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The Diagnostic Yield of Prenatal Genetic Technologies in Congenital Heart Disease: A Prospective Cohort Study

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Keywords
Congenital heart disease · Cardiac · Chromosome microarray · Exome sequencing · Aneuploidy · Prenatal diagnosis · Genetic

Abstract
Introduction: The objective was to evaluate: (i) the proportion of prenatally diagnosed congenital heart disease (CHD) associated with an abnormal quantitative fluorescence-PCR (QF-PCR), chromosome microarray (CMA), and exome sequencing (ES) result; and (ii) the diagnostic yield of these technologies based on CHD category and presence of extracardiac anomalies (ECAs). ***Methods:*** This prospective cohort study was set across 12 UK foetal medicine centres. All cases underwent QF-PCR, CMA, and ES, and the diagnostic yield in $n = 147$ cases of prenatally diagnosed CHD was assessed. ***Results:*** In 34.7% ($n = 51/147$), a genetic diagnosis was obtained. Using a stepwise testing strategy, the diagnostic yield for QF-PCR, CMA, and ES was 15.6% ($n = 23/147$), 13.7% ($n = 17/124$), and 10.2% ($n = 11/107$), respectively. Abnormal QF-PCR/shunt (septal) defects 31.4% ($n = 11/35$), $p = 0.046$, and abnormal CMA/conotruncal anomalies 22.7% ($n = 10/44$), $p = 0.04$, had significant associations. Monogenic variants were commonest in complex CHD 36.4% ($n = 4/11$). Multisystem CHD had a greater diagnostic yield overall compared to isolated OR 2.41 (95% CI, 1.1–5.1), particularly in association with brain and gastrointestinal tract anomalies. The proportion of variants of uncertain significance was 4.7% ($n = 5/107$) with ES, with none in the CMA group. ***Conclusion:*** In the era of prenatal ES, there remains an important role for QF-PCR and CMA. Identification of monogenic pathologic variants further allows delineation of prognosis in CHD.

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Introduction

Congenital heart disease (CHD) affects up to 12 per 1,000 live births and is more prevalent in utero [1]. CHD is a common foetal anomaly, but prognosis is not just dependent upon the structural complexity of the anomaly but also the underlying aetiology [2]. As survival rates continue to improve with advances in paediatric surgical interventions and intensive care, establishing a diagnosis prenatally is associated with improved neonatal survival rates to surgery as well as a reduction in early neurological morbidity [3–5]. Prenatally the type and complexity of CHD, as well as the presence of extra-cardiac anomalies (ECAs) (present in a third of cases) and/or underlying genetic abnormality (present in 35% of cases) compound adverse perinatal outcome [5–9]. The underlying cause of CHD is poorly understood but is proposed to be multifactorial, with a combined environmental-genetic influence and the potential of multiple interacting gene loci of variable penetrance, which makes establishment of a genetic cause challenging [8, 10]. Obtaining a unifying genetic diagnosis is important not only in prospective counselling relating to additional burden of disease but also in informing decisions relating to the pregnancy course and neonatal care. Furthermore, such information may inform recurrence risk rates in future pregnancies and potential pre-implantation or early prenatal testing [11]. The evolution of prenatal genomic testing over the last decade has progressed from the detection of aneuploidy with G-banding karyotype only, to quantitative fluorescence-PCR (QF-PCR), through to submicroscopic chromosomal detection of copy-number variation with chromosome microarray (CMA) and now the potential of identification of monogenic variants with next-generation sequencing (NGS) [12]. While studies have individually attempted to quantify the proportion of CHD attributable to the aforementioned testing strategies, there are limited studies which have provided a unified breakdown from a single cohort which can aid clinicians in the counselling of patients and in development of a prenatal genomic pathway. Hence, the objectives of this study were to evaluate: (i) the proportion of prenatally diagnosed CHD associated with abnormal QF-PCR, CMA, and exome sequencing (ES); and (ii) the diagnostic yield of the aforementioned genomic technologies based upon the category of CHD and the presence of ECAs.

Table 1. Demographics and outcomes of study cohort

Variable	Outcome Mean (±SD) or *N* (%)
Age, years	30.2 (±5.73)
Parity	0.86 (±1.09)
Ethnicity, *N* = 147	
Caucasian	109 (74.1)
African-Caribbean	6 (4.1)
South Asia	32 (21.8)
Prior pregnancy losses, *N* = 119	53 (44.5)
Gestation at testing, wks	21.7 (±4.43)
Singleton pregnancy, *N* = 138	132 (95.7)
Consanguineous, *N* = 135	7 (5.2)
Source of DNA, *N* = 147	
Amniocytes	121 (82.3)
Chorionic villi	15 (10.2)
Foetal blood	5 (3.4)
Foetal tissue	6 (4.1)
Pregnancy outcome, *N* = 137	
Live birth	55 (40.1)
Miscarriage	1 (0.7)
Termination of pregnancy	71 (51.8)
Stillbirth	5 (3.6)
Neonatal death	5 (3.6)

Materials and Methods

This prospective cohort study based upon the extended Birmingham arm of the Prenatal Assessment of Genomes and Exomes (PAGE) study, including probands (as trios) recruited from 12 sites in England and Scotland recruited between October 2014 and June 2017 [13]. PAGE sought to recruit 1,000 trios (foetus and both parents) in instances where there was a foetal structural anomaly (FSA) and karyotype/CMA was "normal." Inclusion criteria included the presence of an FSA (including a nuchal translucency >4 mm: recruitment "capped" at 10% of total) after 11-weeks where invasive testing (amniocentesis or chorionic villous sampling) had been performed. Exclusion criteria were if participants were aged <16 years and where written informed consent was not provided from both parents [13].

All foetal tissue samples underwent genetic testing at their respective regional Genomic Laboratory, where DNA was extracted and then ES which was performed at the Wellcome Sanger Institute. In the first instance, a QF-PCR was performed to assess autosomes 13, 18, 21, and the sex chromosomes. All sample preparation, PCR, and analysis procedures were carried out in accordance with the Association for Clinical Genomic Science best practice guidelines [14, 15]. Testing was carried out on DNA extracted from uncultured amniotic fluid, enzymatically dissociated uncultured chorionic villus cells or occasionally, if required, from cultured cells. Five microsatellite markers for each of chromosomes 13, 18, and 21 were included in the assay, and markers from the X to Y chromosomes are included in a separate assay used to identify sex chromosome aneuploidy. PCR products were separated on an ABI3500 Capillary genetic analyser, and results were analysed us-

ing ABI GeneMapper software. Subsequently depending on local policy, if QF-PCR testing was negative then either CMA was performed (91.7%; n = 11/12 centres) or G-banding karyotype with fluorescence in situ hybridization (FISH) targeting the 22 q11.2 region (single centre). CMA was performed using an array comparative genomic hybridization approach as previously described by our group [15, 16]. ES was performed as outlined in the published PAGE study and used a standard ES approach with a targeted virtual gene panel for developmental disorders including 1,628 genes [13]. Pathogenic variants and variants of uncertain significance (VUS) where the American College of Medical Genetics and Genomics classification had been agreed upon at the clinical review panel were reported and incidental findings were not [17].

The PAGE cohort in the Birmingham arm underwent phenotype filtering for all CHD based upon Human Phenotype Ontology terms [18]. A cardiac defect was included if it could be classified using Human Phenotype Ontology terms for "abnormality of the foetal cardiovascular system"; HP: 0010948 or "abnormality of the cardiovascular system"; HP: 0001626 [18]. In total, n = 147 cases of prenatally diagnosed CHD where invasive testing was performed were included. In all instances, a foetal echocardiogram was performed by a foetal/paediatric cardiologist in a tertiary centre to confirm the anomaly. For the majority of West Midlands centres 58.3% (n = 7/12), this involved referral to the Birmingham Women's Hospital 68.7% (n = 101/147). In this centre, based upon a sample of n = 100 non-PAGE recruited subjects during the study period with a prenatally diagnosed CHD, the uptake of invasive testing was 26% (n = 26). CHD was subsequently categorized into (i) multisystem or isolated and (ii) by subgroup, based upon 2 classifications (1) The American Heart Association/American College of Cardiology (AHA/ACC) criteria ([i] complex; [ii] left-sided obstructive; [iii] right-sided; and [iv] shunt [septal] lesions) and (2) conotruncal and non-conotruncal, which were the most appropriate classifications based upon expert consensus review [19]. Ten of 12 (83.3%) monogenic variants described here were included (without detailed clinical information) in a previous report from the PAGE study [13]. This study obtained ethical approval from the Research and Development offices and Research Ethics Committees at the West Midlands – South Birmingham (ref: 13/WM/1219) and at each institution. All couples provided prospective informed written consent, and the study was performed in compliance with the guidelines for human studies and was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. For the purposes of the analysis outcome, measures included the diagnostic yield of (i) QF-PCR; (ii) CMA – inclusive of a positive karyotype and or FISH; and (iii) ES. As ES also captures copy-number variation (CNV) in instances where karyotype/FISH was performed and a CNV was detected which would have been detectable on CMA, this was reported as a positive CMA result as opposed to ES. Both the overall and stepwise diagnostic yields were calculated for all technologies, the stepwise yield reflecting the proportion of positive QF-PCR, CMA, and ES with the denominator for each represented by the total number of cases of CHD (n = 147) subtracting those who had a positive QF-PCR (in the case of the cumulative CMA yield) and then CMA (in the case of the cumulative ES yield).

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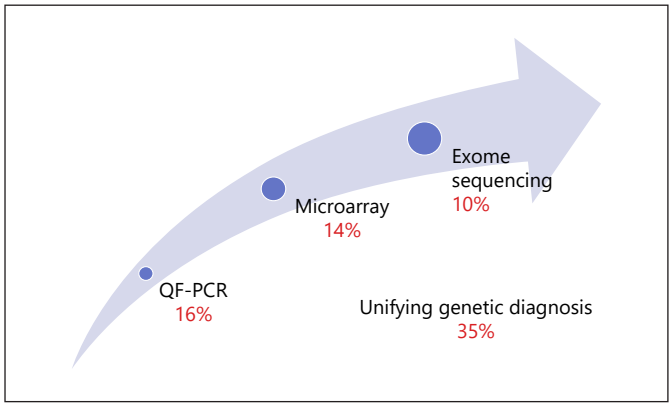

Fig. 1. Diagnostic yield (%) per CHD subtype. CMA, chromosome microarray; ES, exome sequencing; QF-PCR, quantitative fluorescence-PCR; CHD, congenital heart disease.

Results

There were n = 147 cases of CHD diagnosed on foetal echocardiogram which underwent prenatal invasive testing. The demographics and outcomes of the cohort studied are demonstrated in Table 1. There was a high incidence of pregnancy loss (associated with miscarriage and stillbirth), but termination of pregnancy was the commonest outcome 51.8% (n = 71/137), p = 0.001. The categorization of anatomical diagnosis made using prenatal diagnosis is demonstrated in Figure 1. Not demonstrated here were the miscellaneous group; 12.9% (n = 19).

Based on a stepwise testing strategy, the diagnostic yield for each prenatal genetic test for QF-PCR, CMA, and ES was 15.6% (n = 23/147), 13.7% (n = 17/124), and 10.2% (n = 11/107), respectively (Fig. 2). The total proportion of cases of CHD obtaining a unifying genetic diagnosis was 34.6% (n = 51/147). The overall diagnostic yield for all 3 testing strategies was 15.6% (n = 23/147) QF-PCR, 11.6% (n = 17/147) CMA, and 7.5% (n = 11/147) prenatal ES p = 0.09.

Figure 1 demonstrates the diagnostic yield (%) per test per CHD subcategory. For QF-PCR, the yield was highest for shunt lesions (i.e., septal; ventricular septal defects [VSDs], and atrioventricular septal defects [AVSDs]), 31.4% (n = 11/35), p = 0.046. The subcategory with the greatest overall rate of unifying genetic diagnoses was also that of shunt lesions 45.7% (n = 16/35), p = 0.45. In shunt anomalies with an abnormal QF-PCR, AVSDs were commonest (54.5%; n = 6/11), with trisomies 13 and 18 most prevalent (both 36.4%; n = 4/11). An abnormal CMA (most commonly 22 q11.2 microdeletion) was signifi-

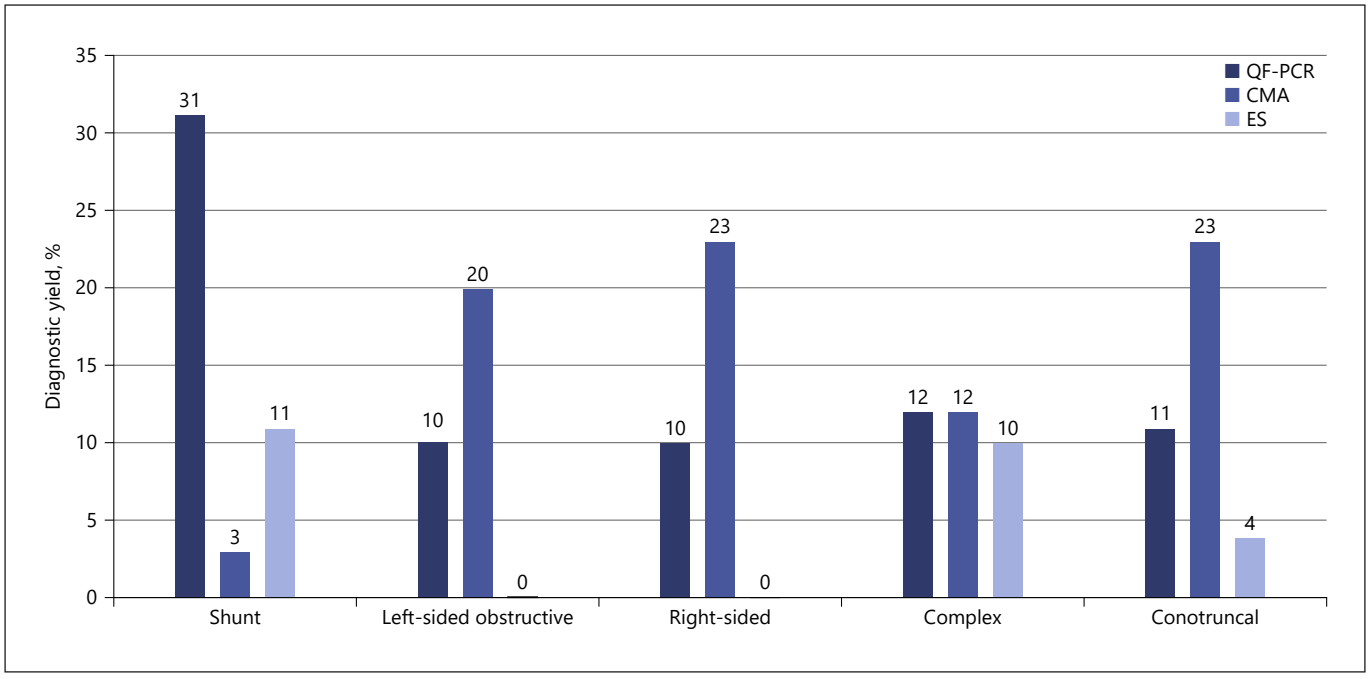

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Fig. 2. The diagnostic yield of genomic technologies in CHD based on a stepwise testing strategy. QF-PCR, quantitative fluorescence-PCR; CHD, congenital heart disease; ES, exome sequencing; CMA, chromosome microarray.

Table 2. Diagnostic yield in isolated and multisystem congenital heart disease

	Isolated CHD stepwise yield, %, *n*	CHD with extra-cardiac defects stepwise yield, %, *n*	Odds ratio (95% confidence interval)	Extra-cardiac defects, %, *n*
QF-PCR	11.9, *n* = 13/109	26.3, *n* = 10/38	2.6 (1.0–6.7)	Extremities 29.1, *n* = 9/23
Chromosome microarray	11.5, *n* = 11/96	21.4, *n* = 6/28	2.1 (0.7–6.3)	Brain and GI tract Both 17.6, *n* = 3/17
ES	9.4, *n* = 8/85	13.6, *n* = 3/22	1.5 (0.4–6.3)	GI tract 27.3, *n* = 3/11
All technologies	29.4, *n* = 32/109	50, *n* = 19/38	2.4 (1.1–5.1)	Brain and GI tract 25.4, *n* = 13/51, and 23.5, *n* = 12/51

GI, gastrointestinal; QF-PCR, quantitative fluorescence-PCR; ES, exome sequencing.

cantly over-represented in the conotruncal category at 22.7% (*n* = 10/44) versus 6.5% (*n* = 6/92) non-conotruncal OR 4.2 (95% CI, 1.4–12.5), with the commonest cardiac defect in this category Tetralogy of Fallot (40%; *n* = 4/10). In the positive ES group, the commonest type of CHD was complex (36.4%; *n* = 4/11), with *n* = 2 cases of right atrial isomerism (one of which was associated with cerebral ventriculomegaly), both of which were subsequently identified as being secondary to primary ciliary dyskinesia. There were a further 2 cases with a positive ES result in the miscellaneous CHD subgroup associated with tricuspid regurgitation (±serous body cavity effusions). Excluding the miscellaneous group from the cohort (*n* = 128), the cumulative diagnostic yield for all 3

technologies was almost identical to that of the original cohort at 16.4% (n = 21/128), 15.9% (n = 17/107), and 10.0% (n = 9/90).

Overall, the commonest chromosomal abnormalities identified by QF-PCR were Trisomy 18 and Trisomy 21 (both 39.1%; n = 9/23, respectively) (see online suppl. Table 1; see www.karger.com/doi/10.1159/000512488 for all online suppl. material). For CMA, the commonest submicroscopic chromosomal anomaly was 22 q11.2 microdeletion 41.2% (n = 7/17), and for ES, primary ciliary dyskinesia (CCDC103 and DNAH11 variants), Noonan syndrome (SOS1 and RIT1 variants), and CHARGE syndrome (CDH7 variant) (all 18.2%; n = 2/11, respectively) (online suppl. Tables 2, 3).

The overall diagnostic yield for genetic testing in isolated and multisystem CHD is presented in Table 2. The commonest ECAs overall associated with a positive genetic diagnosis were those affecting the foetal brain (25.5% [n = 13/51]) and gastrointestinal tract (23.5% [n = 12/51]). The commonest brain anomalies in this category were ventriculomegaly 30.8% (n = 4/13) and cerebellar malformations 23.1% (n = 3/13). The commonest gastrointestinal anomalies in this category were "echogenic bowel" in 25.0% (n = 3/12) and a small or "not visualized foetal stomach," with a presumptive diagnosis of oesophageal atresia (33.3% [n = 4/12]). There were no VUS reported with CMA testing, but 5 were identified with ES (n = 5/107 [4.7%]).

Discussion

Main Findings

Based on a stepwise testing strategy, the diagnostic yield for QF-PCR, CMA, and ES in prenatally diagnosed CHD is 16, 14, and 10%, respectively. This yield is highest in cases of CHD associated with ECAs. The subtype of CHD with the greatest diagnostic yields in relation to genetic testing was shunt lesions with abnormal QF-PCR and conotruncal lesions with abnormal CMA. A positive ES was found most commonly in complex CHD.

The strength of this prospective multicentre cohort study is that it has a concise inclusion criteria, CHD confirmed by a foetal cardiologist and centralized analysis and interpretation of ES results. It is also to our knowledge the largest series assessing prenatal CHD and the yield of the three current genomic testing strategies in one cohort in the era of NGS [20]. Although one of the largest, in relation to ES the numbers with positive findings were too small to demonstrate a significant difference in any CHD subcategory. A further limitation was lack of an internationally agreed prenatal CHD classification system. Hence, categorization in our study was based on postnatal classifications, which are not as relevant as they do not consider the progressive nature of in utero cardiac development. Such classification systems are very much anatomically based and do not account for the foetal haemodynamic circulation or cardiac function [21]. Also, prenatal and postnatal findings can differ as one tries to make a firm diagnosis of a complex 3-dimensional structure based upon 2-dimensional ultrasound images as well as the fact that several complex cardiac anomalies may not make it to beyond delivery and, hence, are not commonly found in postnatal classification systems [22]. Perhaps an embryologically or morphologically based-prenatal classification which aligns more with the biological pathways and genetic mechanisms leading to CHD would be worth considering in future developments [22, 23].

The clinical utility of prenatal ES in CHD (as with other FSAs) is dependent not just on the prospective targeting of phenotypes (i.e., specific anatomical abnormalities of the foetal heart) but also robust bioinformatic filtering of variants within accredited molecular genetic laboratories. The potentially identified "variants" then need clinical multidisciplinary review groups to assess a "causative" association with the phenotype. In addition, it is essential that there is clear accurate and comprehensive pretest counselling. Without such robust bioinformatics and clinical screening of variants, prenatal ES should not be offered in clinical practice. Furthermore, once a causative, pathogenic variant has been identified this needs to be discussed in sensitive way with the parents and the impact on the proband and future pregnancies discussed.

The overall yield of genetic testing in CHD (35%) is concordant with current research, which is primarily based upon postnatal data [8]. One would anticipate that the yield would be lower in the prenatal series due to the potential for less accurate phenotyping although may well be balanced by increased rates of aneuploidy detected antenatally [8, 24]. Despite this, rates of aneuploidy in our cohort were modest compared to other studies, although an abnormal QF-PCR was commonest in shunt lesions, as anticipated [5, 24]. This may be secondary to the uptake of first-trimester combined screening and non-invasive prenatal testing which may select out aneuploidies prior to recruitment into the study. The proportion of pathogenic CNVs was also similar to reported prenatal series, with 22 q11 the commonest CNV in CHD and most commonly associated with conotruncal defects and of an isolated nature [24–27]. The yield of ES is similar to

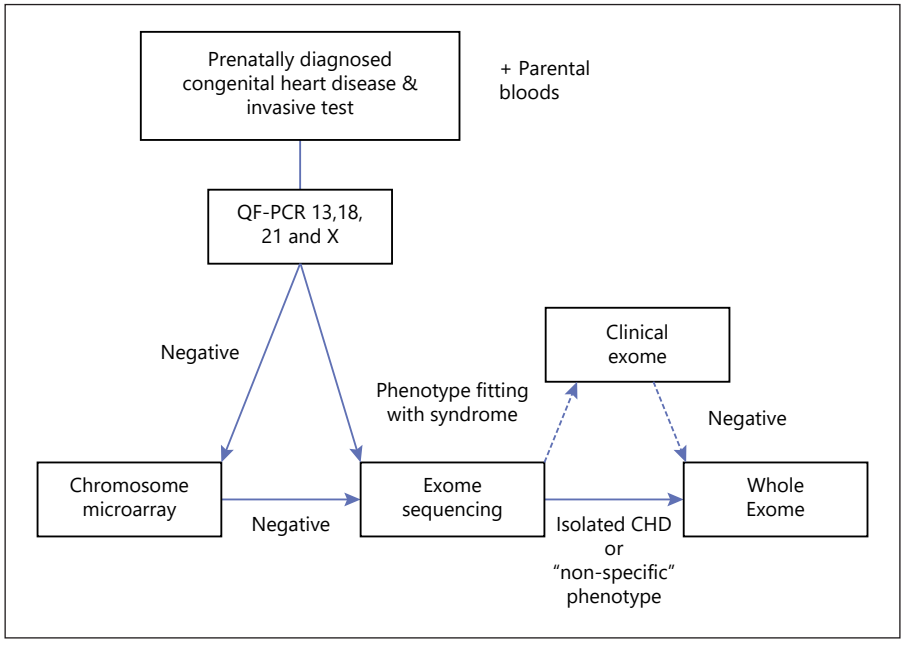

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Fig. 3. Proposed algorithm for prenatal genetic testing in cases of CHD. QF-PCR, quantitative fluorescence-PCR; CHD, congenital heart disease; ES, exome sequencing; CMA, chromosome microarray.

that of larger prenatal series but modest in comparison with smaller series, likely due to potential selection bias of positive cases due to a smaller series of predominantly multisystem CHD for which the ES yield is greater [20, 28–30]. As demonstrated in our own study, most pathogenic variants detected from ES were de novo variants inherited in an autosomal dominant fashion and most were seen in complex CHD, notably in cases of heterotaxy [31]. Heterotaxy shows a strong association with single-gene disorders and a predominance for pathogenic biallelic variants in ciliopathies, as was evident in our own study [32, 33]. The commonest ECAs seen in line with a positive genetic diagnosis in the presence of a CHD were concordant with published research although urogenital and skeletal anomalies were not as significantly represented [9]. However, many important ECAs are missed prenatally, and this is vital in variant interpretation and phenotype-genotype correlation [9].

The uptake of invasive testing by parents after counselling was modest in our population at 26% [5]. This means that many couples do not have the opportunity to obtain further prognostic information in relation to perinatal outcome and are less equipped to make informed decisions regarding the pregnancy or neonatal care [34]. In the wake of prenatal NGS, it is important to educate women and clinicians that the opportunity to make a prenatal genetic diagnosis is greater and failure to do so can have adverse implications. It is anticipated that as sequencing technologies advance and with the instigation of clinical pathways and guidelines, following the roll-out of ES via Genomic Laboratory Hubs in England this year, ES turnaround times will fall and the provision of an ES result within a time frame that can inform the pregnancy can be realistically achieved [12, 35]. A proposed algorithm is demonstrated in Figure 3 with the provision of CMA and ES testing in parallel and selection of the ES sequencing target dependent upon the prenatal phenotype as decided by the multidisciplinary team, that is, if there are ECAs and the prenatal phenotype is suggestive of a specific monogenic disorder then a gene panel or "clinical exome" can be considered. If there is an isolated lesion and no specific phenotype then a "whole ES" approach may be more appropriate [36]. The organization of such services may potentially be provided centrally by the tertiary foetal medicine centre with virtual multidisciplinary team meetings extended to smaller regional foetal medicine centres. It is promising that there was no detectable VUS in the CMA group, in keeping with the low rates seen in our previously published cohort [15]. While there were VUS in the ES group, this is unsurprising as with the initial introduction of new genomic technologies as our understanding of variant interpretation develops, and it is predicted that these rates will fall [35].

One must also consider what the underlying causes for the remaining 65% of cardiac defects are. The aetiology of CHD is complex and further research using CHD models to explore the interaction between genetic and epigenetic modifications and cardiac embryology and morphology

is needed [11]. Whole-genome sequencing aids not only in gene discovery but can also assess for the presence of aneuploidy and structural variation as well as single nucleotide changes, hence, can potentially serve as an "all-in-one-test" in the future once turnaround times and costs reduce [37, 38].

Conclusion

Prenatally diagnosed CHD has an associated diagnostic genetic yield with existing technologies of 35%. As we enter the era of prenatal ES, there remains an important role for QF-PCR and CMA, most notably in the presence of shunt, conotruncal, and multisystem CHD. As evidence and clinical guidance in prenatal genomics evolve, it will become clearer if QF-PCR/CMA and ES should be run in parallel or a stepwise fashion and which CHD cases should avail of ES and by selection of a targeted or whole-exome approach. Ultimately, achieving a prenatal genetic diagnosis is important for prognostication of CHD and in counselling couples and creating a perinatal management plan but is reliant on progression of the technology and service for results to be obtainable within a timely fashion.

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Statement of Ethics

The study 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 20 June 2014. All couples provided prospective informed written consent, and the study was conducted in compliance with the guidelines for human studies and was conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

Conflict of Interest Statement

D.J.M. reports grants for travel expenses from Congenica to attend educational symposia during the conduct of the PAGE study. M.E.H. 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. M.D.K. is a member of Illumina's International Perinatal Advisory Group but receives no payment for this. E.R.M. has received travel expenses, accommodation and consultant fees for participating in an Illumina International Advisory Group after completion of the PAGE study. M.D.K. is funded through the Department of Health, Wellcome Trust, and Health Innovation Challenge Fund (award number HICF-R7-396) for the PAGE and PAGE2 research studies complete August 2019. All other authors declare no competing interests.

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Author Contributions

M.D.K. and F.M. were involved in study conception, while the remaining authors excluding B.K.S. and A.N.S. were involved in the original PAGE concept. M.D.K., F.M., B.K.S., S.H., S.A., A.N.S., and E.Q.J. were involved in study planning with M.E.H., D.J.M., and E.R.M. also responsible for original PAGE planning. All authors were involved at some point in carrying of the study and analysis/interpretation out the study with analysis performed by all. F.M. and M.D.K. wrote the initial draft of the manuscript which was subsequently reviewed and approved by all authors.

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