Integration of Large-scale Genomic Data Sources with Evolutionary History Reveals Novel Genetic Loci for Congenital Heart Disease

Running title: *Fotiou et al.; Ohnologs in congenital heart disease*

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Abstract:

Background - Most cases of congenital heart disease (CHD) are sporadic and non-syndromic, with poorly understood aetiology. Rare genetic variants have been found to affect the risk of sporadic, non-syndromic CHD, but individual studies to date are of only moderate sizes, and none to date has incorporated the ohnolog status of candidate genes in the analysis. Ohnologs are genes retained from ancestral whole-genome duplications during evolution; multiple lines of evidence suggest ohnologs are over-represented among dosage-sensitive genes. We integrated large-scale data on rare variants with evolutionary information on ohnolog status to identify novel genetic loci predisposing to CHD.

Methods - We compared copy number variants (CNVs) present in 4,634 non-syndromic CHD cases derived from publicly available data resources and the literature, and >27,000 healthy individuals. We analysed deletions and duplications independently and identified CNV regions exclusive to cases. These data were integrated with whole-exome sequencing data from 829 sporadic, non-syndromic patients with Tetralogy of Fallot. We placed our findings in an evolutionary context by comparing the proportion of vertebrate ohnologs in CHD cases and controls.

Results - Novel genetic loci in CHD cases were significantly enriched for ohnologs compared to the genome (χ^2-test, $p<0.0001$, OR=1.253, 95% CI:1.199-1.309). We identified 54 novel candidate protein-coding genes supported by both: (i) CNV and whole-exome sequencing data; and (ii) ohnolog status.

Conclusions - We have identified new CHD candidate loci, and show for the first time that ohnologs are over-represented among CHD genes. Incorporation of evolutionary metrics may be useful in refining candidate genes emerging from large-scale genetic evaluations of CHD.

Key words: congenital heart disease; copy number variant; single nucleotide polymorphism; ohnologs, whole-exome sequencing

Non-standard Abbreviations and Acronyms

CHD Congenital heart disease

CNV Copy number variant

DEL Deletion

DUP Duplication

SNV Single nucleotide variant

SSD Small-scale duplication

TOF Tetralogy of Fallot

WES Whole exome sequencing

WGD Whole-genome duplication

Background

Congenital heart disease (CHD) is the most prevalent birth defect in humans, occurring in approximately 8 per 1000 live births, and consisting of malformation of the heart and/or the great vessels[1]. Around 20% of all CHDs can be attributed to chromosomal imbalances such as Down and Turner, and 22q11 deletion syndromes; around 80% occur as sporadic, non-syndromic CHD. In such cases, CHD behaves overall as a genetically complex trait with moderate heritability. Previous genome-wide investigations into CHD have found evidence for rare causative copy number variants (CNVs) and single nucleotide variants (SNVs); and associations with common SNVs in GWAS[2-6]. It has been estimated in previous studies that several hundred genes may be involved in polygenic CHD susceptibility; therefore, many remain to be discovered[7].

CNVs are 1 kilobase (kb) to several megabase (Mb) sized regions of duplication (DUP) and deletion (DEL) in the genome. A 2014 meta-analysis of CNVs in 1694 non-syndromic CHD cases identified 79 chromosomal regions in which 5 or more CHD cases had overlapping imbalances[5]. The estimated prevalence of pathogenic CNVs in non-syndromic CHD patients is 4-14%, whereas in syndromic CHD patients it is 15-20% (the most common being 22q11 deletion syndrome)[3, 8, 9]. There are multiple mechanisms by which a CNV may lead to disease including the disruption of chromosome structure, alteration of gene expression due to disruption of regulatory elements, and changes of the relative amounts of dosage-sensitive genes[10].

The dosage-balance model postulates that, for genes that are in stoichiometric relationships (for example forming protein complexes with other genes), any perturbation in their relative ratios will tend to be deleterious[10]. In the early course of vertebrate evolution, around 500 million years ago, two whole-genome duplications (WGD), during which gene stoichiometry throughout the genome was preserved, as all genes were duplicated, took place.

Periods of gene loss followed each of these events, resulting in the retention of some WGD paralogs in the genome (termed "ohnologs") and the loss of others. The dosage-balance model would predict that ohnologs should be enriched for dosage-sensitive genes. [11] Ohnologs, of which there are around 7,000 in the human genome, have indeed been shown to exhibit characteristics consistent with dosage-sensitivity: for example, ohnologs are enriched for haploinsufficient genes[11,12]; and Makino *et al*. reported, based on CNV data in healthy individuals from the Database of Genomic Variants (DGV), that genomic regions (~2Mb in size) near ohnologs are CNV deserts, indicating that those regions are dosage-balanced[13].

The formation and fixation of gene duplications within the genome is subject to different evolutionary mechanisms –small scale duplications (SSD) involving relatively few genes, and WGD. A strong relationship between the evolutionary mechanism of duplication and phenotypic consequences, including heritable diseases, has been previously shown[14 15, 16]. Ohnologs have a significant association with certain human genetic diseases; for example 12 out of 16 reported candidate genes within the Down syndrome critical region (21q22.12, 21q22.13 and 21q22.2) are dosage-balanced ohnologs[11]. By contrast, genes arising from SSDs lack enrichment for disease association [17]. In addition, ohnologs are enriched for genes involved in signalling and gene regulation, key cardiovascular developmental processes[11]. These considerations led us to hypothesise that ohnologs may be enriched among CHD causative genes.

We tested this hypothesis in a meta-analysis of CNV data including 4,634 non-syndromic CHD cases, and integrated these data with a whole-exome sequencing (WES) study of 829 cases of Tetralogy of Fallot (TOF), the commonest cyanotic CHD phenotype, which has been previously shown to have a significant aetiological contribution from CNVs[6]. These were compared with control data, which were derived from large-scale genomic resources[18-21].

Methods

The appropriate institutional review bodies approved all recruitment of human participants in this study. The study corresponded with the stipulations of the Declaration of Helsinki, and all participants (or their parents, if affected probands were children too young to themselves consent) provided informed consent. Data from consortia were accessed subject to the applicable data-sharing agreements. Summary data, analytic methods, and summary study materials will be made available to other researchers for purposes of reproducing the results or replicating the analyses reported here, on request to the corresponding authors. Full Materials and Methods are available in the Data supplement of the article.

Results

Update of CHD CNV dataset and generation of control CNV dataset

We updated the previous meta-analysis of CNVs in non-syndromic CHD cases[5], in a further 2,882 non-syndromic CHD cases from DECIPHER, ECARUCA and ISCA databases and further published studies investigating the role of CNVs in CHD[4, 20, 22-32]. The updated CHD case CNV dataset consists of 4,634 unrelated individuals of different ancestries (Table 1). The outline workflow to identify candidate genes is shown in Figure 1. Filtering of the CHD case population against DECIPHER known microdeletion/microduplication syndromes resulted in 224 cases being removed; this left 4,410 CHD cases with 3,362 DEL CNVs and 2,540 DUP CNVs which were used for further analysis (Supplementary figure 1).

A control CNV dataset was generated by acquiring CNV data from individuals not explicitly identified as having a developmental disorder, who were enrolled in the 1000 Genome Project Phase 3, DGV, DECIPHER, and published studies[21, 27, 28, 33, 34]. The control CNV dataset

resulted in 256,511 DEL CNVs, 84,343 DUP CNVs and 6,403 BOTH CNVs, i.e. either DEL or DUP. gnomAD CNVs[35] were incorporated into the analysis as they became available, and resulted in an additional 51,420 DUP CNVs and 198,611 DEL CNVs.

Comparison of CHD CNV regions with control CNV regions

All CHD DEL and DUP CNV regions (coordinates hg19) were compared against control DEL and DUP CNV regions, respectively. Any CHD CNV regions overlapping control CNV regions were excluded. As a result, we identified DEL and DUP CNV regions only seen in non-syndromic CHD cases. The genes located in those regions were annotated using the Ensembl database. There were a number of genes that already had an assigned phenotype (OMIM)[36]; among these, 59 had been previously associated with CHD pathogenesis such as *ZIC3, NKX2-6, GATA4, JAG1, GJA1* and *TBX5*. All genes with OMIM assigned phenotypes were excluded from further analysis.

Novel genes found in the CNV regions only seen in CHD cases were then compared to an in-house list of 12,771 genes with novel or rare SNVs (either absent from ExAC or with frequency of <0.01) from WES data in 829 TOF cases[6]. Genes supported by both CNV and WES data were included for further analysis. In total, 3,082 genes in DEL CNVs, 4,297 genes in DUP CNVs and 3,068 in BOTH CNVs (i.e. genes found in DEL and DUP CNVs) were also found in the TOF WES data with either high (nonsense variants, frameshift, splice variants) or medium (missense, splice variants) impact SNVs. This intersection of CNV and WES data led to an overall reduction of ~60% in the number of candidate genes for CHD (Figure 2).

Ohnologs are highly enriched in CHD cases whereas small-scale duplications (SSD) and singleton genes are not.

Ohnologs (N=7,023) were identified using data from Singh *et al* (2015)[37], available at http://ohnologs.curie.fr/. SSDs (N=7,014) were extracted from Ensembl gene trees[12]. Any remaining genes that were neither found in the ohnolog dataset nor identified as having a direct paralog were considered for the purpose of this study to be singletons. The frequencies of ohnologs, SSDs and singletons among the candidate CHD genes were compared with their frequency in the human genome. Novel genes supported by the CNV data in CHD cases were found to be enriched for ohnologs (14.65% vs 12.05%, χ^2 test, p<0.0001, OR=1.253, 95% CI: 1.199-1.309,) (Figure 3A). There were no differences in SSDs (Figure 3B) and an under-representation for singletons (Figure 3C) compared to the human genome. There was a 2.3-fold increased enrichment of ohnologs in the genes supported by both CNV and WES data in CHD cases (χ^2 test, p< 0.0001, OR=3.751, 95% CI: 3.574-3.937). In this instance, SSDs were also enriched in CHD cases compared to the human genome (χ^2 test, p< 0.0001, OR=1.437, 95% CI: 1.356-1.905). However, ohnologs were 2-times elevated compared to SSD genes (33.94% versus 16.43%). Additionally, we assessed our methodology by applying it to a group of genes with strong *a priori* evidence for pathogenicity. The crowd-sourced Genomics England "PanelApp" gene list for CHD (available at https://panelapp.genomicsengland.co.uk/panels/212/), which represents a consensus view of causative genes, was highly enriched for ohnologs (76.6% vs 12.05%, χ^2 test, p<0.0001, OR=23.89, 95% CI: 12.33-46.18). We therefore used ohnolog status as an additional candidate gene filter.

Candidate genes supported by both CNV and WES data of CHD cases

In order to further refine our candidate genes, we integrated additional genomic resources including the top 5% ExAC CNV intolerance scores, probability of haploinsuffieciency (pHI)[38], probability of loss-of-function intolerance (pLI)[19], and RNAseq expression data from mouse embryonic hearts[39]. Lastly, we incorporated ohnolog status. Genes from BOTH CNVs were analysed twice; once with the metrics used for genes from DEL CNVs and once with the metrics used for genes from DUP CNVs (Figure 4).

This led to the identification of 9 candidate genes from DEL and BOTH CNVs: *BRWD1, DIP2C, EYA3, GRB10, HNRNPC, RC3H2, SLIT3, TLN1* and *UBASH3B*. All 9 have the following properties: a) loss-of-function (LoF) variants in the WES data, b) found in DEL or BOTH CNV regions only seen in non-syndromic CHD cases, c) top 5% of ExAC DEL CNV intolerance scores, d) haploinsufficient (pHI≥0.65) and/or unable to tolerate LoF variants (pLI≥0.9), e) in the top 25% of highly expressed genes in mouse heart at E9.5 and/or E14.5, f) ohnolog, g) not present in the list of genes curated from the DDD study, h) not classified as human non-essential genes from the Sudmant study[21] (Table 2).

In addition, we found 45 candidate genes from DUP and BOTH CNVs, which had the following properties: a) high or medium impact SNVs in the WES data, b) found in DUP and BOTH CNV regions only seen in non-syndromic CHD cases, c) top 5% of ExAC DUP CNV intolerance scores, d) in the top 25% of highly expressed genes in mouse heart at E9.5 and/or E14.5, e) ohnolog, f) not present in the list of genes curated from the DDD study, g) not in the list of non-essential human genes from the Sudmant study[21] (Table 2).

Pathway enrichment and gene ontology analysis

We performed pathway enrichment analysis, using the Reactome Pathways Analysis tool[40], on the final 54 candidate genes supported by both CNV and WES data in non-syndromic CHD cases. This resulted in 11 pathways, where >5 of our candidate genes were involved in those pathways (Table 3). The top 3 pathways based on entities ratio (entities found/total entities) from Reactome were ‘’axon guidance’’, ‘’signalling by receptor tyrosine kinases’’ and ‘’cellular responses to external stimuli’’. In addition, Ingenuity pathway analysis (IPA) was also used with the only pathway including >5 genes being ‘’axon guidance signalling’’. Gene ontology analysis[41] of our candidate genes revealed 22 Gene ontology (GO) terms with particular enrichment on 4 GO terms; apoptotic process involved in luteolysis (GO0061364) (FDR corrected p-value= 0.0462), ventricular septum morphogenesis (GO0060412) (FDR corrected p-value=0.00921), ventricular septum development (GO0003281) (FDR corrected p-value=0.0343) and cardiac septum morphogenesis (GO0060411) (FDR corrected p-value=0.036). Both pathway and gene ontology analysis identified processes in which the genes *ABLIM1, ARHGEF12, SLIT2* and *SLIT3* are involved (Figure 5).

***SLIT2* and *SLIT3* variants in CHD**

SLIT2 and *SLIT3* were the most strongly supported genes found both by pathway analysis and gene ontology (Figure 5). Therefore, we further explored the phenotypic associations of these genes within our population.

In the present study, individuals with CNVs including *SLIT3* were reported with malformation of the heart and great vessels (n=1), VSD (n=1), atrial septal defect (n=3) and TOF (n=1) whereas individuals with *SLIT2* CNVs were reported with malformation of the heart and great vessels (n=1), VSD (n=2) and double outlet right ventricle (n=1). In addition, 20 missense

SNVs and 3 splice-site SNVs in *SLIT3* were found in 24 out of 829 TOF cases (2.9%, 95% CI: 1.91%-4.35%) and *SLIT2* had 12 missense SNVs and 2 splice-site SNVs in 14 out of 829 TOF cases (1.7%, 95% CI: 0.9%-2.9%). Probands were available for 12 *SLIT3* variants and 5 *SLIT2* variants which were confirmed by Sanger sequencing. Remaining variants were confirmed to have good coverage using Integrative Genomics Viewer (IGV). Samples from both parents were available for 9 probands with *SLIT3* variants and were analysed for variant inheritance; 2 of the 9 *SLIT3* variants tested were identified as *de novo*. Samples from both parents were available for 5 probands with *SLIT2* variants and were all either maternally or paternally inherited.

Discussion

Here, we performed a large-scale genome-wide meta-analysis study of non-syndromic CHD cases and identified 54 novel candidate genes for CHD. In addition to the large size of our dataset, we incorporated a novel analysis strategy incorporating the evolutionary origin of gene duplications. Ohnologs tend not to be observed in CNVs in vertebrate genomes[13]. Moreover, McLysaght *et al.* have also shown that ohnologs are significantly overrepresented in pathogenic CNVs associated with schizophrenia and neurodevelopmental disorders and that they are the possible cause of the deleterious effects of these rare pathogenic CNVs[14]. Here, we have shown for the first time that genes included in CNVs from CHD cases are significantly enriched for ohnologs compared to the human genome. Due to this significant association between ohnologs and CHD we incorporated ohnolog status in our methodology to identify novel genetic loci associated with CHD.

Pathway analysis and gene ontology analysis concordantly identified the *SLIT2* and *SLIT3* genes, which have recently received increasing attention in heart development[42]. In

vertebrates, the Slit family comprises of 3 known members (SLIT1-3), which are highly conserved secreted proteins that bind to Roundabout (ROBO) receptors. *SLIT2* and *SLIT3* are expressed during mouse embryonic development and interact with ROBO1 and ROBO2[43, 44]. They both encode proteins that consist of 4 LRR domains (leucine-rich repeats) also called D1-D4, 8 EGF repeats (epidermal growth factor) and 1 Laminin-G-like domain[43]. *Slit3* is expressed early in murine cardiogenesis in the cardiac crescent at E7.5 and linear heart tube at E8.5, later expression being restricted to the myocardium of the atria and OFT but not in the cardiac cushions or valves[44, 45]. *Slit2* is strongly expressed in the pharyngeal region at E8.5-E9.5 and later in the ventricular trabecular myocardium, epicardium, aortic semilunar valves and the mesenchyme surrounding the caval veins[44, 46]. *Slit3*$^{-/-}$ mutant mice exhibit ventricular septal defect (VSD), thick atrioventricular valves and hypoplastic posterior aortic semilunar leaflet with Slit2$^{-/-}$ mutant mice exhibiting bicuspid aortic valves and immature semilunar valves[44]. Robo1$^{-/-}$ mutant mice also exhibit VSD with down-regulation of NOTCH signalling, suggesting a potential mechanism for the underlying defects[44]. In another study, *Slit3*$^{-/-}$ mice also exhibit congenital diaphragmatic hernia[47]. Congenital heart defects ranging from bicuspid aortic valves to septal and outflow tract defects are therefore observed in variety of animal models in which genes involved in the SLIT/ROBO pathway have been inactivated.

We identified *SLIT2* and *SLIT3* heterozygous SNVs in 2.9% and 1.7% non-syndromic TOF patients, respectively. All SNVs were novel or rare (either absent from ExAC or with frequency of <0.01) and predicted with *in silico* tools to be pathogenic. The majority of the SNVs in both genes were missense, although a few splice site SNVs were also found. Their functional relevance will be of interest in future studies. Both genes were also present in CNVs in CHD patients with varying phenotypes including septal defects and malformation of the great

arteries. This is the first study to find an association of *SLIT2* and *SLIT3* with predisposition to human CHD, although, of note, a recent study identified *ROBO1* LOF SNVs in cases with TOF and septal defects[48].

Limitations

This study has certain limitations. The databases and publications included in this analysis incorporated different CNV detection platforms and analysis algorithms[49]. Irrespective of the method used in the studies identifying pathogenic CNVs, we only included studies that used the same genotyping method between cases and controls and confirmed their CNV detection by an additional methodology like qPCR, which to a degree addresses this limitation. Another potential limitation is the fact that during our filtering strategy we might have missed some important genes crucial for cardiac development. Though we accept that all important genes will not have been captured, we detected 54 strong candidate genes supported by multiple lines of evidence as having a causative role in non-syndromic CHD. Further research in much larger numbers of comprehensively genetically characterised CHD cases is warranted to establish the magnitude of the contribution of these genes, and to discover novel loci.

In conclusion, we show that ohnologs are over-represented in CHD cases and that incorporation of the evolutionary origins of genes is useful in refining candidate genes emerging from large-scale genetic evaluations of CHD. We also observe that CNVs and SNVs in *SLIT2* and *SLIT3* are associated with CHD involving TOF, septal defects and outflow tract defects, supporting the importance of the SLIT-ROBO signalling pathway in heart development.

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Table 1. Number of cases in previous and current meta-analysis studies as well as controls used in the current study.

Databases for CHD cases	Thorsson *et al.* study	Current study	Databases for controls	Current study
DECIPHER	279	1,252	**1,000 Genome phase 3**	2,504
ISCA	331	1,107	**DGV**	>6,430
Published literature	814	1,900	**gnomAD**	10,738
CHDwiki	328	328	**Published literature**	356
ECARUCA	0	47	**WTCC2**	~6,000
WES TOF (Page *et al.* 2019)	N/A	829	**DDD**	845

DECIPHER= Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources, ISCA= International Standards for Cytogenomic Arrays, ECARUCA= European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations, WES TOF= whole exome sequencing of Tetralogy of Fallot, DGV= Database of Genomic Variants, WTCC2= Wellcome Trust Case Control Consortium 2, DDD= Deciphering Developmental Disorders study

Table 2. Candidate genes supported by both CNV and WES data of CHD cases. 54 protein-coding candidate genes supported by CNV and WES data in non-syndromic CHD cases. All genes in the list are strict ohnologs. Data presented includes the Ensembl ID and the nature of the chromosomal imbalance for which the gene is either deleted (DEL), duplicated (DUP) or deleted/duplicated (BOTH).

ENS gene ID	Gene name	Chr	Start (hg19)	End (hg19)	DEL/DUP/BOTH	TOF LOF var count	TOF HIGH impact var count	TOF MED impact var count	Case CNVs overlap: FULL	Case CNVs overlap: PARTIAL
ENSG00000058668	ATP2B4	1	203595689	203713209	DUP	0	0	13	3	1
ENSG00000064042	LIMCH1	4	41361624	41702061	DUP	6	6	21	4	0
ENSG00000083223	ZCCHC6	9	88902648	88969369	DUP	1	1	8	4	0
ENSG00000092847	AGO1	1	36335409	36395211	DUP	0	0	2	3	0
ENSG00000094916	CBX5	12	54624724	54673886	DUP	0	0	1	2	0
ENSG00000101367	MAPRE1	20	31407699	31438211	DUP	0	0	1	5	0
ENSG00000108387	SEPT4	17	56597611	56618179	DUP	0	0	6	5	1
ENSG00000108389	MTMR4	17	56566898	56595266	DUP	0	0	9	10	0
ENSG00000109332	UBE2D3	4	103715540	103790053	DUP	2	2	0	3	0
ENSG00000109685	WHSC1	4	1873151	1983934	DUP	0	0	12	6	0
ENSG00000112079	STK38	6	36461669	36515247	DUP	0	0	3	5	1
ENSG00000113108	APBB3	5	139937853	139973337	DUP	1	2	8	8	0
ENSG00000122515	ZMIZ2	7	44788180	44809477	DUP	1	1	10	10	0
ENSG00000138641	HERC3	4	89442199	89629693	DUP	0	0	5	14	0
ENSG00000138835	RGS3	9	116207011	116360018	DUP	1	1	19	5	0
ENSG00000140403	DNAJA4	15	78556428	78574538	DUP	0	0	8	9	0
ENSG00000140497	SCAMP2	15	75136071	75165706	DUP	0	0	1	6	0
ENSG00000145147	SLIT2	4	20254883	20622184	DUP	0	0	14	3	0
ENSG00000146463	ZMYM4	1	35734568	35887659	DUP	0	0	12	26	1
ENSG00000179361	ARID3B	15	74833518	74890472	DUP	0	0	6	5	0
ENSG00000185658	BRWD1	21	40556102	40693485	DEL	5	5	16	10	1
ENSG00000151240	DIP2C	10	320130	735683	DEL	1	1	15	4	0
ENSG00000158161	EYA3	1	28296855	28415207	DEL	1	1	2	4	0
ENSG00000106070	GRB10	7	50657760	50861159	DEL	1	2	8	3	0

ENSG00000154127	UBASH3B	11	122526383	122685181	DEL	1	1	5	9	0
ENSG00000092199	HNRNPC	14	21677295	21737653	BOTH	1	1	2	6	0
ENSG00000056586	RC3H2	9	125606835	125667620	BOTH	1	1	8	3	1
ENSG00000184347	SLIT3	5	168088745	168728133	BOTH	2	2	21	3	0
ENSG00000137076	TLN1	9	35696945	35732392	BOTH	4	4	22	9	0
ENSG00000010017	RANBP9	6	13621730	13711796	BOTH	0	0	14	3	0
ENSG00000020577	SAMD4A	14	55033815	55260033	BOTH	0	0	11	8	0
ENSG00000033800	PIAS1	15	68346517	68483096	BOTH	0	0	4	7	1
ENSG00000064726	BTBD1	15	83685174	83736106	BOTH	1	1	7	5	0
ENSG00000083312	TNPO1	5	72112139	72212560	BOTH	0	0	4	5	0
ENSG00000091009	RBM27	5	145583113	145718814	BOTH	0	0	7	4	0
ENSG00000099204	ABLIM1	10	116190872	116444762	BOTH	3	3	14	12	0
ENSG00000100320	RBFOX2	22	36134783	36424473	BOTH	0	0	5	2	0
ENSG00000100330	MTMR3	22	30279144	30426855	BOTH	0	0	11	4	0
ENSG00000100592	DAAM1	14	59655364	59838123	BOTH	0	0	9	14	0
ENSG00000113649	TCERG1	5	145826874	145891524	BOTH	0	0	6	9	0
ENSG00000116191	RALGPS2	1	178694300	178889238	BOTH	0	0	1	2	0
ENSG00000120899	PTK2B	8	27168999	27316903	BOTH	0	0	8	4	0
ENSG00000127022	CANX	5	179105629	179157926	BOTH	0	0	10	2	0
ENSG00000135074	ADAM19	5	156822542	157002783	BOTH	2	2	10	7	1
ENSG00000137573	SULF1	8	70378859	70573150	BOTH	1	1	10	3	0
ENSG00000137962	ARHGAP29	1	94614544	94740624	BOTH	0	0	12	3	0
ENSG00000138107	ACTR1A	10	104238986	104262482	BOTH	0	0	2	2	1
ENSG00000155506	LARP1	5	154092462	154197167	BOTH	2	2	13	6	0
ENSG00000166747	AP1G1	16	71762913	71843104	BOTH	0	1	4	4	1
ENSG00000166888	STAT6	12	57489191	57525922	BOTH	0	0	7	5	0
ENSG00000180340	FZD2	17	42634925	42636907	BOTH	0	0	8	8	0
ENSG00000180776	ZDHHC20	13	21950263	22033509	BOTH	1	1	2	6	0
ENSG00000196914	ARHGEF12	11	120207787	120360645	BOTH	0	0	14	12	0
ENSG00000213079	SCAF8	6	155054459	155155192	BOTH	0	0	13	14	0

TOF= tetralogy of Fallot, var=variants, MED=medium impact

Table 3. Top pathways overrepresented in our 54 candidate genes.

Pathway tool	Pathway name	# Entities found	# Entities total	Entities ratio (%)
Reactome	Axon guidance	8	583	1.372212693
Ingenuity pathway analysis	Axon guidance signalling pathway	7	501	1.397206
Reactome	Signaling by Receptor Tyrosine Kinases	5	521	0.959692898
Reactome	Cellular responses to external stimuli	5	621	0.805152979
Reactome	Signaling by Interleukins	5	641	0.780031201
Reactome	Developmental Biology	8	1177	0.679694138
Reactome	Adaptive Immune System	6	998	0.601202405
Reactome	Cytokine Signaling in Immune system	6	1056	0.568181818
Reactome	Signal Transduction	15	3202	0.468457214
Reactome	Post-translational protein modification	7	1594	0.439146801
Reactome	Immune System	11	2662	0.41322314
Reactome	Metabolism of proteins	9	2354	0.382327952

- number

Figure Legends:

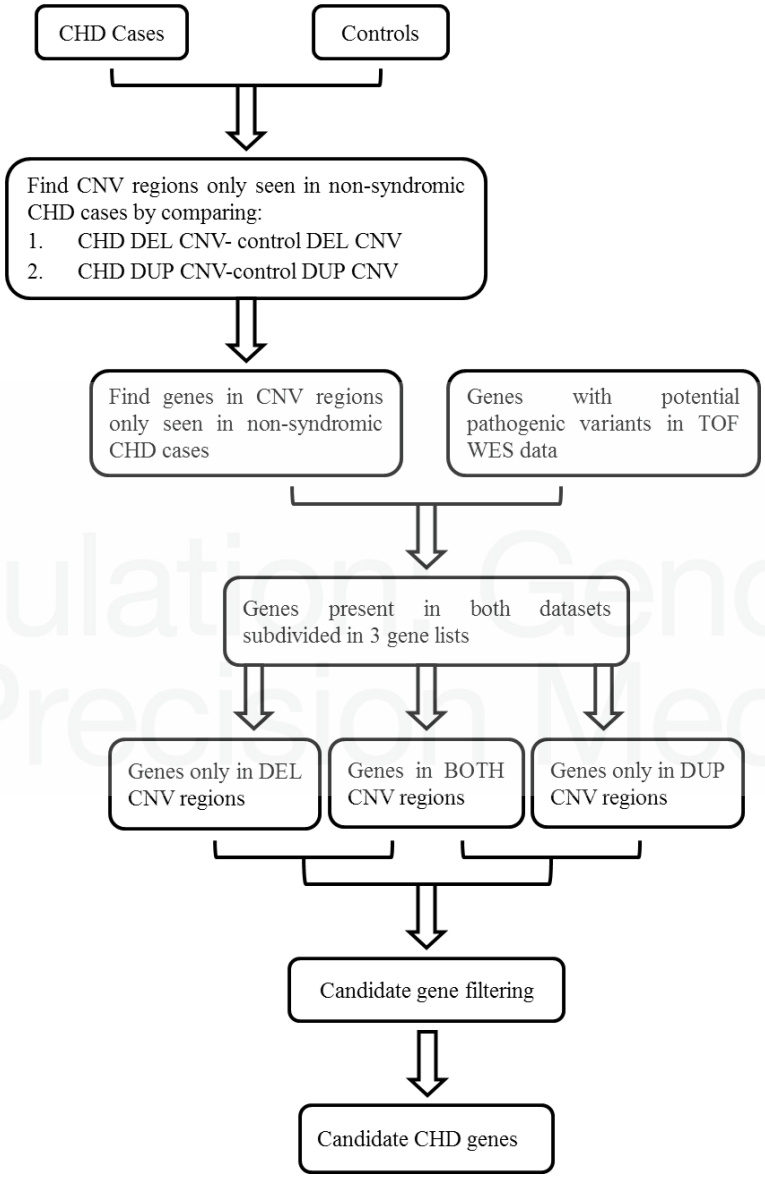
Figure 1: Overall methodology. Flowchart showing the methodology used to identify novel genetic loci for non-syndromic CHD cases. Potential pathogenic variants were novel or rare SNVs (either absent from ExAC or with frequency of <0.01). Candidate genes identified at the end of the workflow were subsequently analysed for ohnolog status.

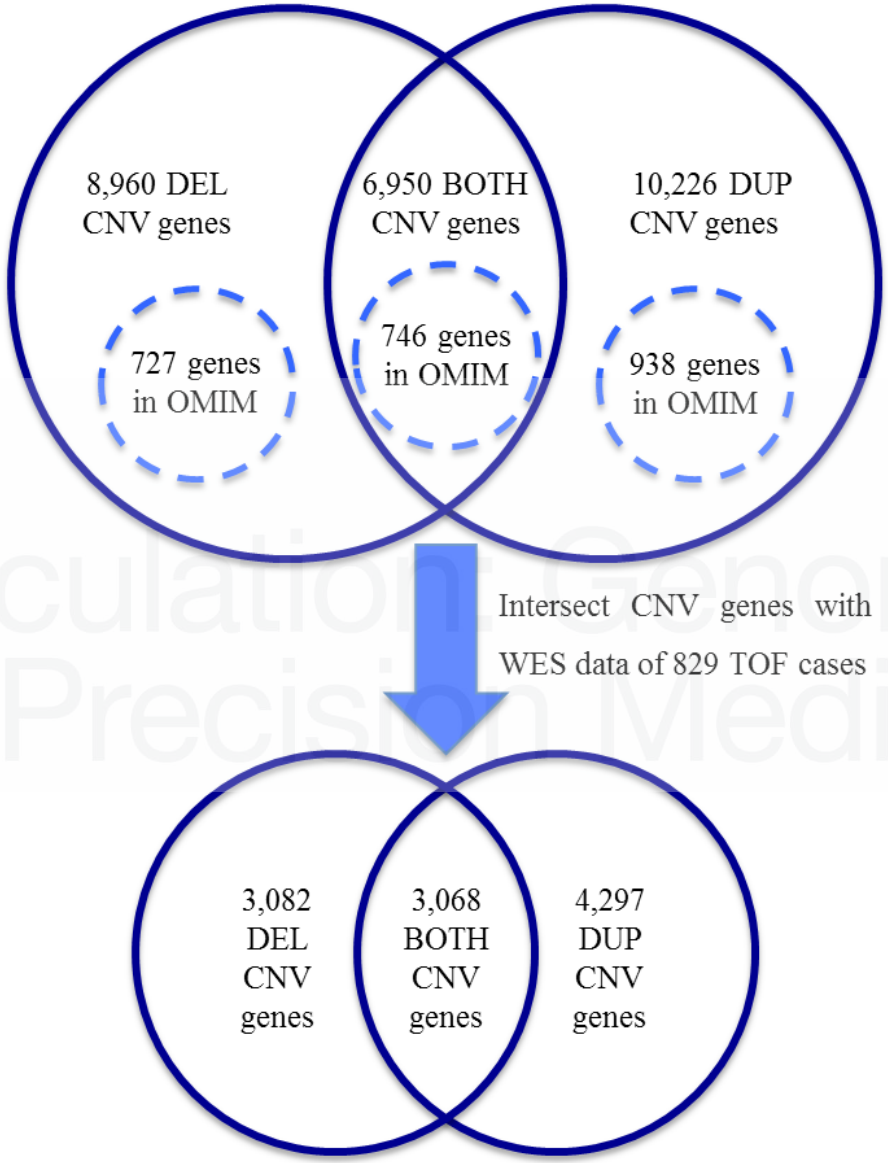
Figure 2: Intersection of CNV and WES data. Numbers of genes involved in the final stages of the workflow depicted in Figure 1 are shown. Genes with assigned phenotypes (circles with dashed line) were excluded from further analysis.

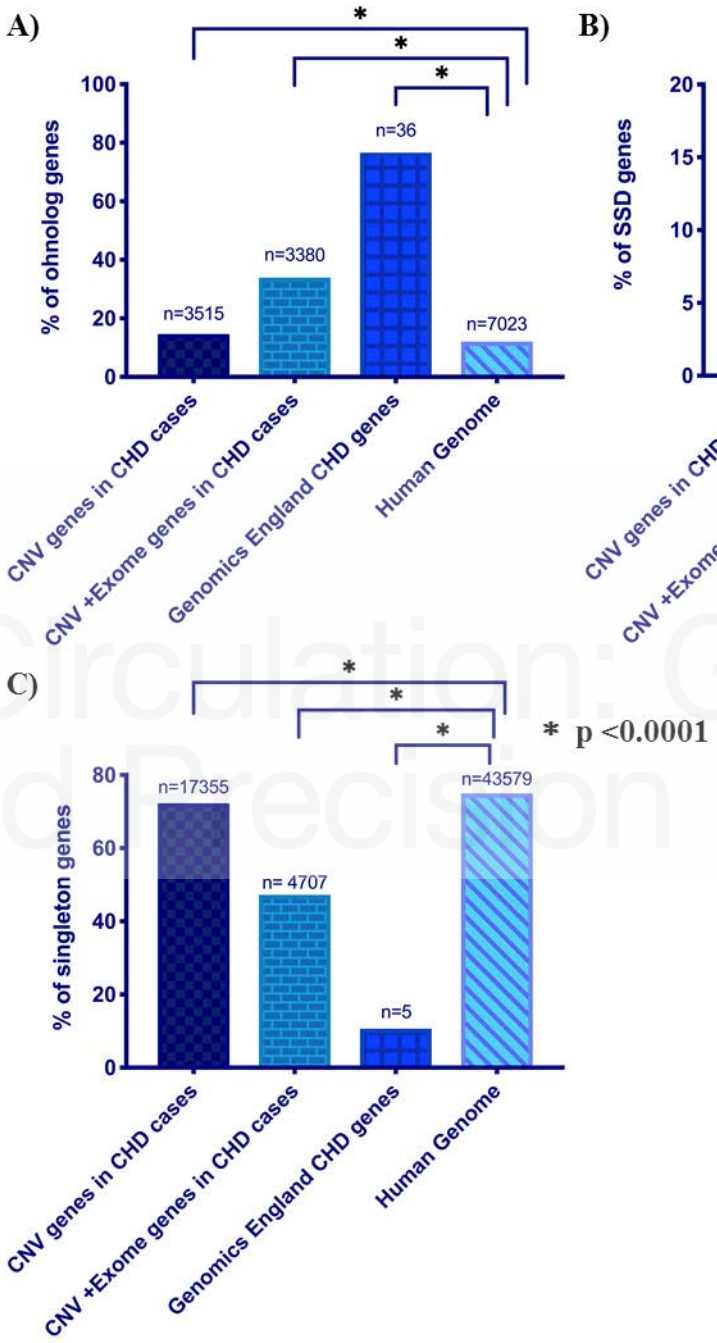
Figure 3: Ohnologs are enriched in CHD cases. Graphs show the percentage of genes that are **A)** ohnologs **B)** small scale duplications (SSD) and **C)** singletons. Statistical significance was tested using two-tailed Chi-square test with Yates's correction, $p<0.05$ was considered statistically significant.

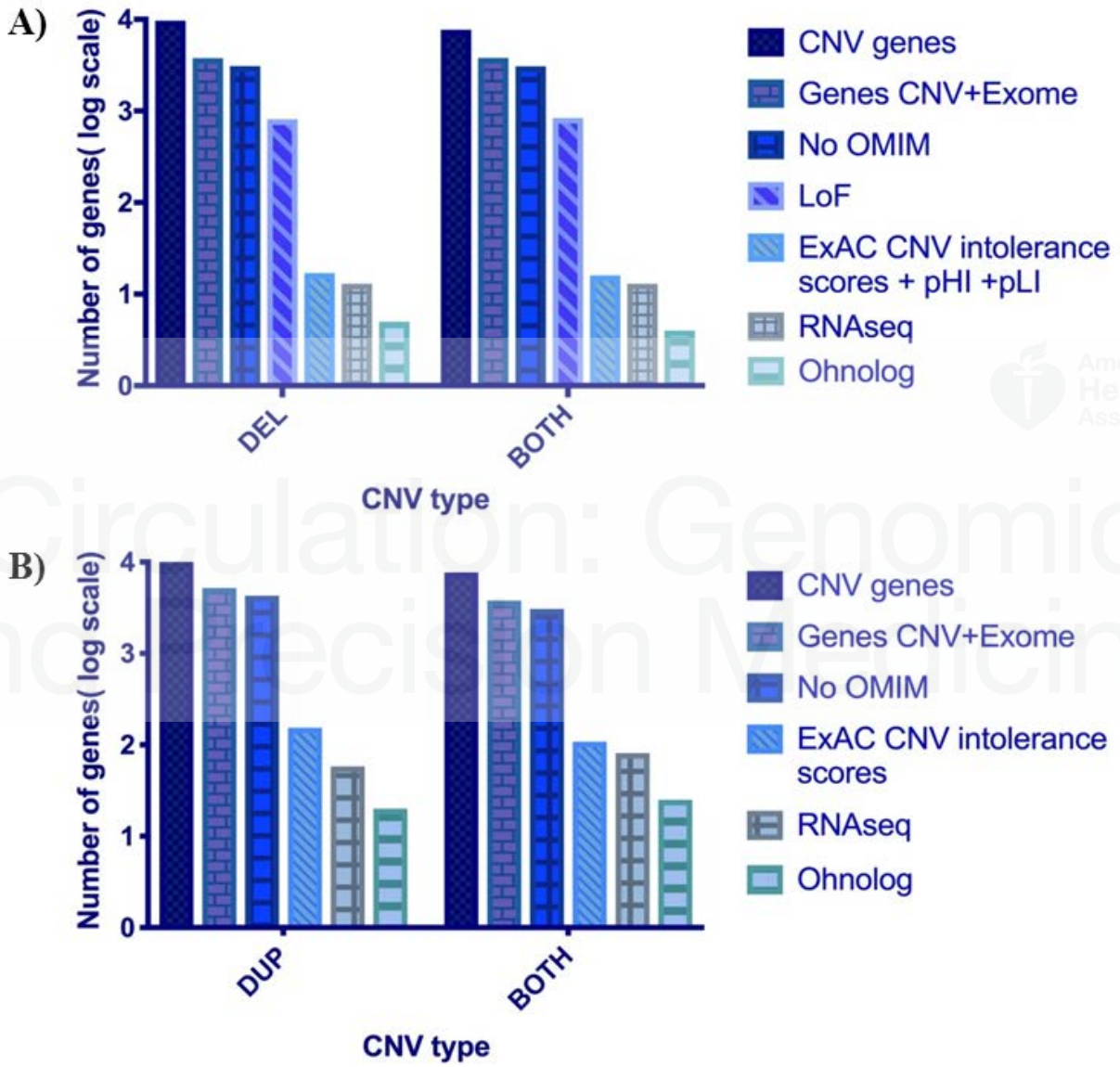
Figure 4: Filtering process using large-scale genomic data resources**.** Both graphs are in logarithmic scale and represent the consecutive filtering of the genes using the different metrics for **A)** deleted (DEL) and both CNV genes **B)** duplicated (DUP) and both CNV genes. There is approximately 70% reduction in the number of candidate genes when we apply the evolutionary duplication metric – ohnolog. Also, none of our candidates were present in the list of homozygous deleted genes (non-essential) from the Sudmant study as well as not present in the list of genes curated from the DDD study.

Figure 5: Genes in the top significant pathways and biological processes**.** *SLIT2* and *SLIT3* genes were supported by multiple lines of evidence.








A)
Number of genes(log scale)
0
1
2
3
4
DEL
BOTH
CNV type
CNV genes
Genes CNV+Exome
No OMIM
LoF
ExAC CNV intolerance scores + pHI +pLI
RNAseq
Ohnolog
B)
Number of genes(log scale)
0
1
2
3
4
DUP
BOTH
CNV type
CNV genes
Genes CNV+Exome
No OMIM
ExAC CNV intolerance scores
RNAseq
Ohnolog