted to validate 31 CNVs (27 deletions and 4 duplications) in 25 cases and 33 CNVs (32 deletions and 1 duplication) in 25 controls (Table S3). Selection criteria included CNVs intersecting ciliome genes, CNVs from the chromosome *14q32.22* (region corresponding to our most significant finding from region-wise analysis), and a random, genome-wide selection of CNVs in cases and controls not found in the Database of Genomic Variants (DGV). We genotyped these samples with the Illumina HumanOmni2.5–8 (Omni2.5) bead chip to validate CNVs located genome-wide (Illumina Inc, CA, USA). CNVs were called with CNVpartition using Illumina Genotyping module v1.8.4. To confirm, the logR plot of each call was visually inspected using Illumina Karyostudio 1.3.

Deletions on chromosome 21 were validated using custom-made high density chromosome 21 aCGH platform (Agilent Technologies, CA, USA). Among the 59 deletions chosen for validation, 23 were previously reported in DGV. Among the 5 duplications chosen for validation, 4 were previously reported in DGV. We successfully validated 91.5% of deletions (54/59 attempted) and 100% of duplications (5/5 attempted), demonstrating that our data quality is high. All non-validated CNVs were excluded from final analyses.

## RESULTS

Copy number variation was assessed in a collection of 210 cases and 242 controls of EA using Affymetrix Genome-wide SNP 6.0 arrays with rigorous quality control and validation as described in Methods. We identified 541 deletions (253 in cases, 288 in controls) and 383 duplications (177 in cases, 206 in controls) genome-wide. Among the 541 deletions, 400 (194 in cases and 206 in controls) were previously reported in the DGV and 141 (59 in cases and 82 in controls) were unreported. Of the 383 duplication events, 310 (147 in cases and 163 in controls) were previously reported in DGV and 73 were novel (30 in cases and 43 in controls). The complete list of CNVs identified along with the DGV status is provided in Tables S4–S7.

### Contribution of Common Copy Number Variants to DS-associated AVSD

We first tested the hypothesis that an increased burden of common CNVs might account for the 2000-fold increased risk of AVSD among individuals with DS. We did not identify any significant difference in the number or size of common deletions or duplications between cases and controls. Burden analyses of large common deletions and duplications also failed to identify any significant differences between our cases and controls (Table 1). Region-based association analyses did not identify any specific gene to be significantly associated with case CNVs after correcting for multiple testing. None of the common CNVs showed a significant association after correction for multiple testing. Under an additive model, using 210 cases and 242 controls we had 80% power to find any common marker that explained more than 4% of the variance, or had an odds ratio greater than ~2.0 at a  $p < 0.00019$  (i.e. 0.05 multi-tested for 263 tests, corresponding to the total number of common CNVs identified among cases and controls). Our findings provide strong evidence that common CNVs do not explain the increased risk of AVSD in children with DS.

### Contribution of Rare Copy Number Variants to DS-associated AVSD

We next asked whether an increased burden of rare CNVs might explain the increased risk of AVSD in DS. We observed an elevated frequency of large rare deletions in cases compared to controls ( $p < 0.01$ , Table 2). Furthermore, these large rare deletions in cases spanned genes more often than did those in controls ( $p < 0.007$ , Table 2). In contrast, we did not observe any statistically significant differences in large, rare duplications between cases and controls.



## Gene Enrichment Analysis

Since the burden analyses revealed an excess of large, rare deletions spanning genes in cases, we next asked whether specific pathways were differentially affected in cases as compared to controls. We performed a gene-set enrichment analysis of different pathways that contribute to normal heart development and/or function. These include Notch, WNT, Jak-Stat, Hedgehog, Epithelial to Mesenchymal transition (EMT), Angiogenesis, Ciliome, and Histone-modifying genes.<sup>11,21,28</sup> We observed a marginally significant trend for enrichment of all deletions and of rare deletions affecting the ciliome pathway in cases as compared to controls (Deletions intersecting ciliome genes are provided in Table S8, complete list of genes in Table S2). Interestingly, this finding parallels the results of a recent gene expression study which identified a statistically significant enrichment of differentially expressed ciliome genes in their DS+AVSD cases as compared to DS controls with a structurally normal heart (Table 3).<sup>21</sup> None of the other pathways tested showed a significant enrichment in our cases as compared to controls. No statistically significant enrichment was observed for duplications in any of the pathways tested.

## Overlap of Case-associated CNVs with Previous Non-DS, Disomic Heart Studies

To understand whether the observed CNV burden among our cases overlapped with that identified in studies of CHD among non-DS cases, we compared our results to published studies of all types of CHD (Table 4).<sup>5,8,10,29-31</sup> We found three rare deletions in our cases at *2q13*, *8p23.1* and *22q11.2*, which overlapped with CNVs identified among isolated non-syndromic CHD patients.<sup>9,29,32</sup> Though we did not see an increased burden of rare duplications in cases compared to controls, we did find two large duplications at *8p23.1* and one at *16p13.11* in cases that overlapped rare CNVs observed in other CHD phenotypes (Table 4).<sup>9,10,30</sup> Among the rare CNVs observed in our cases, only one of the euploid patients with an identified CNV had an AVSD as a part of their phenotypic spectrum (Table 4). In addition, we observed a number of CNVs predominantly in DS+NH controls, which have been previously reported in disomic CHD patients (Table S9).

## DISCUSSION

Approximately 20% of children with Down syndrome (DS) are born with a life-threatening atrioventricular septal defect (AVSD), reflecting a 2000-fold increased risk of AVSDs as compared to the disomic population. Our goal was to uncover genetic variants that distinguish children with DS and an AVSD from those with DS and a structurally normal heart. This is the largest study of this condition to date, surpassing the 1989 Baltimore-Washington heart study which considered 190 cases (DS+AVSD).<sup>14</sup> Here we genetically characterized a carefully phenotyped collection of 210 DS+AVSD cases and 242 DS+NH controls. Our study had sufficient power to detect common variants that explained more than 4% of the variance, or had an odds ratio greater than ~2. Our findings provide strong evidence that common copy number variants do not account for the increased risk of AVSD in children with DS.

Recently Sailani et al.<sup>20</sup> conducted a case-control SNP and CNV association study on a smaller collection of individuals with DS and a variety of CHD phenotypes (cases, n=187; controls, n=151). Their genome-wide association and the SNP based interaction studies failed to detect a significant association in any of the CHD groups. Interestingly, a CNV association analysis using 55 DS cases with AVSD and 53 DS-without CHD controls identified two small CNVs (< 5kb) enriched in cases compared with controls (relative risk ~ 2.2). Both the CNVs were located on previously known CHD loci on chromosome 21 and were confirmed in a replication cohort. The lower marker density of our genome-wide genotyping platform precluded us from replicating these findings. Our strict filtering criteria were designed to ensure accurate detection of large CNVs and we therefore cannot exclude the hypothesis that small, common CNVs that contribute to the risk of AVSD remain undiscovered. We intend to present a replication of SNP and CNV association study reported by Sailani et al. in a future manuscript.

Our genome-wide analysis of CNVs revealed a significant burden of large rare deletions in cases compared to controls. Moreover, the case deletions were more likely to include genes as compared to controls. These findings are consistent with those of Ackerman et al.<sup>4</sup> who conducted targeted sequencing of 26 candidate genes in a subset of samples analyzed here. They found a significant excess of deleterious missense variants in 19% of cases compared with 2.6% of controls ( $p < 0.0001$ ). Many of the potentially damaging variants occurred within VEGF-A pathway genes. Functional studies demonstrating a potentially pathogenic allelic interaction between AVSD-associated gene *CRELD1* and VEGF-A genotype further substantiate the complex interactions among different genetic modifiers to AVSD pathology.<sup>15,33,34</sup> Together these data suggest an etiology whereby the enormous increase in risk of AVSD in children with DS is multifactorial, being influenced by many different genes and environmental exposures in addition to the substantial risk from trisomy 21.<sup>2,22</sup>

We performed pathway analysis to determine the nature of pathways affected by the large, rare deletions observed in our cases. Previous reports from both DS populations<sup>18,21</sup> and disomic populations<sup>8-10</sup> have reported the contribution of different signaling pathways to CHD pathogenesis. Our genetic analysis revealed marginally significant 2-fold enrichment of deletions in ciliome genes in cases (5.2%) as compared to controls (2.9%). Of note, our strongest genetic finding (ciliome) was identical to the most significant gene expression finding reported by Ripoll et al.<sup>21</sup> Using differential gene expression analyses on a subset of DS patients, they identified a significant enrichment for cilia genes in their AVSD and atrial and ventricular septal (ASD [MIM 108800], VSD [MIM 614429])<sup>12</sup> CHD groups. This is intriguing since the association between CHD and defective cilia is well established in the euploid population, in particular laterality defects.<sup>35</sup> In fact, there is a 200-fold higher prevalence of CHD among patients with primary ciliary dyskinesia (PCD [MIM 244400])<sup>12</sup> when compared to the general population, emphasizing the significance of intact cilia for normal heart development.<sup>36</sup> An intact cilium is required for Hedgehog (Hh) signaling for normal embryonic development in vertebrates.<sup>37</sup> Recent developmental studies demonstrated that the cilia-based Hh signaling in the second heart field is required for development of the dorsal mesenchymal protrusion which is a critical component of complete atrioventricular septation. Disruption of Hh signaling could result in AVSD-associated clinical features.<sup>38,39</sup>



Recent studies on mouse models implicate attenuated Hh signaling to be a significant underlying factor behind many DS-associated phenotypes. For example, trisomic granule cell precursors in the cerebellum are less responsive to Hh signaling than their euploid counterparts. This deficit can be rescued using a sonic hedgehog agonist, suggesting the importance of Hh signaling in DS-associated pathology as well as a possible candidate for therapeutic intervention.<sup>40</sup> Our results complement the above studies and suggest an important role for ciliome/hedgehog-genes in the elevated CHD risk in a trisomic background.

We also identified large rare CNVs in our cases at genomic loci previously associated with non-syndromic CHD (Table 4).<sup>9,10,30,32</sup> These previous reports indicate variable penetrance of CHD phenotypes, although the specific defects differ from those seen most often in the DS population. However, the overlap validates the idea that regions identified in DS are relevant to CHD in euploid population. These variations may be easier to observe in the sensitized DS background.

In summary, our data provide strong evidence that large rare deletions increase the risk of DS-associated AVSD, while large common CNVs do not account for the 2000-fold elevated risk of DS-associated AVSD. This is the single largest genetic study of a well-defined homogeneous cardiac phenotype (extreme ends of the phenotypic spectrum) in ethnically matched individuals with DS. The stringent filtering we performed to reduce false positives supports the accurate detection of large CNVs, but may miss smaller CNVs, thus underestimating the total impact of CNVs on DS-associated AVSD. Replication studies with larger sample sizes and more detailed genomic analyses, such as whole-exome or whole-genome sequencing, would be required to get a true assessment of the contribution of rare variants and the intersected genes we have identified to CHD pathology. Launching of DS-Connect (<https://dsconnect.nih.gov/>), the new Down syndrome registry may facilitate DS-associated research in this regard. Given that the prevalence of AVSD, like other forms of CHD varies by sex and ethnic/racial background, a complementary approach would be to test a population at higher risk, such as individuals with DS of African-American ancestry.<sup>22</sup> To confirm the association of ciliome genes to AVSD pathology, large genetic studies and direct functional studies, such as in a mouse model of DS, will be required. A more comprehensive approach to elucidate how the diverse genetic, epigenetic and environmental factors converge at a functional level to create distinct heart phenotypes is of paramount essence to identify potential therapeutic targets and can be expected to influence the life-long medical treatment of individuals with DS.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**Table 1**

Results of burden analyses of common (&gt;0.01) CNVs &gt; 100kb

Test	Cases	Controls	<i>P</i> value <sup>a</sup>
Number of deletions	98	140	
Deletion rate per person	0.46	0.56	0.96
Proportion of individuals with at least 1 deletion	0.41	0.47	0.91
Number of genes spanned by deletions	2.70	3.63	0.98
Number of deletions with at least 1 gene	0.37	0.44	0.95
Number of duplications	78	111	
Duplication rate per person	0.37	0.45	0.92
Proportion of individuals with at least 1 duplication	0.33	0.38	0.90
Number of genes spanned by duplications	2.03	2.24	0.69
Number of duplications with at least 1 gene	0.28	0.34	0.94

<sup>a</sup>*P* values in each category were estimated from 1 million permutations.

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**Table 2**

Results of burden analysis of rare (&lt;0.01) CNVs &gt; 100kb in size

Test	Cases	Controls	<i>P</i> value <sup>a</sup>
Number of deletions	94	75	
Deletion Rate per person	0.45	0.30	0.01
Proportion of individuals with at least 1 deletion	0.32	0.26	0.10
Number of genes spanned by deletions	0.87	0.37	0.007
Number of deletions with at least 1 gene	0.19	0.17	0.25
Number of duplications	67	76	
Duplication Rate per person	0.32	0.32	0.49
Proportion of individuals with at least 1 duplication	0.26	0.24	0.33
Number of genes spanned by duplications	1.02	1.23	0.71
Number of duplications with at least 1 gene	0.22	0.21	0.46

<sup>a</sup>*P* values in each category were estimated from 1 million permutations.

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**Table 3**

Results of gene set enrichment analyses (*p*-values based on 1e6 permutation tests)

Pathway Tested	Notch	Wnt	Jak-Stat	Hedgehog	Angiogenesis/cardiogenesis	Ctrome	Epithelial-to-Mesenchymal (EMT)	Histone Modifying genes
Deletions	0.60	0.26	1.0	0.95	0.73	0.10	1.0	0.91
Deletions (<0.01)	0.64	0.36	1.0	0.96	0.89	0.13	1.0	0.93
Duplications	0.68	0.23	0.70	1.0	1.0	0.75	1.0	0.81
DE genes in AVSD <sup>a</sup>	0.62	0.28	0.26	0.91	0.70	0.00017	0.67	ND

<sup>a</sup>Included here are results from Ripoll et al.<sup>21</sup> that indicate the same pattern of enrichment of differentially expressed (DE) genes among their case and control groups; ND: Not Determined.

Table 4

Rare CNVs observed only among cases, that overlap with CNVs identified among non-DS CHD cohorts published elsewhere

Genomic location in DS+AVSD cases (size of CNV)	DS+AVSD CNV	Count in DS+AVSD cases	Non-DS CHD CNV	Non-DS CHD phenotypes observed <sup>a</sup>	Genes intersected <sup>b</sup>
<i>2q13</i> (583.3 - 559.3kb)	del	2	del	CoA, cardiovascular <sup>9,32</sup>	<i>MIR4267</i>
<i>8p23.1</i> (79.1kb)	del	1	del	TOF, AVSD, cardiovascular <sup>9,32</sup>	<i>XKR5, DEFB1</i>
<i>22q11.21</i> (957.8kb)	del	1	dup	VSD, tricuspid valve dysplasia, right ventricular hypoplasia <sup>29</sup>	<i>PPM1F, TOP3B</i>
<i>2p21</i> (584.5kb)	dup	1	dup	DORV, cardiovascular <sup>9,32</sup>	<i>LINC01121, SRBD1, PRKCE</i>
<i>2q12.3</i> (784.7kb)	dup	1	del	LS-CHD <sup>31</sup>	<i>LIMS1</i>
<i>8p23.1</i> (251.2 - 524.7kb)	dup	2	dup	BAV, VSD <sup>9</sup>	<i>CTSB, DEFB134, DEFB136, DEFB135</i>
<i>15q13.3</i> (514.7kb)	dup	1	del	IL, PDA, PS, cardiovascular <sup>9,32</sup>	<i>CHRNA7</i>
<i>16p13.11</i> (1.54Mb)	dup	1	del, dup	ASD-SEC, HLHS, TOF, CoA <sup>9,10,30</sup>	<i>MPV17L, C16orf45, KIAA0430, NDE1, MIR484, MYH11, ABCC1, ABCC6</i>

<sup>a</sup>The following abbreviations are used: ASD-SEC- Atrial septal defect secundum; AVSD- Atrioventricular septal defect; BAV- Bicuspid aortic valve; CoA- Coarctation of the aorta; DORV- Double outlet right ventricle; HLHS- Hypoplastic left heart syndrome; IL- Left isomerism; LS-CHD- Congenital left sided heart defect; PDA- Patent ductus arteriosus; PS- Pulmonary valve stenosis; TOF- Tetralogy of fallot; VSD- Ventricular septal defect; del- deletion; dup- duplication.

<sup>b</sup>Genes that are commonly intersected by the current study and the corresponding references