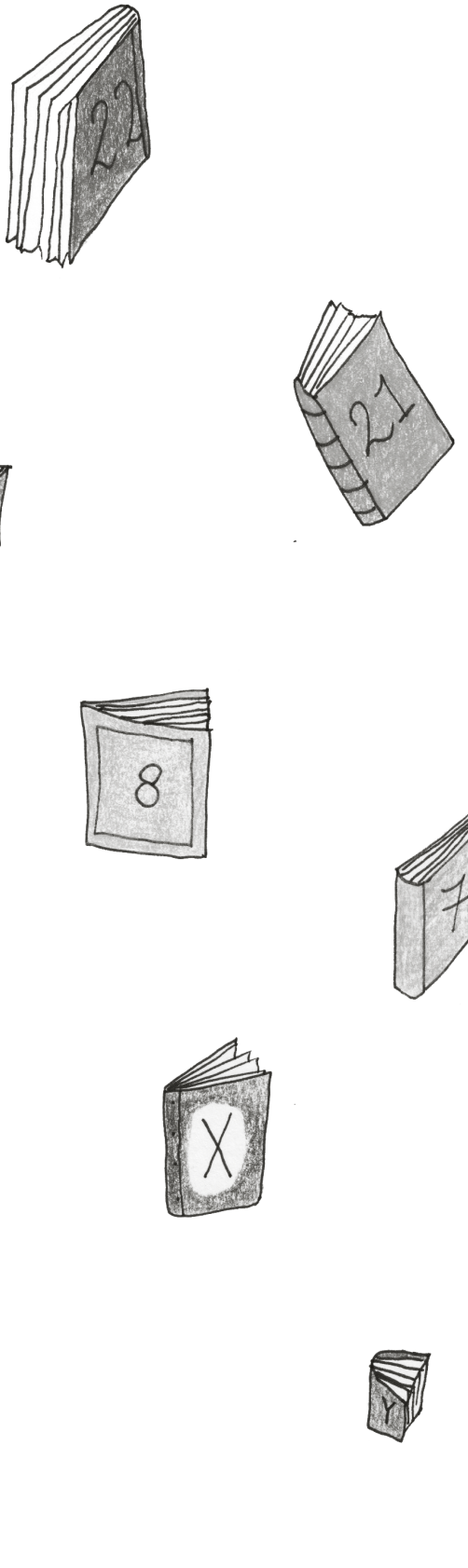
Are isolated heart defects really isolated? A prenatal view on submicroscopic genetics and brain development

Jansen, F.A.R.


22
21
12
8
7
X
Y

CHAPTER 3

Chromosomal abnormalities and copy number variations in fetal left sided congenital heart defects

Prenatal Diagnosis 2016; 36: 177–185

F.A.R. Jansen
M.J.V. Hoffer
C.L. van Velzen
S. Klingeman Plati
M.E.B. Rijlaarsdam
S.A.B. Clur
N.A. Blom
E. Pajkrt
S.L. Bhola
A.C. Knegt
M.A. de Boer
M.C. Haak

ABSTRACT

Objectives

To demonstrate the spectrum of copy number variants (CNVs) in fetuses with isolated left sided congenital heart defects (CHDs), and analyze genetic content.

Methods

Between 2003 and 2012, 200 fetuses were identified with left sided CHD. Exclusion criteria were chromosomal rearrangements, 22q11.2 microdeletion and/or extra-cardiac malformations (n=64). We included cases with additional minor anomalies (n=39), such as single umbilical artery. In 54 of 136 eligible cases, stored material was available for array analysis. CNVs were categorized as either (likely) benign, (likely) pathogenic or of unknown significance.

Results

In 18 of the 54 isolated left sided CHDs we found 28 rare CNVs (prevalence 33%, average 1.6 CNV per person size 10.6kb – 2.2Mb). Our interpretation yielded clinically significant CNVs in two of 54 cases (4%) and variants of unknown significance in three other cases (6%).

Conclusions

In left sided CHDs that appear isolated, with normal chromosome analysis and 22q11.2 FISH analysis, array analysis detects clinically significant CNVs. When counselling parents of a fetus with a left sided CHD it must be taken into consideration that aside from the cardiac characteristics, the presence of extra-cardiac malformations and chromosomal abnormalities influence the treatment plan and prognosis.

INTRODUCTION

Congenital heart defects (CHDs) are the most prevalent congenital malformations and occur in 6-8 per 1000 neonates[1]. The collective term CHD is used for a combined group of different cardiac lesions that can be anatomically heterogeneous. Abnormalities of the left ventricular outflow tract constitute roughly 10% of all neonatal CHDs and 20% of all CHDs detected prior to birth[2]. The spectrum of left sided CHDs varies from a bicuspid aortic valve, without clinical symptoms, to hypoplastic left heart syndrome (HLHS), leading to neonatal death if left untreated. Children with HLHS require a single ventricle palliation associated with considerable mortality and long-term morbidity[3]. Other left sided CHDs, like critical aortic valve stenosis or coarctation of the aorta, call for immediate postnatal intervention but, if treated in time, have a better prognosis.

CHDs in general present as either an isolated anomaly or as part of a malformation syndrome with chromosomal and/or extra-cardiac malformations. The rates of association with genetic syndromes vary, depending on the type of CHD. In children with HLHS it has been described that 5-12% of cases are associated with chromosomal or syndromic abnormalities[3;4], including Turner syndrome (monosomy X), 22q11.2 microdeletion syndrome and Jacobsen syndrome (11q deletion). Providing information about the association of CHDs with these syndromes is important when counselling future parents, given the influence of genetic conditions on surgical success and long-term outcome[5;6]. Most syndromes are detectable after birth and/or display multiple malformations. However, prenatal ultrasound cannot identify all signs of syndromes such as dysmorphic features, nor can it predict developmental delay. Therefore, prenatal genetic assessment by amniocentesis is routinely offered in cases with a fetal CHD. Chromosome analysis (karyotyping) using fetal cells can detect aneuploidy and chromosome rearrangements. However, it has a limited resolution (5-10 Mb), requires operator dependent microscopic analysis, and has a relatively slow turn-around time. Chromosome analysis can be supplemented by FISH analysis of the 22q11.2 region.

Recent studies suggest that instead of chromosome analysis, detection of copy number variants (CNVs) by array analysis could be more informative[7;8]. Array analysis has a much higher resolution and it is an automated molecular technique that detects chromosomal imbalances throughout the whole genome. It has proven to be clinically valuable in the pediatric population, especially in the setting of multiple malformations or developmental delay[9]. Experience gained from postnatal cohorts has encouraged the use of this diagnostic tool for prenatal diagnosis and it is increasingly performed if fetal abnormalities are diagnosed by ultrasound[10]. Nowadays, array analysis has become the

standard procedure for prenatal genetic analysis, and it is commonly preceded by rapid aneuploidy detection (RAD) to exclude common aneuploidies first[11-13].

The prevalence of clinically significant CNVs in prenatal CHDs is described in a few cohorts[14-20]. As mentioned, CHD are a very heterogeneous group of lesions. The prenatal cohorts that have been published in recent years, focus on CHDs in general, but not at the level of the specific defect. These cohorts are not large enough, have significant selection bias, had no postnatal confirmation of the CHDs, or are otherwise unsuitable to extract the prevalence on the level of specific heart defects[21]. Thus, from a clinical point-of-view, our aim was to assess the presence and spectrum of clinically significant CNVs or variants of unknown significance (VOUS) by performing array analysis in a group of isolated fetal left sided CHDs.

MATERIALS AND METHODS

Cases with a prenatal diagnosis of a left sided CHD were selected from the CAHAL database. This is a regional cohort of fetuses with severe CHD born between 2002 and 2012 in the northwest region of the Netherlands. Methods of data collection are previously reported[2]. We extracted left sided CHD from this cohort, and subsequently excluded cases with additional CHD such as abnormal positioning of the great vessels. Ultrasound data were reviewed and cases were grouped as either 'isolated' or 'non-isolated' (defined as the presence of significant extra-cardiac malformations, hydrops or hygroma colli). Soft markers, minor additional findings, growth restriction, amniotic fluid pathology and/or single umbilical artery were not considered as significant extracardiac abnormalities. These cases are included in the 'isolated' group (see table S3). The presence and outcome of genetic analysis was assessed.

Cases with a prenatal diagnosis of an isolated left sided CHD, with a normal karyotype or rapid aneuploidy detection (RAD) result and absence of 22q11.2 microdeletion were eligible for array analysis Array was performed if frozen amniocytes, chorionic mesoderm, or isolated DNA was available in storage. Samples were anonymously processed. Affymetrix Cytoscan HD array or Agilent CGH 180K oligo array (Amadid 023363) was used as array platform and performed according to manufacturer's instructions. Data analysis was performed using Chromosome Analysis Suite (ChAS) 2011 version CytoB-N1 2.0.232 (r4280), Nexus Copy Number versions 5.0, 6.1 and 7.0 or Genomic Workbench 6.5, and interpreted using Cartagenia BENCH 4.0 Feb-2012 (genome build hg19). Standard settings for SNPs in ChAS were adjusted: gain- size of 20 kb, marker count of 10, and a confidence of >85 and

for loss-size of 10 kb, marker count of 10 and a confidence of >85. Standard settings for CNVs in Nexus were adjusted: threshold for probe median: gain 0.3 and loss -0.3. Minimal probes for a call: 20 per segment. Only samples meeting the quality criteria, i.e. QC >15, MapD <0.25 and a WavinessSD <0.12, were analyzed. For the oligo arrays analyzed with genomic workbench an aberration was defined as at least 3 consecutive probes with log2 ratio ≤ -0.4 or ≥0.4. The interpretation of CNVs has been done according the criteria as described by Gijsbers et al[22]. If parental material was available, we analyzed trios to assess whether rare CNVs were *de novo* or inherited. Various available online platforms were used, including the UCSC Genome Browser, Ensembl Genome Browser, the Toronto DB of Genomic Variants (DGV) and Decipher. Common polymorphic CNVs were considered as benign, with the exception of CNVs that are known as (possible) susceptibility factors, such as 15q11.2 BP1-BP2 microdeletions[23;24] and Xp22.31 microduplications[25;26], and maternally inherited CNVs on the X chromosome in male fetuses. The remaining variants were included for consideration for clinical significance. Inherited CNVs from parents were also considered as rare CNVs to account for CNVs with a possible reduced penetrance. To assess the function of the genes involved, we consulted PubMed and the OMIM database, as well as genecards.org (consulted between July and November 2015). Statistical analysis was performed using SPSS version 20.0.0.

RESULTS

The database contained 200 cases of prenatally diagnosed left sided CHDs. In table 1 the anatomic subgroups of the CHD, the rates of invasive testing, and rates of residual material available are summarized. A significant extra-cardiac malformation, detected by prenatal ultrasound, was present in 55 fetuses (27.5%), such as multiple soft markers, cerebral malformations, abdominal wall defects, or severe hydrops/hygroma colli. In 145 fetuses (72.5%) no significant extra-cardiac defects were present; 11 of these (7.6%) had a single umbilical artery and 28 (19%) had a single soft marker, minor malformation, growth abnormality and/or amniotic fluid pathology. In 67 of 145 cases (46%) with an 'isolated' left sided CHD the child was live born; in 67 cases (46%) a termination of pregnancy was performed (table S1). The CHD was confirmed by either postnatal ultrasound or post-mortem analysis in 100 of 145 'isolated' cases (69%). In 45 cases (31%), the diagnosis was only ascertained by prenatal ultrasound. Further details on survival in both groups are summarized in the supplemental table S1. Rates of chromosome abnormalities and 22q11.2 microdeletions, of the isolated and non-isolated groups, are summarized in table S2. Large chromosomal abnormalities or 22q11 microdeletion were present in 8% (95% CI 3-14%) of 'isolated' left sided CHDs.

Table 1: Rates of invasive testing, genetic analysis in total and number of arrays performed in fetuses with isolated and non-isolated left sided CHDs

type of left sided CHD	n	PND (%)	genetic analysis postnatal	genetic analysis total	cases with left over material (array performed)
Isolated left sided CHD					
HLHS	104	73 **(70%)**	8	81 (78%)	43
Coarctation of the aorta	22	11 **(50%)**	4	15 (68%)	7
Aortic stenosis	10	5 **(50%)**	-	5 (50%)	2
other left sided CHD*)	9	5 **(56%)**	-	5 (56%)	2
TOTAL isolated	145	94 **(65%)**	12	106 (73%)	54
Non-isolated left sided CHD					
HLHS	40	34 **(85%)**	1	35 (88%)	
Coarctation of the aorta	5	5 **(100%)**	-	5 (100%)	
Aortic stenosis	4	3 **(75%)**	-	3 (75%)	
other left sided CHD*)	6	5 **(83%)**	1	6 (100%)	
TOTAL non-isolated	55	47 **(85%)**	2	49 (89%)	
TOTAL overall	**200**	**141 (71%)**	**14**	**155 (78%)**	

*) includes cases with Shone syndrome, aortic arch hypoplasia and small left ventricle not otherwise specifiedAbbreviations: CHD congenital heart defect; PND prenatal invasive procedure; HLHS hypoplastic left heart syndrome;

The inclusion process for array analysis is displayed in figure 1, resulting in 54 inclusions of 136 eligible cases (40%) for array analysis. Details of these 54 cases are available in table S3. Of the 54 cases, 36 (67%) were performed on the Affymetrix Cytoscan and 18 (33%) were performed on the Agilent CGH.

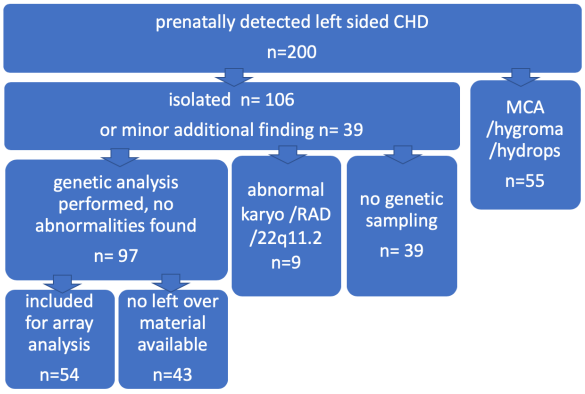


FIGURE 1: Inclusion for array analysis

Abbreviations: CHD congenital heart defect; MCA multiple congenital anomalies; karyo karyogram; RAD rapid aneuploidy detection; 22q11.2 microdeletion

Table 2 lists the encountered rare CNVs, the clinical implications, the locus on the chromosome, and the corresponding genes pertaining to that locus. We found 28 rare CNVs in 18 cases accounting for a prevalence of 33% with an average of 1.6 rare CNVs per person. The size of the CNVs ranged between 10.6 kb and 2.2 Mb. Our analysis and interpretation yielded clinically significant CNVs in 2 of 54 cases (4%; 95%CI 0 - 9%). In case 7 we found a ~10% mosaicism for trisomy 2, which remained undetected by previous chromosome analysis because at that time not enough cells (n=16) were analysed to detect the very low mosaicism. Because amniocytes were the cells used for the initial diagnosis, this result was not caused by a confined placental mosaicism. This aberration is known to be associated with cardiac defects and multiple congenital malformations[27;28]. Follow-up is unavailable because the pregnancy was terminated without post-mortem analysis. In case 48 we identified a 2.2Mb *de novo* 10q25 deletion, associated with multiple congenital malformations[29;30]. Genes include: *DUSP5*, associated with susceptibility to vascular anomalies, *SMC3*, associated with mild Cornelia de Lange syndrome 3, *RBM20*, associated with dilated cardiomyopathy, *SHOC2*, associated with Noonan-like disease, and *ADRA2A*, associated with cardiac hypertrophy and diminished contractility. Currently three years old, the child has dysmorphic features, a horseshoe kidney (missed antenatally), and appears to be developing normally compared to peers.

In the above mentioned two cases, as well as the 16 other cases, we also found 26 smaller CNVs. Most of these are unlikely to be clinically relevant or possibly causative, because the genetic involvement appears to be unrelated to critical developmental processes. Parental samples were not available for comparison in 14 of the 18 cases, therefore it is uncertain if 20 of the 26 found rare CNVs were inherited or *de novo*. Analysis of the involved genes demonstrated genes possibly related to abnormal cardiac development in only 1 case: In case 5 array analysis demonstrated a duplication including the 3'part of the *AAK1* gene; this gene interacts with the activated form of *NOTCH1a*[31]. The clinical implications of this duplication are uncertain (VOUS). The parents were not tested, and the pregnancy was terminated without post-mortem analysis.

In case 38 we found a maternally inherited 4q21.23 deletion in a region including the *WDFY3* gene. This deletion has previously been reported as a possible risk factor for autism spectrum pathology[32]. This child died 3 weeks after birth due to cardiovascular complications.

In case 43 we found a maternally inherited Xp22.31 duplication in a region including the *STS* gene in a male fetus. This gain has been reported as a possible risk factor for neurodevelopmental delay[25;26]. This child died after surgery due to cardiovascular complications.

Table 2: Copy number variants encountered in the isolated left sided CHDs group

Case	Platform	Array results (rare CNVs)	Array abnormality *)	Type	Size	Genes included	Clinical significance	Parental material available
2	A	3 CNVs	1p11.2(120,825,175-121,125,362)	loss	300kb	*FAM72B FCGR1B*	Likely benign	no
			4q27(121,589,739-121,797,841)	gain	208kb	*PRDM5*	Likely benign	
			11p15.1(21,068,742-21,464,425)	gain	396kb	*NELL1*	Likely benign	
5	C	2 CNVs	1p33(49,477,344-49,738,282)	loss	261kb	*AGBL4*	Likely benign	no
			2p13.3(69,490,034-69,737,401)	gain	247kb	*GFPT1 NFU1 AAK1*	AAK1: uncertain, probably benign. Binds to and stabilizes the activated form of NOTCH1, increases its localization in endosomes and regulates its transcriptional activity.	
7	C	mosaic trisomy 2, 1 small CNV	1p34.2 (40,961,609-41,002,340)	loss	40.7kb	*ZFP69 EXO5 ZNF684*	Likely benign	no
			mosaic trisomy 2			>20genes	Clinically significant, associated with cardiac defects and multiple anomalies	
11	A	1 small CNV	Xq28 (154,310,086-154,444,066)	gain	134kb	*BRCC3*	Likely benign	no
12	C	1 small CNV	5q33.1 (151,264,063-151,366,169)	gain	102kb	*GLRA1*	Likely benign	no
21	C	1 small CNV	1p22.3(87,029,391-87,039,945)	loss	10.6kb	*CLCA4*	Likely benign	no
22	C	1 small CNV	5q14.1(80,494,619-80,539,078)	loss	44.5kb	*RASGRF2 CKMT2*	Likely benign	no
24	C	2 CNVs	3q13.31(116,789,790-116,964,183)	loss	174.4kb	*LSAMP*	Likely benign	no
			4q13.3(73,813,058-74,048,508)	gain	235.5kb	*COX18 ANKRD17*	Likely benign	
26	A	1 CNV	20q13.33 (61,209,209 -61,392,119)	gain	183kb	*SLCO4A1 NTSR1*	Likely benign	no
32	C	2 CNVs	5q21.1(102,167,747-102,434,937)	loss	282.1kb	*PAM GIN1*	Likely benign	no
			20q11.22(34,312,988-34,378,069)	gain	65.1 kb	*RBM39 PHF20*	Likely benign	
33	C	1 small CNV	12q13.2(55,815,028-55,831,512)	loss	16.5kb	*OR6C76*	Likely benign	no
34	C	1 small CNV	4q22.1 (93,681,949-93,780,269)	loss	98.3kb	*GRID2*	Likely benign	no
37	C	2 small CNVs	8p22(13,974,602-14,118,431)	gain	143.8kb	*SGCZ*	Likely benign	no
			Xp11.3(46,311,914-46,342,995)	gain	31.1kb	*KRBOX4*	Likely benign	

Case	Platform	Array results (rare CNVs)	Array abnormality *)	Type	Size	Genes included	Clinical significance	Parental material available
38	C	2 CNVs	4q21.23(85,784,554-85,825,218) x1 mat	loss	40.7kb	*WDFY3*	Uncertain, seen in non-affected mother, possible risk factor autism spectrum pathology	yes
			4q32.1(158,941,501-159,158,531) x1 pat	loss	217kb	*FAM198B TMEM144*	Likely benign	
43	A	2 CNVs	Xp22.31(6,457,403-8,131,810)mat	gain	1.7Mb	*STS*	Uncertain, seen in non-affected mother, possible risk factor developmental delay	yes
			11p11.2 (43,858,992-44,033,281) mat	gain	174.2kb	*HSD17B12 ALKBH3*	Likely benign	
44	A	1 CNV	21q21.3 (29,922,501-30,308,957)	gain	368.6kb	*N6AMT1 LTN1*	Likely benign	no
48	C	2 CNVs	10q25(111,473,160-113,665,727)dn	loss	2.2 Mb	*XPNPEP1 ADD3 MXI1 SMNDC1 DUSP5 SMC3 RBM20 PDCD4 BBIP1 SHOC2 ADRA2A*	Clinical significant; associated with cardiac defects and multiple anomalies. (DUSP5 associated with susceptibility to vascular anomalies SMC3 is associated with mild Cornelia de Lange syndrome, RBM20 is associated with dilated cardiomyopathy, SHOC2 is associated with noonan like disease, ADRA2A is associated with cardiac hypertrophia and diminished contractility)	yes
			12q24.33(131,536,220-131,893,995)pat	gain	357.8kb	*FBRSL1 P2RX2 POLE PXMP2 PGAM5 ANKLE2 GOLGA3*	Likely benign, seen in non-affected father	
54	C	1 CNV	1q31.3(198,042,714-198,267,273) mat	gain	224.6kb	*NEK7*	Likely benign, seen in non-affected mother	yes

*) Genome build hg19. A small CNV is defined as <150kb.

Abbreviations: CNV copy number variation; platforms: A Agilent CGH 180K, C Cytoscan HD array, CHD congenital heart defect; pat inherited from father; mat inherited from mother; kb x10^3 basepair; Mb x 10^6 basepair

As deduced from table S3, minor additional findings were present in 17 of 54 'isolated' cases (35%), including enlarged nuchal translucency/neck cysts (n=4), ascites/pericardial effusion (n=6), single umbilical artery (n=4), and other minor findings (n=3). Additionally, two fetuses were postnatally identified with extra-cardiac malformations (horseshoe kidney in cases 23 and 48), where one had a clinically significant CNV (case 48). These 19 fetuses with prenatally detectable (although missed in 2 cases) additional malformations did not differ in the frequency of rare CNVs from fetuses that are 'truly' isolated, without additional findings (both 31%). Furthermore, one child with normal array results currently displays neurodevelopmental delay (case 3). Another child with a normal array result developed hydrocephalus of an unknown cause (case 52). Both fetuses with a clinically significant CNV had an additional finding (cases 7 and 48), however in case 48 the extra-cardiac anomaly was only detected after birth. This results in 1/17 (6%) clinically significant array findings in fetuses with additional findings and 1/37 (3%) clinically significant array findings in prenatal isolated appearing cases (independent samples T test p=0.6).

DISCUSSION

Congenital heart defects (CHDs) are known to be associated with chromosomal abnormalities and 22q11.2 microdeletion[4]. This is confirmed by our study (table S2). Furthermore, our study shows that array analysis can yield clinically significant abnormalities in 4% of euploid fetuses without a 22q11.2 microdeletion. Thus, in the absence of ultrasonographically detected significant extra-cardiac malformations, and with a normal karyotype/FISH 22q11.2 result, array can in some cases predict if fetuses with a left sided CHD are at risk for a more severe phenotype. In our study, the risk of array abnormalities appears to be unrelated to the presence of minor additional malformations such as enlarged nuchal translucency. In two cases additional malformations (horseshoe kidney) remained undetected prior to birth.

Previous reports on the incidence of submicroscopic chromosomal abnormalities in fetal CHDs focus on CHDs in general, or analyze postnatal cohorts[21;33-35], which is impractical in prenatal counselling. As the diagnostic accuracy of prenatal ultrasound increases, targeted information concerning the specific diagnosis will also need to emerge. The current study determines the specific incidence of genetic abnormalities in the subgroups of isolated and non-isolated left sided CHDs. Left sided CHD are generally considered not to be associated with genetic syndromes, if they appear isolated on prenatal ultrasound. Compared to other CHD, tetralogy of Fallot for example, which is highly associated with syndromic and chromosomal anomalies, physicians may be

more reluctant to stress the need for fetal genetic sampling in absence of other fetal abnormalities. Thus, with our data, physicians are able to counsel parents more tailored to this specific condition. A great strength in our study is the large rate of postnatal confirmation (69% in isolated cases), thus analyzing a sharply defined phenotype of left sided CHD only.

Our array data confirms a previously reported additional yield of 6% with clinically significant submicroscopic chromosomal abnormalities in two large cohorts of euploid fetuses with isolated malformations in general[36;37]. When focusing on left sided CHD only, our findings are in concordance with Shaffer, who reported a subgroup with isolated HLHS in a large cohort of fetuses with various ultrasound abnormalities[14]. Shaffer found 4 (9.5%) significant findings (all < 10Mb) in 42 isolated HLHS fetuses. This study, however, does not provide follow-up data to validate the prenatal findings with regard to postnatal outcome, nor does it elaborate on the details of the array abnormalities and inheritance. Hitz et al. stated that in 10% of left sided CHDs, CNVs play a causative or contributing role[38]. Though this study included a well-focused phenotype, Hitz studied families with postnatally proven isolated left sided CHDs, excluding known syndromes and dysmorphic features. As this information is not available in the prenatal setting, the data of Hitz are not applicable for parental counselling in a fetal diagnosis.

Our study is the first to report the detection of rare CNVs, in a prenatal cohort. Our data demonstrate an average of 1.6 rare CNVs per person in 33% of fetuses with left sided CHDs. Our data coincide with findings in postnatal similar patient groups with similar array resolution: Hitz found 1.35 rare CNVs per person in 31% of children with left sided CHD (n= 54/174) with a resolution of 10kb, and Iascone found 1.32 rare CNVs per person, in 47% of postnatal HLHS cases (n= 25/53), with an average resolution of 20kb[38;39]. Payne reported on the frequency of small CNVs (<60kb), not likely to be disease-causing in 43 postnatal isolated and non-isolated cases of HLHS. Their found average (1.49 CNVs per person) was significantly higher when compared to 16 healthy controls[39]. In comparison to Hitz[38] and Iascone[39], the availability of parental material is somewhat lower in our dataset. Considering the fact that we found a similar number, or fewer, patients with rare CNVs, we do not expect this to have resulted in a high number of false CNVs calls.

The interpretation of CNVs remains controversial and prone to differences between centers. The identification of clinically significant CNVs is subject to variations in the used platform and the consulted genomic databases. The clinical (in)significance of variants of unknown significance (VOUS) are increasingly unveiled. Our interpretation of the CNVs yielded two array anomalies with clinical significance. Both anomalies are

known to be associated with cardiac defects and multiple congenital malformations[27-30]. However, these findings include some ambiguity. The degree of mosaicism trisomy 2 and affected tissues cannot be predicted (case 7). However, it would trigger suspicion of additional fetal congenital abnormalities. Interestingly, the 10q25 deletion case (case 48) did present with an additional structural abnormality, but neurodevelopment is normal.

Three VOUS were identified that were of interest. In the duplication of chromosome 2 in case 5, *AAK1* appears to be an interesting gene due to its interaction with the activated form of *NOTCH1*[31]. However only the 3'part of the gene is duplicated; further investigation is needed to determine whether this duplication will disrupt this gene and subsequently has an effect on the gene function . The second and third VOUS are maternally inherited variants. The Xp22.31 duplication in case 43, including the *STS* gene is a variant that is present at a low frequency in the population, but is still considered clinically significant because it is found at higher frequency in affected individuals. Although this variant will not explain the HLHS, it could be a risk factor for neurodevelopmental delay[25]. The 4q21.23 deletion in case 38, including the *WDFY3* gene, has been correlated to cerebral changes in mice that could be characteristic for autism spectrum disorders and epilepsy. The implications of both variants are unclear, and both children died at very young age due to cardiac complications. As our study was done on banked samples, it is unclear how these findings would have influenced the prenatal counselling.

Previous studies have implicated several loci and genes in left sided CHDs (mainly HLHS), including *NOTCH1, NKX2.5, NKX2.6, HAND1, HAND2, SNAI2, GATA6, GJA1, FGF8, FOXC1, FOXC2, FOXH1* and *FOXL1*[40-45]. Identifying a new candidate gene or combination of genes responsible, however, remains difficult, mainly due to variable penetrance[41]. In isolated left sided CHDs, there appears to be no single genetic cause. Familial recurrence does occur, but left sided CHDs are considered to be genetically heterogeneous. Embryological blood flow alterations also seem to play an important role in the etiology[46;47]. The reported genes were not found in any of the CNV regions we identified. However, the platforms we used either lacked or had few probes specific for the following genes: *HAND1, HAND2, SNAI2, NKX2.5 FOXC1 NKX2.6 FOXH1* and *FGF8;* intragenic insertions or deletions could have been missed.

Our study has some limitations. Due to the retrospective nature of our study, segmental analysis of the development of the CHDs was not available in all cases. It is complicated to provide a link between a CNV or a candidate gene and the observed phenotype[39;48]. In left sided CHDs it is even more difficult because the anomaly itself displays high rates of anatomic variation[49]. Clinical classifications of left sided CHDs are focused on

a functional outcome. In HLHS, as an end stage development product, it is not always possible to identify the developmental cause of the observed anomaly. In our cohort, segmental developmental analysis was only possible in a small group, mainly in those that underwent postmortem dissection after termination of pregnancy. In the live born cases specific developmental details, regarding the presence of mitral or aortic valve hypo- or aplasia, as cause of HLHS were not always identifiable.

Furthermore, only 22% of our samples were analysed as trios, so information regarding the presence or absence of identified CNVs in parents is lacking in the remaining 42 cases. The importance of information regarding inheritance is evidenced by the findings of Warburton, where *de novo* rare CNVs occurred in 12.7% of their 71 postnatal HLHS cases versus 2% in their cohort of healthy controls[50]. The history of familial occurrence of cardiac defects was not always available in our cohort, and parents were generally not tested for the presence of mild left sided CHDs such as a bicuspid aortic valve. Familial segregation analysis (linkage studies) and subsequent speculation on other potentially contributing CNVs, labelled in our study as clinically not significant, is therefore not possible. Thus, we are unable to rule out a possible influence of a yet unknown, common CNV as a susceptibility factor. Known susceptibility factors, such as 15q11.2 BP1-BP2 microdeletions, were not found in our study. Furthermore, the resolution of the used array method is restricted to 10kb in deletions and 20 kb in duplications; smaller intragenic deletions or duplications could not have been detected by this test.

Also, genetic material was not available in all eligible cases. As we have demonstrated in table 1, parents typically opted for an invasive procedure when additional malformations were present. Also, in 53 cases, genetic material was unavailable due to logistic challenges, absence of stored material and failure of cell culture. Therefore a selection bias cannot be ruled out.

Despite the limitations, our data serves as guide in focused prenatal counselling when genetic analysis is offered in left sided CHDs. Considering the fact that the long-term outcome may also be dominated by non-iatrogenic neurological impairment, even in apparently isolated CHDs, attempting to identify beforehand which cases are at highest risk for a more severe phenotype is important[51,52]. As mentioned, our data also confirm reports that left sided CHDs are associated with chromosome abnormalities and 22q11.2 microdeletion syndrome[4], detecting these aberrations in as many as 57% of fetuses with left sided CHDs in the presence of significant extra-cardiac malformations in this study. Left sided CHDs which seem to be isolated on prenatal ultrasound also carry a 7% risk of clinical significant chromosome abnormalities and 22q11.2 microdeletions in our cohort.

Together with clinically significant CNVs found in 4%, the yield of genetic analysis could be as high as 11% when using karyotyping and array analysis combined. However, all of the significant chromosome abnormalities found in our study with karyotyping (table S2) are also identifiable by array. It is advisable therefore to perform array analysis as a first tier test. Depending on local policies and costs deliberations, array analysis can be preceded by RAD to exclude common aneuploidies first. However, our study also shows that array analysis cannot predict all cases that display adverse (neurodevelopmental) outcome. Furthermore, as discussed, the significant array findings include some ambiguity. Therefore, while array analysis would have identified individual cases where the search for additional phenotypic abnormalities would be warranted, counselling may still involve some uncertainty. In the future, if whole exome or genome sequencing becomes widely available in the prenatal setting, this effect might even be stronger. To attach consequences to subtle array abnormalities, such as refusal of certain palliative interventions, has to be avoided until evidence of adverse outcome can be ascertained.

In conclusion, our data show that performing array analysis in a high resolution in cases of prenatal left sided CHD could aid parental counselling. It could identify some fetuses that are at high risk for a more severe phenotype, because of its capability to demonstrate unbalanced submicroscopic chromosome abnormalities and low mosaic aneuploidies. As the first to explore this in a prenatal setting, our research supports the use of array analysis as a first tier diagnostic test in isolated left sided CHD[53]. Left sided CHD are usually considered to have a low risk for genetic anomalies, if not accompanied by additional congenital anomalies, leading to lower rates of invasive procedure performed. This study however confirms that fetal ultrasound misses certain additional lesions, thus emphasizing the importance of fetal genetic analysis. Because array analysis is also able to detect 22q11.2 microdeletion, it can be performed instead of FISH analysis, preceded by RAD (or karyotyping). The relative small size of our cohort, however, attenuates our findings.

REFERENCES

1. van der Linde D, Konings EE, Slager MA et al. Birth prevalence of congenital heart disease worldwide: a systematic review and meta-analysis. *J Am Coll Cardiol* 2011;58:2241-2247.
2. van VC, Clur S, Rijlaarsdam M et al. Prenatal detection of congenital heart disease-results of a national screening programme. *BJOG* 2015.
3. Barron DJ, Kilby MD, Davies B, Wright JG, Jones TJ, Brawn WJ. Hypoplastic left heart syndrome. *Lancet* 2009;374:551-564.
4. Ferencz C, Neill CA, Boughman JA, Rubin JD, Brenner JI, Perry LW. Congenital cardiovascular malformations associated with chromosome abnormalities: an epidemiologic study. *J Pediatr* 1989;114:79-86.
5. Simsic JM, Coleman K, Maher KO, Cuadrado A, Kirshbom PM. Do neonates with genetic abnormalities have an increased morbidity and mortality following cardiac surgery? *Congenit Heart Dis* 2009;4:160-165.
6. Cramer JW, Bartz PJ, Simpson PM, Zangwill SD. The Spectrum of Congenital Heart Disease and Outcomes After Surgical Repair Among Children With Turner Syndrome: A Single-Center Review. *Pediatr Cardiol* 2013.
7. Evangelidou P, Alexandrou A, Moutafi M et al. Implementation of high resolution whole genome array CGH in the prenatal clinical setting: advantages, challenges, and review of the literature. *Biomed Res Int* 2013;2013:346762.
8. Park SJ, Jung EH, Ryu RS et al. Clinical implementation of whole-genome array CGH as a first-tier test in 5080 pre and postnatal cases. *Mol Cytogenet* 2011;4:12.
9. Miller DT, Adam MP, Aradhya S et al. Consensus statement: chromosomal microarray is a first-tier clinical diagnostic test for individuals with developmental disabilities or congenital anomalies. *Am J Hum Genet* 2010;86:749-764.
10. Van den Veyver IB, Patel A, Shaw CA et al. Clinical use of array comparative genomic hybridization (aCGH) for prenatal diagnosis in 300 cases. *Prenat Diagn* 2009;29:29-39.
11. Lichtenbelt KD, Knoers NV, Schuring-Blom GH. From karyotyping to array-CGH in prenatal diagnosis. *Cytogenet Genome Res* 2011;135:241-250.
12. Callaway JL, Shaffer LG, Chitty LS, Rosenfeld JA, Crolla JA. The clinical utility of microarray technologies applied to prenatal cytogenetics in the presence of a normal conventional karyotype: a review of the literature. *Prenat Diagn* 2013;33:1119-1123.
13. Vanakker O, Vilain C, Janssens K et al. Implementation of genomic arrays in prenatal diagnosis: the Belgian approach to meet the challenges. *Eur J Med Genet* 2014;57:151-156.
14. Shaffer LG, Rosenfeld JA, Dabell MP et al. Detection rates of clinically significant genomic alterations by microarray analysis for specific anomalies detected by ultrasound. *Prenat Diagn* 2012;32:986-995.
15. Yan Y, Wu Q, Zhang L et al. Detection of submicroscopic chromosomal aberrations by array-based comparative genomic hybridization in fetuses with congenital heart disease. *Ultrasound Obstet Gynecol* 2014;43:404-412.
16. Liao C, Li R, Fu F et al. Prenatal diagnosis of congenital heart defect by genome-wide high-resolution SNP array. *Prenat Diagn* 2014;34:858-863.
17. Donnelly JC, Platt LD, Rebarber A, Zachary

J, Grobman WA, Wapner RJ. Association of copy number variants with specific ultrasonographically detected fetal anomalies. *Obstet Gynecol* 2014;124:83-90.

18. Mademont-Soler I, Morales C, Soler A et al. Prenatal diagnosis of chromosomal abnormalities in fetuses with abnormal cardiac ultrasound findings: evaluation of chromosomal microarray-based analysis. *Ultrasound Obstet Gynecol* 2013;41:375-382.
19. Schmid M, Stary S, Blaicher W, Gollinger M, Husslein P, Streubel B. Prenatal genetic diagnosis using microarray analysis in fetuses with congenital heart defects. *Prenat Diagn* 2012;32:376-382.
20. Chen M, Yang YS, Shih JC et al. Microdeletions/duplications involving TBX1 gene in fetuses with conotruncal heart defects which are negative for 22q11.2 deletion on fluorescence in-situ hybridization. *Ultrasound Obstet Gynecol* 2014;43:396-403.
21. Jansen FA, Blumenfeld YJ, Fisher A et al. Array comparative genomic hybridization and fetal congenital heart defects: a systematic review and meta-analysis. *Ultrasound Obstet Gynecol* 2015;45:27-35.
22. Gijsbers AC, Schoumans J, Ruivenkamp CA. Interpretation of array comparative genome hybridization data: a major challenge. *Cytogenet Genome Res* 2011;135:222-227.
23. Cox DM, Butler MG. The 15q11.2 BP1-BP2 microdeletion syndrome: a review. *Int J Mol Sci* 2015;16:4068-4082.
24. Soemedi R, Topf A, Wilson IJ et al. Phenotype-specific effect of chromosome 1q21.1 rearrangements and GJA5 duplications in 2436 congenital heart disease patients and 6760 controls. *Hum Mol Genet* 2012;21:1513-1520.
25. Esplin ED, Li B, Slavotinek A et al. Nine patients with Xp22.31 microduplication, cognitive deficits, seizures, and talipes anomalies. *Am J Med Genet A* 2014;164A:2097-2103.
26. Li F, Shen Y, Kohler U et al. Interstitial microduplication of Xp22.31: Causative of intellectual disability or benign copy number variant? *Eur J Med Genet* 2010;53:93-99.
27. Sago H, Chen E, Conte WJ et al. True trisomy 2 mosaicism in amniocytes and newborn liver associated with multiple system abnormalities. *Am J Med Genet* 1997;72:343-346.
28. Chen CP, Chen YY, Chern SR et al. Prenatal diagnosis of mosaic trisomy 2 associated with abnormal maternal serum screening, oligohydramnios, intrauterine growth restriction, ventricular septal defect, preaxial polydactyly, and facial dysmorphism. *Taiwan J Obstet Gynecol* 2013;52:395-400.
29. Gil-Rodriguez MC, Deardorff MA, Ansari M et al. De novo heterozygous mutations in SMC3 cause a range of Cornelia de Lange syndrome-overlapping phenotypes. *Hum Mutat* 2015;36:454-462.
30. Stark Z, Bruno DL, Mountford H, Lockhart PJ, Amor DJ. De novo 325 kb microdeletion in chromosome band 10q25.3 including ATRNL1 in a boy with cognitive impairment, autism and dysmorphic features. *Eur J Med Genet* 2010;53:337-339.
31. Gupta-Rossi N, Ortica S, Meas-Yedid V et al. The adaptor-associated kinase 1, AAK1, is a positive regulator of the Notch pathway. *J Biol Chem* 2011;286:18720-18730.

32. Orosco LA, Ross AP, Cates SL et al. Loss of Wdfy3 in mice alters cerebral cortical neurogenesis reflecting aspects of the autism pathology. *Nat Commun* 2014;5:4692.
33. Erdogan F, Larsen LA, Zhang L et al. High frequency of submicroscopic genomic aberrations detected by tiling path array comparative genome hybridisation in patients with isolated congenital heart disease. *J Med Genet* 2008;45:704-709.
34. Greenway SC, Pereira AC, Lin JC et al. De novo copy number variants identify new genes and loci in isolated sporadic tetralogy of Fallot. *Nat Genet* 2009;41:931-935.
35. Breckpot J, Thienpont B, Arens Y et al. Challenges of interpreting copy number variation in syndromic and non-syndromic congenital heart defects. *Cytogenet Genome Res* 2011;135:251-259.
36. Wapner RJ, Martin CL, Levy B et al. Chromosomal microarray versus karyotyping for prenatal diagnosis. *N Engl J Med* 2012;367:2175-2184.
37. Shaffer LG, Dabell MP, Fisher AJ et al. Experience with microarray-based comparative genomic hybridization for prenatal diagnosis in over 5000 pregnancies. *Prenat Diagn* 2012;32:976-985.
38. Hitz MP, Lemieux-Perreault LP, Marshall C et al. Rare copy number variants contribute to congenital left-sided heart disease. *PLoS Genet* 2012;8:e1002903.
39. Iascone M, Ciccone R, Galletti L et al. Identification of de novo mutations and rare variants in hypoplastic left heart syndrome. *Clin Genet* 2012;81:542-554.
40. Reamon-Buettner SM, Ciribilli Y, Inga A, Borlak J. A loss-of-function mutation in the binding domain of HAND1 predicts hypoplasia of the human hearts. *Hum Mol Genet* 2008;17:1397-1405.
41. Wessels MW, Willems PJ. Genetic factors in non-syndromic congenital heart malformations. *Clin Genet* 2010;78:103-123.
42. Maitra M, Schluterman MK, Nichols HA et al. Interaction of Gata4 and Gata6 with Tbx5 is critical for normal cardiac development. *Dev Biol* 2009;326:368-377.
43. Stankiewicz P, Sen P, Bhatt SS et al. Genomic and genic deletions of the FOX gene cluster on 16q24.1 and inactivating mutations of FOXF1 cause alveolar capillary dysplasia and other malformations. *Am J Hum Genet* 2009;84:780-791.
44. Yamagishi H, Yamagishi C, Nakagawa O, Harvey RP, Olson EN, Srivastava D. The combinatorial activities of Nkx2.5 and dHAND are essential for cardiac ventricle formation. *Dev Biol* 2001;239:190-203.
45. Elliott DA, Kirk EP, Yeoh T et al. Cardiac homeobox gene NKX2-5 mutations and congenital heart disease: associations with atrial septal defect and hypoplastic left heart syndrome. *J Am Coll Cardiol* 2003;41:2072-2076.
46. Hinton RB, Martin LJ, Rame-Gowda S, Tabangin ME, Cripe LH, Benson DW. Hypoplastic left heart syndrome links to chromosomes 10q and 6q and is genetically related to bicuspid aortic valve. *J Am Coll Cardiol* 2009;53:1065-1071.
47. Kodo K, Yamagishi H. A decade of advances in the molecular embryology and genetics underlying congenital heart defects. *Circ J*

2011;75:2296-2304.

48. Marino B, Digilio MC. Congenital heart disease and genetic syndromes: specific correlation between cardiac phenotype and genotype. *Cardiovasc Pathol* 2000;9:303-315.
49. Tchervenkov CI, Jacobs ML, Tahta SA. Congenital Heart Surgery Nomenclature and Database Project: hypoplastic left heart syndrome. *Ann Thorac Surg* 2000;69:S170-S179.
50. Warburton D, Ronemus M, Kline J et al. The contribution of de novo and rare inherited copy number changes to congenital heart disease in an unselected sample of children with conotruncal defects or hypoplastic left heart disease. *Hum Genet* 2014;133:11-27.
51. Miller SP, McQuillen PS, Hamrick S et al. Abnormal brain development in newborns with congenital heart disease. *N Engl J Med* 2007;357:1928-1938.
52. Sanchez-Valle A, Pierpont ME, Potocki L. The severe end of the spectrum: Hypoplastic left heart in Potocki-Lupski syndrome. *Am J Med Genet A* 2011;155A:363-366.
53. Carey AS, Liang L, Edwards J et al. Effect of copy number variants on outcomes for infants with single ventricle heart defects. *Circ Cardiovasc Genet* 2013;6:444-451.

SUPPLEMENTARY FILES

Table S1: Details of survival and postnatal confirmation of the CHD in fetuses with isolated and non-isolated left sided CHD

Type of left sided CHD	number	CHD confirmed*		TOP	IUFD	loss to follow up	Live born		Currently alive†	
Isolated left sided CHD§										
HLHS	104	64	62%	61	4	2	37	36%	17	47%
Coarctation of the aorta	22	21	95%	2	-	1	19	90%	17	89%
Aortic stenosis	10	7	70%	3	1	1	5	56%	5	100%
other left sided CHD*	9	8	89%	1	2	-	6	67%	3	50%
TOTAL isolated	145	100	69%	67	7	4	67	46%	42	63%
Non-isolated left sided CHD§										
HLHS	40	10	25%	27	8	1	4	10%	0	
Coarctation of the aorta	5	2	40%	3	1	-	1	20%	1	
Aortic stenosis	4	3	75%	1	-	-	3	75%	0	
other left sided CHD*	6	5	83%	5	-	-	1	17%	0	
TOTAL non-isolated	55	20	36%	36	9	1	9	16%	1	
TOTAL overall	**200**	**120**	**60%**	**103**	**16**	**4**	**76**	**39%**	**43**	**57%**

* includes cases with Shone syndrome, aortic arch hypoplasia, absent left AV-connection, and small left ventricle not otherwise specified

† by either post-mortem analysis or postnatal ultrasound

‡ percentage of live born cases

§ please note that non-isolated is defined as no extracardiac anomalies present on fetal ultrasound; some of the aneuploidies are therefore included in the isolated group, if presented by only a CHD before birth.

Abbreviations: CHD congenital heart defect; HLHS hypoplastic left heart syndrome; TOP termination of pregnancy; IUFD intra uterine fetal demise

Data available online:

Table S2: prevalence of large chromosomal abnormalities and 22q11 microdeletion in fetuses with isolated and non-isolated left sided CHD.

Table S3: details of all 54 cases undergoing array analysis.