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COngenital heart disease and the Diagnostic yield with Exome sequencing (CODE Study): A prospective cohort study and systematic review

SHORT TITLE: Exome sequencing in congenital cardiac anomalies

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KEY WORDS : CARDIAC; CONGENITAL HEART DISEASE; EXOME SEQUENCING; FETUS;

PRENATAL DIAGNOSIS; NEXT GENERATION SEQUENCING

CONTRIBUTION

What are the novel findings of this work?

This is the first systematic review assessing the incrementaldiagnostic yield of antenatal exome sequencing over chromosome microarray/karyotype in prenatally diagnosed congenital heart disease.

What are the clinical implications of this work?

Dependent on the presence of robust pathways, eExome sequencing mayshould be considered in prenatal congenital heart disease, with particular consideration for to offering it in not just those with extra-cardiac abnormalities but in those of an isolated nature.

ABSTRACT

OBJECTIVES: To determine the yield of antenatal exome sequencing (ES) over chromosome microarray (CMA) / conventional karyotyping in; (i) any prenatally diagnosed congenital heart disease (CHD); (ii) isolated CHD; (iii) multi-system CHD and; (iv) CHD by phenotypic subgroup.

METHODS: A prospective cohort study of 197 trios undergoing ES followingand CMA/karyotype because CHD was identified prenatally and a systematic review of the literature was performed. MEDLINE, EMBASE and CINAHL (2000–Oct 2019) databases were searched electronically. Selected studies included those with; (i) >3 cases; (ii) initiation of testing based upon a prenatal phenotype only and; (iii) where CMA/karyotyping was negative. PROSPERO No. CRD42019140309

RESULTS: In our cohort ES gave an additional diagnostic yield in; (i) all CHD; (ii) isolated CHD and; (iii) multi-system CHD of 12.7% (n=25/197), 11.5% (n=14/122) and 14.7% (n=11/75) (p=0.81). The pooled incremental yields for the aforementioned categories from 18-studies (n=636) were 21% (95% CI, 15-27%), 11% (95% CI, 7-15%) and 37% (95% CI, 18%-56%) respectively. This did not differ significantly when sub-analyses were limited to studies including >20 cases. In instances of multi-system CHD in the primary analysis, the commonest extra-cardiac anomalies associated with a pathogenic variant were those affecting the genitourinary system 44.2% (n=23/52). Cardiac shunt lesions had the greatest incremental yield, 41% (95% CI, 19-63%), followed by right-sided lesions 26% (95% CI, 9-43%). In the majority of instances pathogenic variants occurred *de novo* and in autosomal

dominant (monoallelic) disease genes (68/96; 70.8%). The commonest monogenic syndrome identified was Kabuki syndrome (n=19/96; 19.8%).

CONCLUSIONS: Despite the apparent incremental yield of prenatal exome sequencing in congenital heart disease, the routine application of such a policy would require the adoption of robust bioinformatic, clinical and ethical pathways. ~~In the setting of robust bioinformatic, clinical and ethical pathways, prenatal exome sequencing should be considered when cardiac abnormalities are detected.~~ Whilst the greatest~~high~~ ~~diagnostic~~ yield is with multi-system anomalies, consideration may ~~should be~~ also be given to performing~~offering~~ ES in the presence of isolated cardiac abnormalities.

INTRODUCTION

Congenital heart disease (CHD) ~~occurs in~~complicates 1% of live-born ~~infants~~ neonates and is associated with significantly high rates of perinatal morbidity and mortality.[1,2] Prenatal detection of CHD and establishment of a unifying genetic diagnosis can inform prenatal management, optimise post-natal outcome and aid in the counselling of parents in both index and subsequent pregnancies.[3] Of all prenatally diagnosed CHD, 2/3 tends to be isolated while 1/3 can be associated with extra-cardiac anomalies (ECAs).[4] Aneuploidy is present in between 28-45% of prenatally diagnosed CHD, with at least one ECA present in as many as 98% of such cases.[3] Copy number variation (CNV) can be present in a further 2-25%.[3] The additional proportion of CHD caused by monogenic Mendelian disorders is traditionally thought to be ~5% although results vary.[3] Since the introduction of exome sequencing (ES), large prospective studies suggest that this proportion is greater.[5,6] It has been proposed that a significant number of identified variants in CHD within the pediatric population are *de novo* in nature, most notably when there are co-existing neurodevelopmental and ECAs.[7,8] There are a paucity of studies which have formally assessed the diagnostic yield offered from ES over standard chromosome microarray(CMA)/karyotype in prenatally diagnosed CHD and there is no evidence to suggest which phenotypic CHD sub-types have the greatest diagnostic yield.[9,10,11] Hence, the objectives of this prospective cohort study, systematic review and meta-analysis were to determine the yield of ES over CMA/karyotype in; (i) any prenatally diagnosed CHD; (ii) isolated CHD; (iii) CHD associated with ECAs and; (iv) CHD dependent on phenotypic subgroup.

METHODS

Extended PAGE Cohort

CODE assessed the extended cohort of the published Prenatal Assessment of Exomes and Genomes (PAGE) study which included 850 trios (fetus and parents) that underwent ES analysis when a fetal structural anomaly was detected on ultrasound.[5] This prospective extended cohort study recruited between October 2014 and May 2018 across 34 fetal medicine centres in England and Scotland, using the West Midlands Genetic Research Laboratory (WMGRL) as their laboratory hub and then through the Wellcome Trust Sanger Institute (for exome sequencing).[5] Eligibility criteria included; (i) prenatal detection of an anomaly after 11-weeks' gestation including an ~~elevated~~ increased nuchal translucency (NT) (≥4mm); (ii) an invasive test having been performed; (iii) informed written consent obtained from both parents for testing and both were >16-years and; (iv) negative CMA or karyotype testing. Study methodology is as documented in the original published study but briefly utilized a standard ES approach with variant interpretation based upon a targeted virtual gene panel for developmental disorders encompassing 1628 genes.[5] Phenotypes of all cases were classified using Human Phenotype Ontology (HPO) terms and those which were cardiac related were selected. Following manual review of free-text descriptions, miscoded terms ~~and cases of 'single umbilical artery' or 'lymphatic malformations' were removed from the analysis,~~ and~~as were~~ small muscular ventricular-septal defects (VSDs) were removed. CHD was initially further classified into 'isolated' and 'multi-system' with a HPO

approach to coding additional ECAs, including fetal growth restriction, single umbilical artery and nuchal thickening but not an elevated first trimester NT. Cardiac phenotypes were described by fetal medicine specialists and sonographers and confirmed by fetal cardiologists using the Viewpoint® Version 5.6.16 GE Healthcare, 2012 and were subsequently coded using the American Heart Association/American College of Cardiology (AHA/ACC) criteria as; (i) shunt lesions; (ii) left-sided obstructive lesions; (iii) right-sided lesions and; (iv) complex lesions.[12] Two clinicians reviewed each classification for concordance (F.M. and M.D.K). Pathogenic variants and variants of uncertain significance (VUS) where the American College of Medical Genetics classification had been agreed upon at the clinical review panel were included in the final list of variants.[13] Incidental findings (IFs) were not reported. The study was approved by the Research and Development offices and Research Ethics Committees at each institution and obtained ethical approval from the Research and Development offices and Research Ethics Committees at the West Midlands – South Birmingham (ref: 13/WM/1219) and each institution.

Data Sources

A systematic review was conducted in a standardized fashion in line with PRISMA guidance.[14] A systematic electronic search of MEDLINE, CINAHL, EMBASE and clinicaltrials.gov was performed from January 2000 (as ES was not available prior to this) until October 2019. MeSH keywords with word variations of the terms 'exome sequencing' and 'prenatal' were used in an attempt to capture as many relevant studies as possible. Alternative terms for ES included 'exome sequencing, whole'; 'exome sequencing,

complete'; 'whole genome sequencing' and 'sequence analysis, DNA'. Alternative terms for prenatal included 'fetal'; 'fetus' and 'antenatal'. Experts were also contacted and bibliographies of all relevant papers were searched. Studies not in the English language were translated. The search strategy is available from the corresponding author on request. This systematic review was registered prospectively with PROSPERO No. CRD42019140309.

Eligibility criteria for study selection and data extraction

All study abstracts were screened by two reviewers (F.M. and M.D.K.) and full text articles were subsequently reviewed where further information was required. Studies were selected if; (i) they included three or more cases of CHD undergoing ES; (ii) testing was initiated based upon a prenatal ultrasound-based phenotype and; (iii) CMA/ karyotype testing was negative. In cases where ES was initiated postnatally, these were only included where testing was based upon the prenatal phenotype. Data extracted from studies where obtainable included: ultrasound phenotype, ES approach, genomic variants, source of fetal DNA, turnaround time for testing, fetal outcome, maternal age and gestation at testing. An ES result was deemed positive only if it was graded IV to V 'likely pathogenic' or 'pathogenic' and determined to be causative of the phenotype. VUS and IFs were reported separately.[13]

Quality assessment and data synthesis

The incremental yield or risk difference of ES over CMA/karyotype was calculated for each study with 95% confidence intervals and as a meta-analysis for; (i) all CHD; (ii) subgroup analyses of isolated and multisystem CHD with only studies included in the latter when the presence or absence of CHD were available from the data. Cases were stratified as per the

aforementioned cohort study. Risk differences from each study were pooled using a random effects model throughout to estimate the overall yield and the yield for isolated and multi-system CHD using RevMan version 5.3.4 (Review Manager, The Cochrane Collaboration, Copenhagen, Denmark) via a previously published method which facilitated calculation of the incremental yield with adjustment for 'zero' values from negative CMA testing which was applicable to all included studies.[15] Findings were displayed as forest plots with corresponding 95% confidence intervals. Heterogeneity was assessed graphically and statistically (Higgins' I^2) and a sub-analysis was performed including studies with >20 cases to determine if results differed significantly. Publication bias was assessed graphically using funnel plots (also generated by RevMan version 5.3.4 and demonstrated as Supplementary Figure 1a-c). Quality assessment of studies was assessed using a modified Standards for Reporting of Diagnostic Accuracy (STARD) criteria. The quality criteria deemed most important to optimise accuracy were; (i) if trio analysis was performed; (ii) ACMG criteria for variant interpretation and; (iii) Sanger validation of variants.[13] Due to the limited number of studies available, beyond the pre-defined inclusion criteria, quality assessment could not be incorporated into the analysis so as the optimise the number of cases included.[13,16, 17]

RESULTS

Extended PAGE Cohort

Of 850 fetuses undergoing trio ES with prenatally detected structural anomalies, there were n=197 (23.2%) CHD cases in total, of which 61.9% (n=122) were isolated and 38.1% (n=75) associated were with ECAs. Where documented (n=190), the source of fetal DNA was; a) chorionic villi 15.8% (n=30); b) amniocytes 81.1% (n=154) or; c) lymphocytes 3.2% (n=6). G-banding karyotype was performed 3.0% (n=6) of cases, with CMA in the remainder. The diagnostic yield of ES in each group (excluding VUS) was 12.7% (n=25/197) all CHD, 11.5% (n=14/122) isolated CHD and 14.7% (n=11/75) in multisystem CHD respectively (p=0.81). In instances of multi-system CHD with a pathogenic variant, the commonest systems affected were those affecting growth, the nervous system and face (all 45.5% n=5/11). There were not enough cases to identify a dominant sub-classification of CHD hence this was explored further in the systematic review. The overall incidence of VUS was 5.1%. ~~0.06 per CHD respectively.~~

Systematic review and meta-analysis

In all instances where a study was suitable for inclusion but data was incomplete, the corresponding author was contacted (n=6), of which three responded and two provided complete data.[6,18] Authors of the second largest included study, the Petrovski, *et al*.

Columbia University-based study, provided a completed dataset on their CHD cohort as an extended version of their original study.[6] In addition to both the extended PAGE cohort study and the extended Petrovski, *et al*. study[6], a further 16 studies met the overall selection criteria, leading to a total of 18 studies, as demonstrated in Figure 1.[5,6, 9-11, 18-30] Table 1 outlines the study characteristics and Figure 2 outlines the overall quality assessment of all studies included. There was one study where ES was targeted using a CHD panel while the remainder used a whole ES approach.[9] Not all studies broke CHD down into isolated/multi-system or distinctive phenotypes as demonstrated or described the cardiac phenotype [Table 1].

Combined cohort outcomes

18-studies were included, encompassing n=636 CHD cases undergoing ES, of which n=529 stated whether CHD was isolated or associated with ECAs. Hence, 54.4% (n=288/529) of cases were isolated and 45.6% (n=241/529) multi-system CHD. Where available, the mean maternal age and gestation at the time of testing was 30 (+/-3.5 SD) years and 22 (+/-4.7) weeks. The primary genetic test performed prior to ES was CMA 98.0% (n=623/636) with the predominant source of fetal DNA from amniocytes 54.6% (n=322/590). Of the n=18 studies included, information regarding the originally recruited cohort prior to CMA/karyotype results were stated for n=5 studies.[5,6,9,11,24] These revealed that there was an abnormal CMA/karyotype in 21.0% (n=1109/5285) of cases. Where stated (n=261), the median turnaround time for ES was 42 (range 7-82) days and pregnancy outcome was reported in n=341, of which livebirth 47.8% (n=163) and termination of pregnancy 46.3%

(n=158) were the commonest outcomes. Where reported, the pooled incremental yields of VUS and IFS were 26% (95% CI, 14-39% p=0.0001) and 8% (95% CI, 0-17% p=0.0001).

Incremental yield of pathogenic variants

The pooled incremental yields (excluding VUS) from all 18-studies are illustrated in the forest plots for (i) all ; (ii) isolated and; (iii) multi-system CHD [Figure 3(a-c)]. In the cases of (ii) and (iii) 13 and 15-studies included relevant cases for inclusion. Incremental yields for the aforementioned groups were 21% (95% CI, 15-27% p=0.0006), 11% (95% CI, 7-15% p<0.00001) and 37% (95% CI, 18%-56% p<0.00001) respectively. The sub-analysis of studies with >20-cases (n=8) is demonstrated in Supplementary Figures 2a-c with corresponding funnel plots (Supplementary Figures 3a-c). Findings did not differ significantly from the primary analysis, apart from multi-system CHD, where the incremental yield was greater at 49% (95% CI, 17-80% p=0.003). Where gestational age was recorded in isolated CHDs the incremental yield for those diagnosed after 15-weeks' gestation was greater than for all cases at 24% (95% CI, 7%-41%, p=0.002, I^2=68%). In instances of multi-system CHD in the primary analysis, the commonest ECAs associated with a pathogenic variant were those affecting the genitourinary system 44.2% (n=23/52), nervous system 34.6% (n=18/52) and face 34.6% (n=18/52). In multisystem CHDs, where a pathogenic variant was detected and the specific ECA was documented (82.7%, n=43/52), there was one instance (2.3%, n=1/43) where a 'minor ECA' was present (single umbilical artery), with the remainder being major or affecting two or more systems.

On classification as per AHA/ACC criteria for all CHD, shunt lesions (septal anomalies and total anomalous pulmonary venous drainage) had the greatest pooled incremental yield of pathogenic variants 41% (95% CI, 19-63% p=0.003), followed by right-sided 26% (95% CI, 9-43%, p=0.001), complex 23% (95% CI, 9-36%, p=0.001) and left-sided obstructive lesions 18% (95% CI, 0-35% p=0.02). Where documented, pathogenic variants are described in Supplementary Table 1. Where pathogenic variants were documented (n=96/111; 86.5%), the commonest genetic syndromes identified were those of Kabuki syndrome (n=19/96; 19.8%), CHARGE (Coloboma-Heart defects-Atresia choanae-Retardation of growth-genital abnormalities-ear abnormalities) syndrome (n=8/96; 8.3%), Noonan syndrome (n=6/96; 6.3%) and Primary Ciliary Dyskinesia (n=6/96; 6.3%). In syndromes where CHD was typically described as being multi-system in nature, in 54.1% (n=20/37) of such syndromes only an isolated CHD was detected prenatally e.g. Adams-Oliver, CHARGE, Kabuki and Simpson-Golabi-Behmel syndrome. In the majority of instances pathogenic variants occurred *de novo* and in autosomal dominant (monoallelic) disease genes (68/96; 70.8%) [Supplementary Table 1].

DISCUSSION

This is the first systematic review assessing the yield of antenatal ES in prenatally diagnosed CHD in which CMAchromosome microarray/karyotype testing was negative. The results of this study show an apparent incremental yield of ES in CHDsupport the use of ES in the investigation of prenatally detected CHD. The diagnostic, yield is particularly high for shunt lesions and multi-system CHD. Most pathogenic variants occurred *de novo* and in autosomal dominant (monoallelic) disease genes with a high incidence of Kabuki syndromsyndrome. Thee majority were . A high number of pathogenic variants were reported in syndromes which typically present with ECAs yet presented with an isolated CHD.

The diagnostic yield from our own cohort study (12.7% all CHD) was modest compared to other studies included in the meta-analysis (range 0-40% all CHD). This is potentiallylikely to be secondary to several factors; (i) bias in case selection – while some studies in the review such as PAGE and Petrovski, *et al*.[5,6] presented both positive and negative ES results, smaller series may have had an element of selection bias only selecting cases with where there were positive results;[31] (ii) the proportion of multi-system CHD – the greater the proportion, n of these then the higher the overall yield and; (iii) the sequencing approach used e.g. targeted or whole exome; the series from Hu *et al*. (n=44 CHD cases)[9] revealed a high diagnostic yield when a targeted 77 cardiac gene panel approach was used (n=7; 15.9%). Of the 77 genes, only 5 genes were not included in the PAGE study panel, none of which were found to be causative of CHD in the Hu, *et al* study.[9] While use of targeted gene panels potentially have potential to provide a greater yield in a shorter time frame, users must

exert caution as they are primarily based upon postt~~t~~-natal and not prenatal phenotypes ~~which can differ from the prenatal phenotype where the diagnosis may be less definitive~~.[31]

The greater incremental yield with ES associated with multi-system vs. isolated CHD is similar to the pattern seen with aneuploidy and CNV, as is the case with shunt lesions and left-sided obstructive lesions.[15] Shunt lesions tend to be associated with ECAs which is probably why the diagnostic yield with ES in this group is most significantly enriched.[3,4] The predominance of *de novo* variants ~~occurring~~ in ~~autosomal dominant (~~monoallelic~~)~~ disease genes is also in keeping with published ~~published~~ evidence.[3,7,8,32] It is interesting that the most common syndromes unveiled in this study were those of Kabuki and CHARGE. Kabuki syndrome has a highly variable phenotype ~~with characteristic facies, abnormal growth, developmental delay and cardiac and renal anomalies~~.[33] There is limited evidence with regards the prenatal presentation and the high incidence as seen in this study has not been previously reported, although an overall association with postnatally diagnosed left-sided CHD ~~cardiac lesions~~ has been established.[33-35] Both CHARGE and Kabuki syndromes are caused by pathogenic variants in genes encoding proteins implicated in chromatin function and gene regulation.[36] ~~DNA methylation profiles are altered in both disorders[36] and epigenetic dysregulation was the commonest pathway linked to genetically characterised CHD in our own series and in the systematic review.~~ There~~There~~ is a potential link between these syndromes with an association between DNA methylation targets in their gene-specific signatures.[36] This reflects that epigenetic dysregulation is the commonest pathway responsible for the greatest proportion of CHD where pathogenic ~~single gene~~ variants were uncovered in this series.[36]

The strength of this study is the robust and systematic methodology utilised so that all available studies ~~of both a positive and negative nature~~ were included to limit selection bias. International collaboration between the two groups publishing the two largest series ~~to date~~ of prenatal congenital anomalies and ES has optimised the numbers ~~included~~. By excluding studies where phenotypes were based on ~~a~~ postnatal examination, our study is specific for prenatal ES testing focusing on ultrasound detected CHD. The quality of included studies based upon pre-specified criteria was optimal due to the high number ~~of studies~~ which had an ES approach to testing, variant interpretation based upon ACMG criteria and~~with~~ Sanger sequencing validation which meant ~~that~~ most ~~many of the studies included~~ had a uniform and hence comparable approach.[13]

The main study limitation ~~of the analysis~~ was high heterogeneity~~, notably in the multi-system group~~. This was likely caused by differing platforms used, as well as small-study effects~~, as~~ reflected in asymmetry within the funnel plots. However, limiting the inclusion of studies to those with >20 cases did n'~~o~~t show a significant difference in incremental yield. There is currently no recognised classification system for prenatal CHD hence ~~and in our study,~~ we selected an adult-based ~~classification~~ system.[12] This meant that rare CHD associated with high instances of perinatal ~~or in utero~~ demise ~~e.g. heterotaxy~~ could not be appropriately classified. Alternative classification systems were considered and experts were consulted, however ~~it was felt that~~ the categories included were too broad which mean that due to a restricted number of cases where the phenotype was described, relevant associations would not be identified.[37,38]

The challenges of ES in prenatally diagnosed CHD include; (i) the limited phenotype available from ultrasound imaging. Although ~~the~~ concordance is generally high, more information is typically gathered from detailed post-natal examination~~.~~[1,39,40]; (ii) whether targeted panels or a whole ES approach should be used and; (iii) that CHD tends to be a highly heterogenous group of anomalies with multi-gene and multifactorial pathologies which may not be unveiled with genomic testing.[3] Further novel gene discovery may lie in epigenomic or genomic changes encoding proteins involved in chromatin re-modelling, the RAS signalling pathway, ciliary function and sarcomere achitecture.[2] A further challenge with ES in pregnancy is the time constraint which it poses. ~~Turn-around time for prenatal ES was of limited value from the systematic review.~~ Several studies made an *a priori* decision to report the results after the end of the pregnancy and thus the clinical/laboratory pathways were~~are~~ not accelerated to achieve real time results to individual members of the study. However, several fetal ES studies have reported delivering results in a timely fashion to inform pregnancy management,[28] and a rapid fetal ES service will shortly be introduced in the English National Health Service for the diagnosis of monogenic disorders. As well as turnaround time, the clinical utility of ES in CHD ~~(as with other structural anomalies)~~ is dependent not just on the prospective targeting of phenotypes but also robust bioinformatics filtering within accredited ~~molecular~~ genomic~~etic~~ laboratories and ~~then~~ detailed analysis by clinical multidisciplinary review groups to assess and determine ~~assess variants and decide if they are~~ causative variants ~~of the phenotype~~. ~~In addition,~~ Ppre-test counselling must be accurate, clear and comprehensive with consideration given to ethical challenges. Without such robust bioinformatics and clinical screening of variants, prenatal ES should~~-~~ not~~not~~ be offered or used in clinical practice.[41,42]

In conclusion, despite the apparent incremental yield of prenatal ES in CHD, the routine application of such a policy would require the adoption of robust bioinformatic, clinical and ethical pathways. Whilst the highest yield is with multi-system anomalies, consideration may also be given to performing ES in the presence of isolated CHDs. ~~In conclusion, ES should be considered in CHD. Whilst the highest diagnostic yield is in cases with multisystem abnormalities, consideration should be given to offering it when CHD is isolated.~~ Further work is required to explore the benefits and challenges of delivering targeted or whole exome analysis. Clinical guidelines must be introduced to ensure that testing is correctly implemented.

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CONFLICT OF INTEREST

RYE and JL reports grants from the Health Innovation Challenge Fund during the conduct of the PAGE study. DJM reports grants for travel expenses from Congenica to attend educational symposia during the conduct of the PAGE study. MEH reports grants from the Wellcome Trust and the UK Government Department of Health during the conduct of the study and personal fees from Congenica, outside the submitted work. MDK is a member of Illumina's International Perinatal Advisory Group but receives no payment for this. ERM has received travel expenses, accommodation and consultant fees for participating in an Illumina International Advisory Group after completion of the PAGE study. MDK is funded

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LEGENDS FOR ILLUSTRATIONS

Figure 1 - Flowchart demonstrating included studies

Figure 2 – Quality assessment for studies in the systematic review (n=18) using modified STARD criteria

Figure 3 - Forest plots of incremental yield by exome sequencing over karyotype/microarray in fetuses with prenatally detected cardiac anomalies in (a) all; (b) isolated and; (c) multi-system cardiac anomalies. Only first author of each study is given. [CMA = chromosome microarray; M–H = Mantel–Haenszel].

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Study	ES Approach	Number of Cardiac anomalies		
		All cardiac	Isolated cardiac	Multi-system cardiac
Aarabi *et al.** [26]	WES Trio 20,000 gene panel 60-140X coverage	4	2	2
Boissel *et al.* [20]	WES Trio 110X coverage Agilent capture + Illumina HiSeq 2000 or 2500	11	2	9
Carss *et al.* [21]	WES Trio 103X coverage Agilent capture + Illumina HiSeq	3	2	1
Daum *et al.** [22]	WES Mainly proband only Agilent capture+ Illumina HiSeq 2500	5	1	4
De Koning *et al.* [30]	WES Trio 1128 genes 80X coverage Agilent capture + NextSeq 500	10	2	8
Drury *et al.** [23]	WES Mainly proband only TruSeq Exome + Illumina HiSeq 1000 or Illumina Nextera Rapid Exome kit + HiSeq 2500	3	1	2
Fu *et al.* [24]	WES Mainly proband only 120X coverage Agilent capture+ Illumina HiSeq 2500	34	29	5
Hu *et al.* [9]	CE Proband only 77 genes NimbleGen SeqCap EZ targeted capture Illumina Hiseq 2500 98.9% coverage of targeted region	44	N/S	N/S
Leung *et al.* [18]	WES Trio 100X coverage TruSeq Rapid Exome Library Prep Kit Illumina sequencing	7	4	3
Lord *et al.* [5]	WES Trio 1628 genes Agilent capture + Illumina Hi-Seq 2500 98.3% of the bait regions covered at a minimum depth of 5X	197	122	75
Normand *et al.* [28]	WES Trio Coverage 150X Roche NimbleGen capture Illumina Genome Analyzer IIx platform or HiSeq 2000	37	N/S	N/S
Petrovski *et al.* [6]	WES Trio Nimblegen SeqCap EZ capture + Illumina Hiseq 2500 Average read coverage 89.3 reads Bioinformatic signatures	143	50	93
Stals *et al.* [25]	WES Parents only 80X coverage Agilent capture + Illumina HiSeq 2500 or NextSeq500	8	2	6

	Only include het rare (MAF<0.001) variants in same gene in both parents			
Sun *et al.** [11]	WES Trio Agilent capture + Illumina Hiseq 4000 or Novaseq	66	55	11
Vora *et al.** [29]	CE and WES Trio Illumina Hi-Seq 2500	3	0	3
Westerfield *et al.* [27]	WES Trio 130X coverage Roche NimbleGen capture + Illumina Genome Analyzer IIx or HiSeq 2000	5	0	5
Westphal *et al.* [10]	WES Trio 20,000 genes 150X coverage	30	16	14
Yates *et al.* [19]	WES Trio 140X coverage Agilent capture + Illumina HiSeq 2000 or 2500	26	N/S	N/S

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655 Table 1- Study characteristics and rates of pathogenic variants and variant of uncertain

656 significance [CE=Clinical Exome; N/S = not-stated; WES=Whole exome sequencing *coverage

657 not stated]

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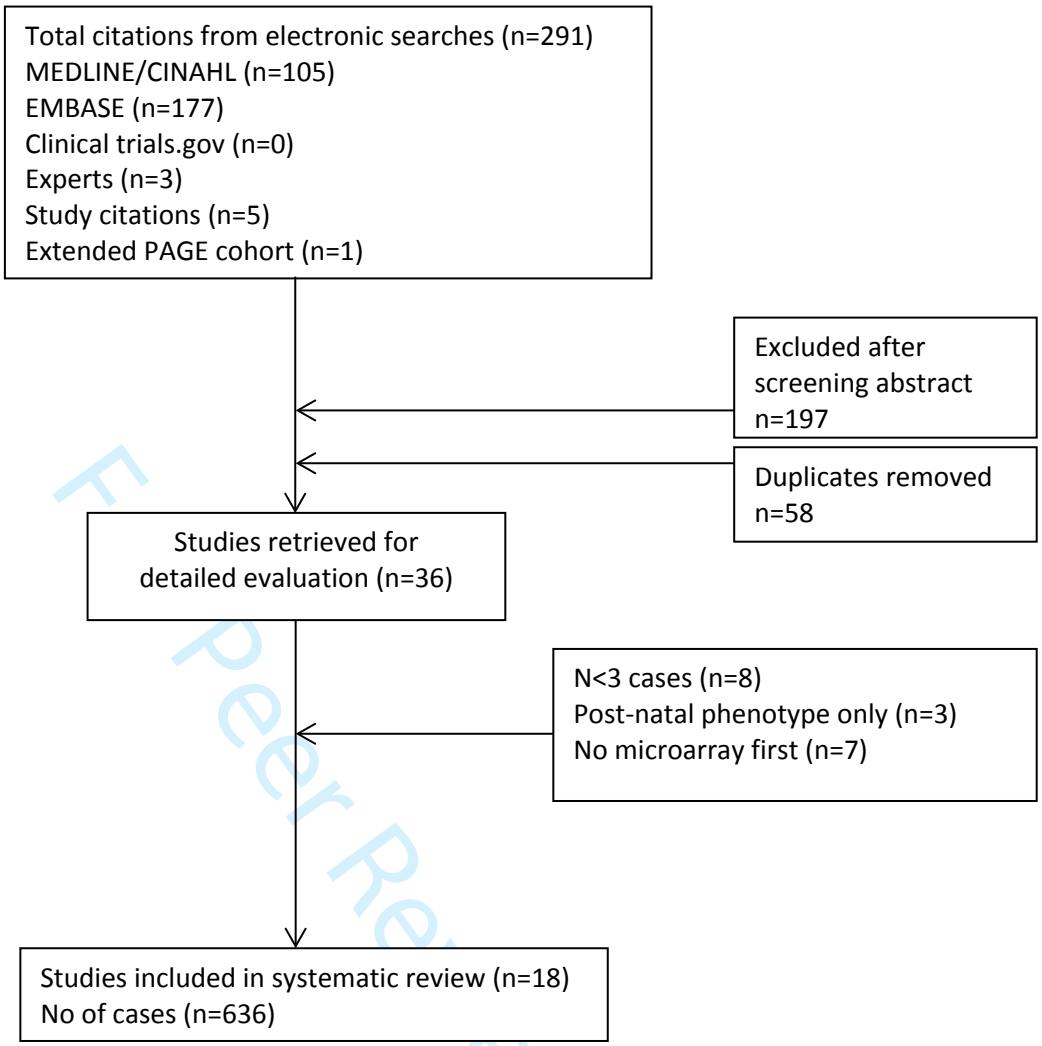

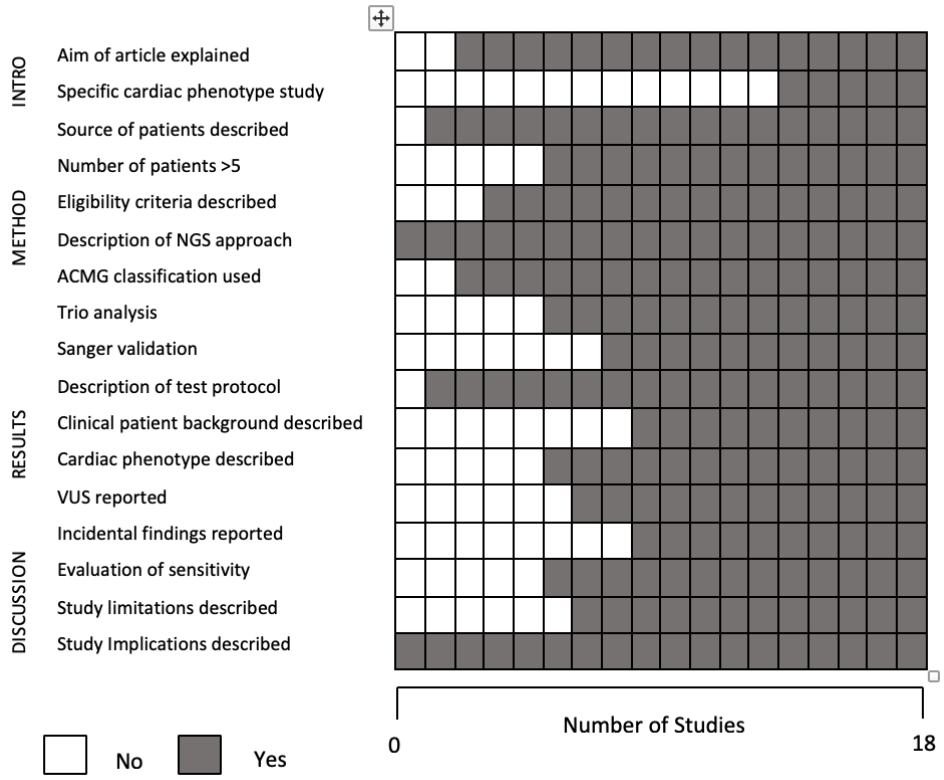

Quality assessment for studies in the systematic review (n=18) using modified STARD criteria

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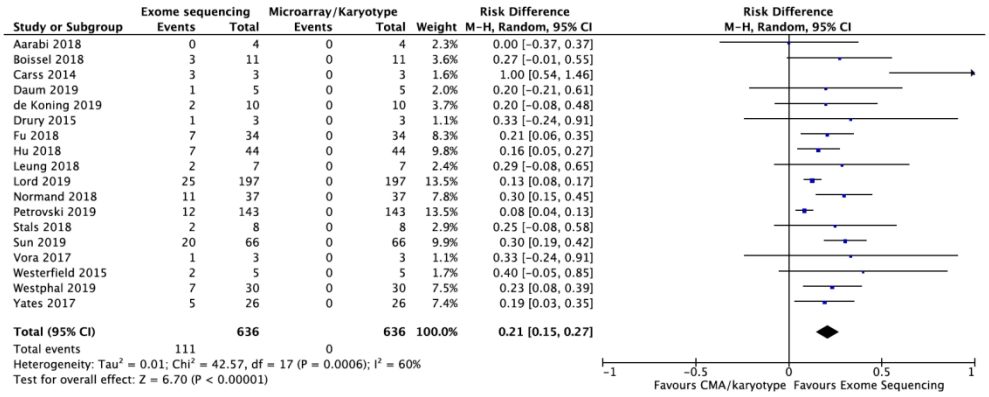

Figure 3 - Forest plots of incremental yield by exome sequencing over karyotype/microarray in fetuses with prenatally detected cardiac anomalies in (a) all; (b) isolated and; (c) multi-system cardiac anomalies. Only first author of each study is given. [CMA = chromosome microarray; M–H = Mantel–Haenszel].

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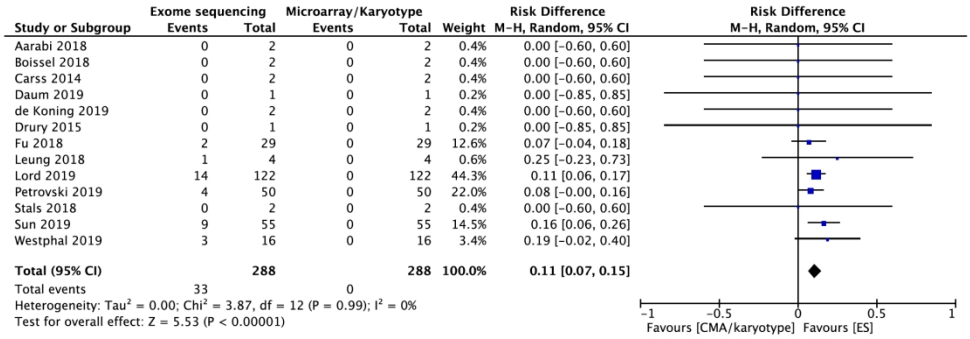

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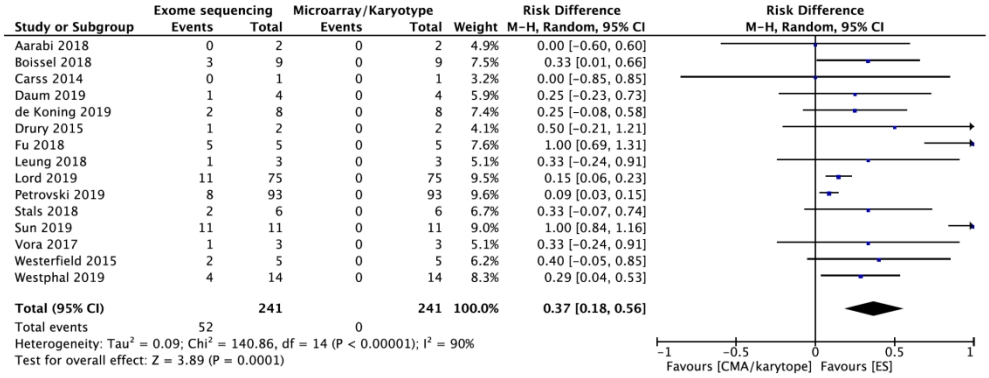

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ID	Cardiac Phenotype	Additional systems	AHA/ACC Class	Variant	Zygosity	Monoallelic (M) or Biallelic (B)	Clinical syndrome	Reference
SR-001	ASD, PLSVC	Brain, Face, GU	1	CHD7 c.2362C>T (p.Gln788Ter*)	Het	M	CHARGE	22
SR-007	PA dilatation, PLSVC	Extremities, Face	3	TGFBR1 c.605_606insGAGAACTATTGT (p.A202delinsARTIV)	Het	M	Loeys-Dietz syndrome 1	20
ST-008	VSD	GU, Thorax, GI	1	FRAS1 c.370C>T (p.R124X)	Hom	B	Fraser 1	20
SR-009	TOF	GI	3	CHD7 c.5428C>T (p.R1810X)	Hom	M	CHARGE	20
SR-019	COA	Skeleton, Thorax	2	C5orf42 c. 8167C > T (p.Gln2723*) + c.8628C > T (p.Ser2876Ser)	Comp het	B	Oral facial digital type VI	23
SR-024	TOF	Extremities, Face	3	ASPH (p.X226E)	Hom	B	Traboulsi	24
SR-025	Single atrium, single ventricle, PS, RA isomerism		4	DNAH11 c.3426-1G>A	Hom	B	PCD 7, with or without situs inversus	24
SR-026	TGA	GU, Skeleton	4	NEK8 IVS10-1G>A	Hom	B	Renal–hepatic–pancreatic dysplasia 2 [615415]/nephronophthisis 9	24
SR-027	TOF	Face	3	IL11RA (Q159X)	Hom	B	Cariosynostosis and dental anomalies	24
SR-028	VSD		1	ANKRD11 (p.S1271X)	Het	M	KBG	24
SR-029	VSD	Brain	1	MRPS22 IVS5+1G>A (p.Q337X)	Comp het	B	Combined oxidative phosphorylation deficiency 5	24
SR-030	Univentricular	Brain	4	AHI1 (p.E1086G)	Hom	B	Joubert syndrome 3	24
SR-059	Heterotaxy		4	DNAH11 c.13288G>A p.(Gly4430Glu) and c.8533_8536delinsATCCG	Comp het	B	PCD 7, with or without situs inversus	18
SR-060	PA		3	CHD7 c.2957+1G>A	Het	M	CHARGE	18
SR-066	TOF		3	CHD7 c.2550_2554delGA GAA (p.K850Nfs*6)	Het	M	CHARGE	9
SR-067	ASD, VSD		1	CITED2 c.574_579delAGC GGC (p.S192_G193del)	Het	M	ASD 8, VSD2	9
SR-068	Single atrium, single ventricle, AA		4	MYH6 c.2168+1G>A	Het	M	ASD 3; cardiomyopathy, dilated, 1EE; cardiomyopathy, familial hypertrophic, 14; sick sinus syndrome	9
SR-069	Cardiac anomaly	GU	6	KMT2D c.11248C>T (p.Q3750*)	Het	M	Kabuki 1	9
SR-070	Extracorporeal heart, VSD	GU	5	ZFPM2 c.2107A>C (p.M703L)	Het	M	Diaphragmatic hernia 3; TOF	9
SR-071	VSD	GU	1	KMT2D c.12140_12168del GGCCGTTAGCAAT AGGAACTACCCCTGAG (p.G4047Vfs*5)	Het	M	Kabuki 1	9
SR-072	Cardiac anomaly	Skeleton	6	JAG1 c.1078 T>G (p.C360G)	Het	M	TOF, Alagille syndrome	9

ID	Cardiac Phenotype	Additional systems	AHA/ACC Class	Variant	Zygosity	Monoallelic (M) or Biallelic (B)	Clinical syndrome	Reference
SR-115	HPV, MGA, TA, VSD.	GI	4	MYH7 c.1727A>G (p.His576Arg)	Comp het	B	Hypertrophic cardiomyopathy 1	10
SR-117	AVSD, DV agenesis	Brain, Face, Skin	1	PTPN11 c.214G>A (p.Ala72Thr)	Het	M	Noonan 1	10
SR-119	DORV, PAPVC	GI	4	DNAI1 c.1003G>T (p.Val335Phe) and c.1543G>A (p.Gly515Ser)	Comp Het	B	PCD, 1, with or without situs inversus	10
SR-126	SYPCA, left SVC, PA, VSD	Face, Extremities, Skin	3	Microdeletion 9q34.3 (approx. chr9:139252466-139418430, including NOTCH1)	Het	M	Adams-Oliver 5	10
SR-127	PA, SYPCA, VSD		3	c.385G>A (p.Glu129Lys)	Het	M	Tetralogy of Fallot	10
SR-128	PA, UV, VSD	GI, Skin	3	c.1372C>T (p.Arg458*) and c.281G>C, (p.Arg94Pro)	Comp Het	B	Heterotaxy, visceral 7	10
SR-130	AA, HRV, MGA		4	PUM1 c.1738C>T (p.Arg580*)	Het	M		10
SR-133	HLHS		4	KMT2D c.11093dup (p.Phe3699Leufs*14)	Het	M	Kabuki 1	10
SR-149	Hypertrophic cardiomyopathy	Brain, Skin, Thorax	5	MRPS22 p.[(Arg170His)];[?] c.[509G>A];[878+1G>T]	Comp Het	B	Combined oxidative phosphorylation deficiency 5	25
SR-150	Hypertrophic cardiomyopathy	GU, Thorax	5	FRAS1 c.[5530-2A>C];[6010G>A] (p.[?];[Gly2004Ser])	Comp Het	B	Fraser 1	25
SR-151 †	VSD, overriding aorta,	Brain, Extremities, Face, GI, Spine	1	PORCN c.90G>A (p.Trp30Ter)	Het	M		5
SR-152*	TR, ECF, PA atresia, HAA, aberrant retro-oesophageal left subclavian artery, dilated left ventricular chamber	Face, Skin, Spine	4	NRAS c.34G>C (p.Gly12Arg)	Het	M	Noonan 6	5
SR-153*	ECF, TR	GU, Skeleton, Skull	5	TCTN2 c.1506-2A>G	Hom	B	Joubert 24	5
SR-154*	Dilated heart, pericardial effusion	GI, Growth	5	COQ9 c.730C>T (p.Arg244Ter)	Hom	B	Coenzyme 10 deficiency	5
SR-155 †	TOF	Brain, GI, Growth, Skin, Extremities	3	FGFR3 c.749C>G (p.Pro250Arg)	Het	M	Thanatophoric dysplasia	5
SR-156*	Truncus arteriosus	Brain, Face, Extremities	4	CHD7 c.988C>T (p.Gln330Ter)	Het	M	CHARGE	5
SR-157*	Cardiac anomaly	Skeleton	6	EVC2 c.3637_3638insTT (p.Trp1213PhefsTer11)	Hom	B	Ellis-van Creveld	5
SR-158*	Bilateral SVCs	Extremities, Skeleton	5	FLNB c.4750G>C (p.Ala1584Pro)	Het	M		5
SR-159*	TOF	Brain, Extremities, Face, Growth, GU	3	RAB23 c.434T>A (p.Leu145Ter)	Hom	B	Carpenter	5

ID	Cardiac Phenotype	Additional systems	AHA/ACC Class	Variant	Zygosity	Monoallelic (M) or Biallelic (B)	Clinical syndrome	Reference
SR-160*	Anomalous pulmonary vessel connection, VSD	Face	1	CHD7 c.757del (p.Val253CysfsTer52)	Het	M	CHARGE	5
SR-161*	TGA, R aortic arch		4	SOS1 c.796_797insAAG (p.Thr266delinsLysAla)	Het	M	Noonan 4	5
SR-162*	Rhabdomyomas		5	PKD1/TSC2 41.2kb deletion	Het	M	Tuberous sclerosis 2	5
SR-163 †	HLHS		4	KMT2D c.11848C>T (p.Gln3950Ter)	Het	M	Kabuki 1	5
SR-165*	AVSD		1	DNAH11 stopped gain	Hom	B	PCD 7, with or without situs inversus	5
SR-166*	AVSD		1	GATA4 frameshift variant	Het	M		5
SR-167*	AS		2	RIT1 c.335G>C (p.Gly112Ala)	Het	M	Noonan 8	5
SR-168*	AVSD		1	ANKRD11 c.5957_5958del (p.Arg1986IlefsTer45)	Het	M	KBG	5
SR-169*	Cardiac anomaly		6	NR2F2 c.745T>C (p.Trp249Arg)	Het	M	Congenital heart defects, multiple types	5
SR-170*	Right atrial isomerism		4	CCDC103 c.461A>C (p.His154Pro)	Hom	B	PCD	5
SR-172*	Cardiac anomaly		6	KMT2D c.673+1G>A	Het	M	Kabuki 1	5
SR-173*	Cardiac anomaly		6	CHD7 c.656dup (p.Leu220ProfsTer67)	Het	M	CHARGE	5
SR-338 †	TOF		3	GPC3 c.677del (p.Thr226IlefsTer8)		M	Simpson-Golabi-Behmel 1	5
SR-341*	Cardiac anomaly		6	TAB2 c.1407_1408del (p.Pro470GlnfsTer2)	Het	M	Congenital heart defects, non-syndromic 2	5
SR-347 †	TOF		3	DNAH5 frameshift variant	Hom	B	PCD 3, with or without situs inversus	5
SR-351	VSD	GU, thorax	1	NIPBL c.459-2A>G	Het	M	Cornelia de Lange type 1	27
SR-354	VSD	Extremities, GU, Skin, Thorax	1	WDR19 c.275>G (p.L92X) and c.880G>A (p.G294R)	Comp Het	B	Short rib thoracic dysplasia, 5, with or without polydactyly	27
SR-357	MGA	Extremities, GU Skull	4	DYNC2H1 c.10594C>T (p.Arg3532Ter) and c.8012T>C (p.Met2671Thr)	Com Het	B	Short rib polydactyly, 3, with or without polydactyly	29
SR-361	DORV and RAA	GU	4	CHD7 c.7890T>A (p.Cys2360*)	Het	M	CHARGE	30
SR-370	left heart obstruction (Shone's complex)	Growth, GU	2	KTM2D c.207T>A (p.Cys69*)	Het	M	Kabuki 1	30
SR-374	Complex cardiac anomaly		4	KMT2D c.6617dupC (p.A2207fs)	Het	M	Kabuki 1	28
SR-375	Complex cardiac anomaly	GU	4	KMT2D c.1967delT (p.L656fs)	Het	M	Kabuki 1	28
SR-376	Complex cardiac anomaly	GU	4	KMT2D c.15680_15693dup (p.I5232fs)	Het	M	Kabuki 1	28
SR-377	Complex cardiac anomaly	GU	4	KMT2D c.5705C>T (p.R10903X)	Het	M	Kabuki 1	28
SR-378	Cardiac anomaly	Skeleton	6	COL1A2 c.2576G>A (p.G859D)	Het	M	OI types 2-4 and Ehlers Danlos type 7B and cardiac valvular	28
SR-379	Cardiac anomaly	Brain, GU, Skeleton	6	DVL1 c.1519delT (p.W507fs)	Het	M	Robinow autosomal dominant 2	28

ID	Cardiac Phenotype	Additional systems	AHA/ACC Class	Variant	Zygosity	Monoallelic (M) or Biallelic (B)	Clinical syndrome	Reference
SR-411	Cardiomyopathy	Brain, Skin	5	MRPS22 c.768_769 and p.R170H	Comp Het	B	MRPS22-related mitochondrial dysfunction	19
SR-412	Cardiomegaly	Skin	5	CYP11A1 (p.R120X)	Hom	B	Adrenal insufficiency, congenital, with 46XY sex reversal, partial or complete	19
SR-413	Cardiac axis deviation	Brain, Extremities	5	FANCB c.987_990del	Hemi	M	Fanconi anaemia, complementation group B	19
SR-414	Cardiac anomaly	Brain, Extremities, Face, Skull	6	AMER1 c.705delT	Hemi	M	Osteopathia striata with cranial sclerosis	19
SR-415	Cardiac anomaly	Brain, Skin	6	RIT1 p.F82C	Het	M	Noonan 8	19
SR-416	Cardiac anomaly	Brain	6	PIK3R2 p.K564E	Het	M	Megalencephaly-polymicrogyria-polydactyly-hydrocephalus	19
SR-437	COA	Face, GI, GU	2	KMT2D c.8430dupA (p.Gln2811Thrfs*34)	Het	M	Kabuki 1	11
SR-438	HLHS		4	KMT2D c.15920_15921+2delC (p.Leu5380Alafs*36)	Het	M	Kabuki 1	11
SR-439	Aortic valve atresia		2	KMT2D c.8074_8075delCG (p.Arg2692Alafs*31)	Het	M	Kabuki 1	11
SR-440	HLHS		4	KMT2D c.1845_1846del (p.Leu617Phefs*5)	Het	M	Kabuki 1	11
SR-441	Mitral atresia	Face	2	KMT2D c.6595delT (p.Tyr2199Ilefs*65)	Het	M	Kabuki 1	11
SR-442	HLHS		4	KMT2D c.8159G>A (p.Trp2720*)	Het	M	Kabuki 1	11
SR-443	HLHS		4	KTM2D c.16489_16491del (p.Ile5497del)	Het	M	Kabuki 1	11
SR-444	COA		2	NOTCH1 c.3643+1G>A	Het	M	Adams-Oliver 5	11
SR-445	HLHS		4	NOTCH1 c.4015-2A>G mat	Het	M	Adams-Oliver 5	11
SR-446	AS		2	NOTCH1 c.4837C>T (p.Gln1613*)	Het	M	Adams-Oliver 5	11
SR-447	HLHS		4	NOTCH1 c.2452dupC (p.Leu818Profs*10)	Het	M	Adams-Oliver 5	11
SR-448	COA	Thorax	2	MYRF c.789delC (p.Ser264Alafs*8)	Het	M	Cardiac-urogenital syndrome	11
SR-449	HLHS	Thorax	4	CRB2c.2029C>T (p.Arg677Cys) and c.3076_3077insTGGCGCGGCCCCGGCCCGGCGCGGCCCC (p.Arg1038Alafs*45)	Het	B		11
SR-545	Rhabdomyomas	Brain	5	TSC2 Chr 16, 2120571, C→T 1831C→T, Arg611Trp	Het	M	Tuberous sclerosis 2	6
SR-546	VSD	GU	1	PKD1 Chr 4, 88983135, ACT→A 2101_2102delTC, Ser701ArgfsX9	Het	M	MPKD	6
SR-557	COA	Growth, GU, Spine	2	KMT2D Chr 12, 49443635, TAG→T 3734_3735delCT, Ser1245TyrfsX4	Het	M	Kabuki 1	6
SR-561	VSD		1	MYL2 484G→A (p.Gly162Arg)	Het	M	Familial hypertrophic cardiomyopathy	6

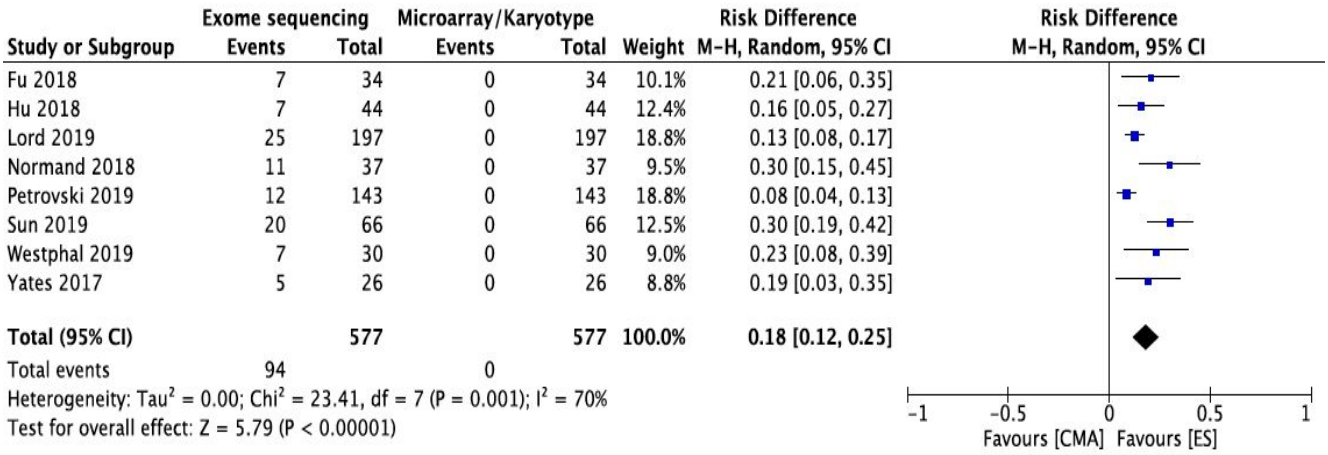
ID	Cardiac Phenotype	Additional systems	AHA/ACC Class	Variant	Zygosity	Monoallelic (M) or Biallelic (B)	Clinical syndrome	ID
SR-569	TGA	Brain	4	COL4A1 Chr 13, 110830552, C→G 2485G→C (p.Gly829Arg)	Het	M		6
SR-576	Rhabdomyomas		5	TSC2 Chr 16, 2138293, CCGGCTCCGCCACATCAAG→C 5037_5054delC_A, His1679_Arg1684del	Het	M	Tuberous sclerosis 2	6
SR-592	TOF		3	NIPBL variant	Het	M	Cornelia de Lange 1	6
SR-598	Cardiac anomaly	Neck/Skin	6	NR2F2 variant	Het	M	Congenital heart defects, multiple, type 4	6
SR-612	Cardiac anomaly	Face, Skeleton, Thorax	6	SCN2A variant	Het	M	Epileptic encephalopathy, early infantile, 11	6
SR-613	Cardiac anomaly		6	LZTR1 variant	Comp Het	B	Noonan 2	6
SR-635	TOF	GU	3	KMT2D variant	Het	M	Kabuki 1	6

Table S1 – Diagnostic variants identified from the systematic review

[Abbreviations; AA = aortic atresia; ADPKD; Autosomal dominant polycystic kidney disease; AHA/ACC = American Heart Association/American College of Cardiology; AS = aortic stenosis; ASD = atrial septal defect; AVSD = atrial-ventricular septal defect; B = Biallelic; COA = coarctation of the aorta;; DOLV = Double outlet left ventricle; DORV = double outlet right ventricle; DV = ductus venosus; ECF = echogenic cardiac focus; GI = Gastrointestinal; GU = Genitourinary; HAA = hypoplastic aortic arch; HLHS = hypoplastic left heart; HPV; hypoplastic pulmonary veins; HRHS = hypoplastic right heart; HRV = hypoplastic right ventricle; MGA = malposition of great arteries; PA = pulmonary atresia; PAPVC = partial anomalous pulmonary venous connection; PCD = Primary Ciliary Dyskinesia; PLSVC = partial left superior vena cava; PS = pulmonary stenosis; SVC = superior vena cava; TA = tricuspid atresia; TGA = transposition of the great arteries; TOF=Tetralogy of Fallot; TR = tricuspid regurgitation; UV = univentricular; VSD = ventricular septal defect] *previously reported in PAGE study publication; † unreported in PAGE study publication. AHA/ACC Criteria 1= Shunt lesions; 2= left-sided obstructive lesions; 3= right-sided lesions and; 4= complex lesions; 5=miscellaneous; 6=uncategorised).

Figure S2 – Forest plots of studies with >20 cases reporting on reporting on incremental yield of exome sequencing over microarray/karyotyping in fetuses with congenital heart disease (CHD)

S2a – All CHD

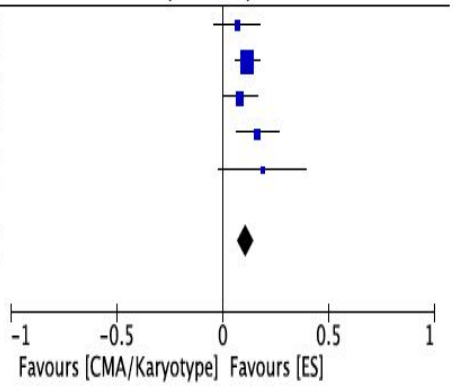


S2b – Isolated CHD

Study or Subgroup	Exome sequencing Events	Total	Microarray/Karyotype Events	Total	Weight	Risk Difference M-H, Random, 95% CI
Fu 2018	2	29	0	29	13.0%	0.07 [-0.04, 0.18]
Lord 2019	14	122	0	122	45.7%	0.11 [0.06, 0.17]
Petrovski 2019	4	50	0	50	22.7%	0.08 [-0.00, 0.16]
Sun 2019	9	55	0	55	15.0%	0.16 [0.06, 0.26]
Westphal 2019	3	16	0	16	3.6%	0.19 [-0.02, 0.40]
Total (95% CI)		**272**		**272**	**100.0%**	**0.11 [0.07, 0.15]**
Total events	32		0			

Heterogeneity: $Tau^2 = 0.00$; $Chi^2 = 2.80$, df = 4 (P = 0.59); $I^2 = 0\%$

Test for overall effect: Z = 5.53 (P < 0.00001)



S2c – Multi-system CHD

Study or Subgroup	Exome sequencing Events	Total	Microarray/Karyotype Events	Total	Weight	Risk Difference M-H, Random, 95% CI
Fu 2018	5	5	0	5	17.8%	1.00 [0.69, 1.31]
Lord 2019	11	75	0	75	21.3%	0.15 [0.06, 0.23]
Petrovski 2019	8	93	0	93	21.4%	0.09 [0.03, 0.15]
Sun 2019	11	11	0	11	20.5%	1.00 [0.84, 1.16]
Westphal 2019	4	14	0	14	19.0%	0.29 [0.04, 0.53]
Total (95% CI)		**198**		**198**	**100.0%**	**0.49 [0.17, 0.80]**
Total events	39		0			

Heterogeneity: $Tau^2 = 0.12$; $Chi^2 = 137.35$, df = 4 ($P < 0.00001$); $I^2 = 97\%$

Test for overall effect: Z = 3.00 (P = 0.003)

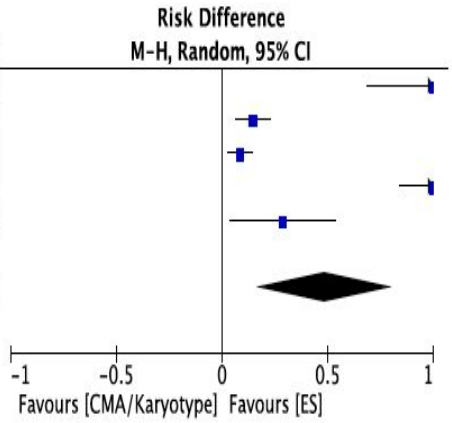
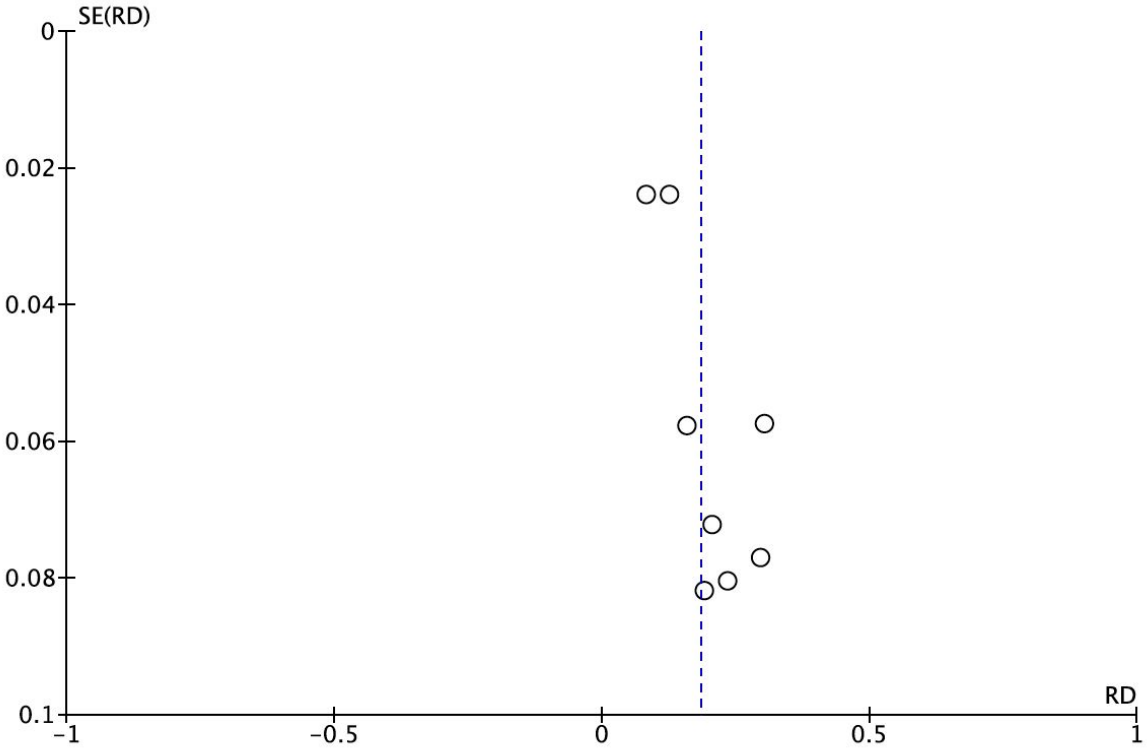


Figure S3 Funnel plots of studies with >20 cases reporting on incremental yield of exome sequencing over microarray/karyotyping in fetuses with congenital heart disease (CHD)



S3a – All CHD

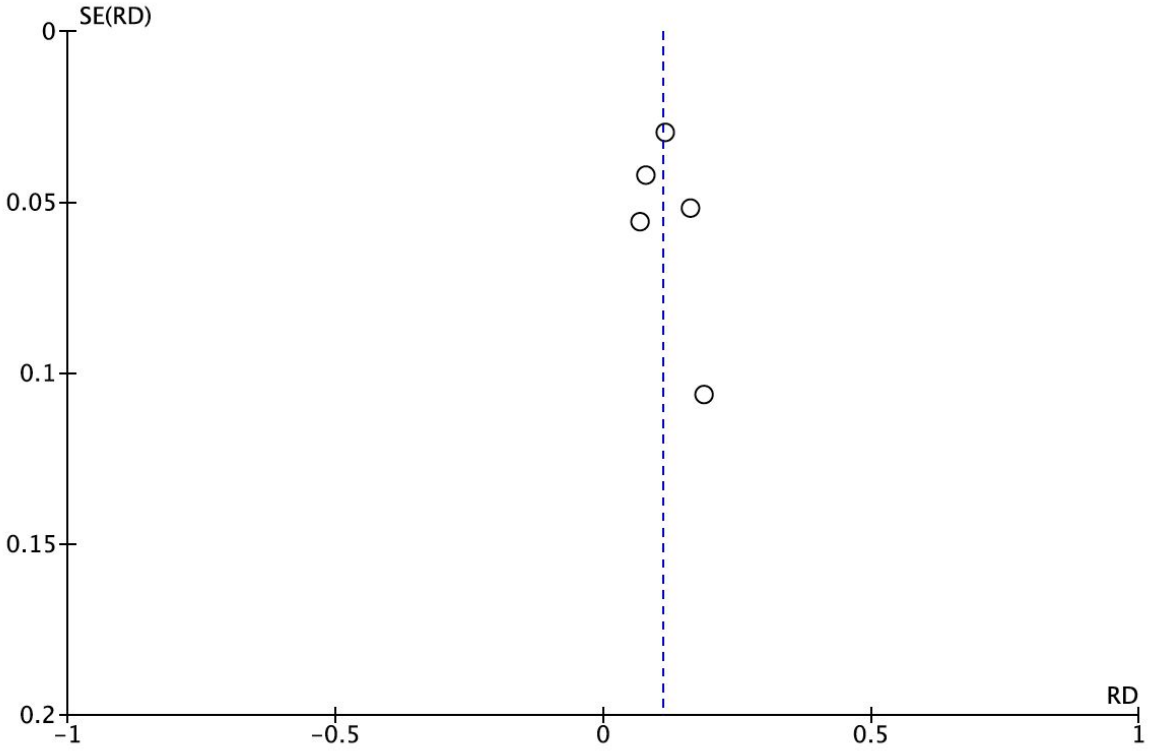
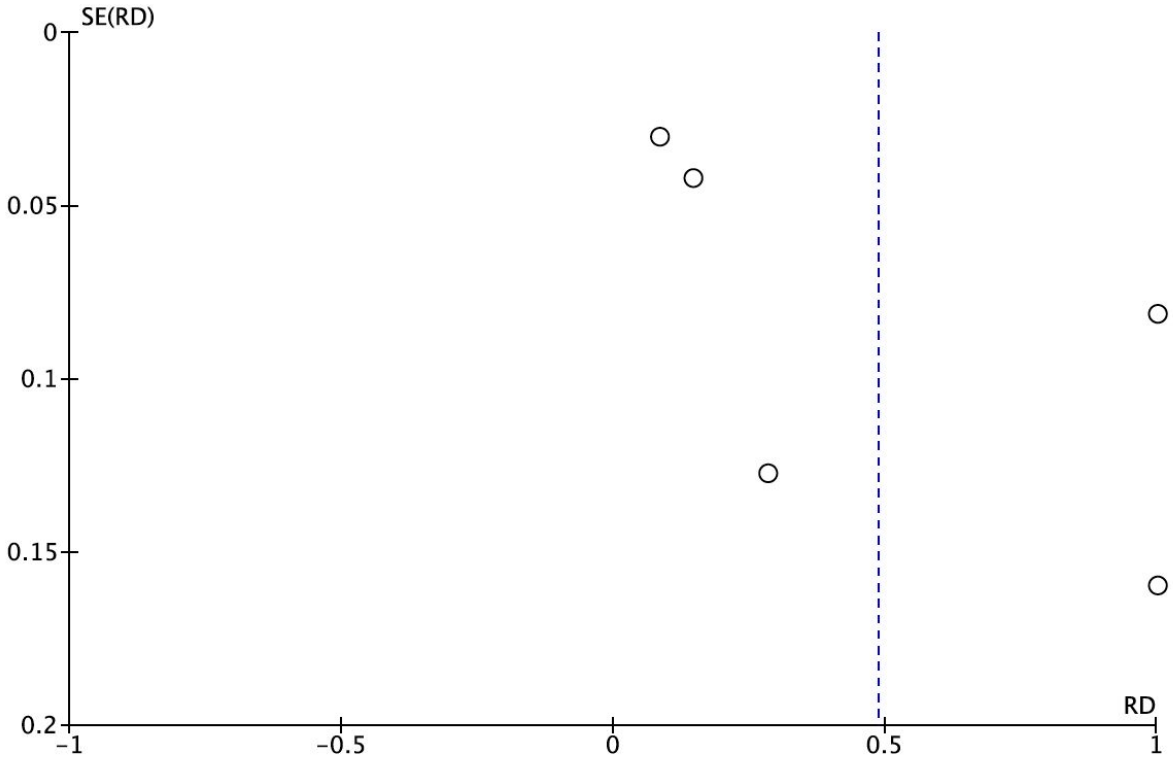


Figure S3b – Isolated CHD



S3c – Multi-system CHD

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	3
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	6
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	6-7
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	6
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	6
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	6
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	6
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	6
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	7-8
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	7-8
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	7-8

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	7-8
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	7-8
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	Fig 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	10
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	Fig 3, 14
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	Fig 3
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	10
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	10-11
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	10-11
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	12-14
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	14
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	15
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	16

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

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