THE USE OF PRENATAL CHROMOSOMAL MICROARRAYS WHEN PERFORMED FOR A FETUS WITH STRUCTURAL ABNORMALITIES ON ULTRASOUND SCAN

By

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ABSTRACT

Fetal chromosomes are examined conventionally by G-band karyotyping. More recently Prenatal Chromosomal Microarray (CMA) has been used to look for fetal chromosomal abnormalities. Advantages of CMA include its higher detection rate. Disadvantages include its detection of Variants of Unknown Significance (VOUS).

I recruited a prospective cohort of 243 women with structural abnormalities on fetal ultrasound scan. A 1Mb targeted BAC array was performed in addition to G-band karyotyping. In 62 cases from this cohort an additional higher resolution 60K oligonucleotide array was used. A health economic analysis, by use of a decision tree, was performed. Finally qualitative work determined women's feelings about testing.

The 1Mb BAC cohort found a 4.1% increase in fetal chromosomal abnormalities over karyotyping, with a low detection rate of VOUS (0.4%). The 60K sub-cohort noted an extra 4.8% pathogenic chromosomal anomalies but, in addition, a 13% increase in VOUS. The health economic analysis indicated that when CMA is £360 (per test) and the Willingness To Pay (WTP) for a "positive diagnosis" is £9768; then CMA is cost effective over karyotyping. Qualitative analysis showed that couples were keen for as much information as possible. They struggled to recall and retain information conveyed at the time of the testing.

DEDICATION

To my parents for their love and ever continuing support of my career, and the endless hours of childcare they have provided. To my son Alistair and daughter Elizabeth for being a welcome distraction and for their unconditional love. Lastly for my husband John for his support, love, advice, and on many occasions being a voice of reason. Thank you.

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LIST OF DEFINITIONS AND ABBREVIATIONS

aCGH	Array Comparative Genomic Hybridisation
bp	base pair
BAC	Bacterial Artificial Chromosome
CEAC	Cost Effectiveness Analysis Curve
CMA	Chromosomal microarray
CNV	Copy Number Variation
FISH	Fluorescent In Situ Hybridisation
HCP	Health Care Professional
ICER	Incremental Cost Effectiveness Ratio
kb	kilobase (1,000 base pairs)
Mb	Megabase (1,000,000 base pairs)
QFPCR	Quantitative Fluorescence Polymerase Chain Reaction
QUALY	Quality Adjusted Life Year
USS	Ultra Sound Scan
VOUS	Variant of Unknown Significance
WMGL	West Midlands Genetics Laboratory
WTP	Willingness To Pay

CHAPTER 1 INTRODUCTION AND AIMS

INTRODUCTION

During the 1860s both Charles Darwin and Gregor Mendel proposed their hypotheses of heritability . By the middle of the nineteenth century it was largely agreed that hereditary information was located within the nucleus of a cell, but the nature of this hereditary material was unknown. German anatomist Walter Flemming was the first to discover “stainable material” or chromatin using innovative staining techniques. The term chromosome was later coined by Heinrich Waldeyer. It was not until 1956 that the correct numbers of human chromosomes were estimated by Joe Hin Tjio and Albert Levan as 46.

Chromosomes are “packages” of DNA tightly coiled around proteins called histones, supporting the chromosomes structure. It is during cell division that chromosomes have a key role in ensuring DNA is copied and distributed accurately. During cell division and the metaphase stage of the cell cycle, chromatin condenses and chromosomes become visible as two sister chromatids joined by a centromere with a short p arm (for petit) and long q arm (for queue).

Chromosomal anomalies are a major cause of perinatal morbidity and mortality (1-3). Since the late 1960’s full conventional G-band karyotyping has been the mainstay of excluding and diagnosing structural karyotypic abnormality. In order for chromosomes to become visible under a light microscope chemical dyes need to be applied. G-banding is the most commonly used and involves treating chromosomes with trypsin (to degrade proteins, relax the chromatin and allow access of the

Giemsa stain) and then staining with Giemsa. Heterochromatic regions (which are adenine and thymine rich DNA and relatively gene poor) stain more darkly. Less condensed regions (guanine-cytosine rich and gene rich DNA) incorporate less stain and appear as light bands. Chromosome preparation and G-band quality can be affected by many factors relating to quality include the sample type (blood often yields a better preparation than amniotic fluid or a Chorionic Villus Sample) and whether the sample has clotted or has been delayed in transit. Laboratory factors include a change in temperature, a problem with reagents, and analysis is dependent on the skill of the operator. Despite significant improvements in cytogenetic resolution over the last 25 years, conventional full karyotyping can only detect anomalies down to a resolution of 5-10 Mb (4).

In the 1990's the use of QFPCR (Quantitative Fluorescence Polymerase Chain Reaction) to target specific whole chromosomal anomalies (typically Trisomy 13, 18, 21, and sex chromosome anomalies X and Y) provided a relatively cheap and rapid result (within 48-76 hours) when a prenatal screening test was reported as "high risk" (5).

QFPCR analysis is performed by amplifying and detecting small tandem repeats (genetic markers). Fluorescently labelled primers are used for PCR amplification and the copy number of the marker is representative of the copy number of the chromosome. A normal diploid sample has a contribution from each of the two chromosomes. Two alleles of a specific marker are represented by two peaks at a 1:1 ratio if heterozygous and one large peak double the size if homozygous. The

detection of 3 peaks 1:1:1 ratio or 2:1/1:2 indicates an additional chromosome (as in Trisomy).

Many laboratories are now providing this test alone for karyotyping after a high risk screening result and only performing further conventional full karyotyping using G-banding when the risk of more complex chromosome anomalies is high (i.e. structural differences on fetal ultrasound scan).

Fluorescence In Situ Hybridisation (FISH) is a useful adjunct to full conventional karyotyping when a high degree of suspicion of a specific chromosome anomaly is present (e.g. Di George syndrome and a cardiac anomaly) or when clarification of a difference seen on karyotype is sought. This technique may be used to identify interstitial submicroscopic microdeletions and microduplications or subtelomeric deletions and duplications (6). FISH uses fluorescent probes that bind to complementary parts of the chromosomes. Microscopy is then used to find out where the probe is bound to the chromosomes. Probes are constructed and tagged with fluorophores. An interphase or metaphase preparation of chromosomes is produced and repetitive sequences of DNA blocked (usually by adding short DNA fragments to the sample). The probe is then added and the sample hybridised. Results are visualised using a microscope that can excite the dye and record the images.

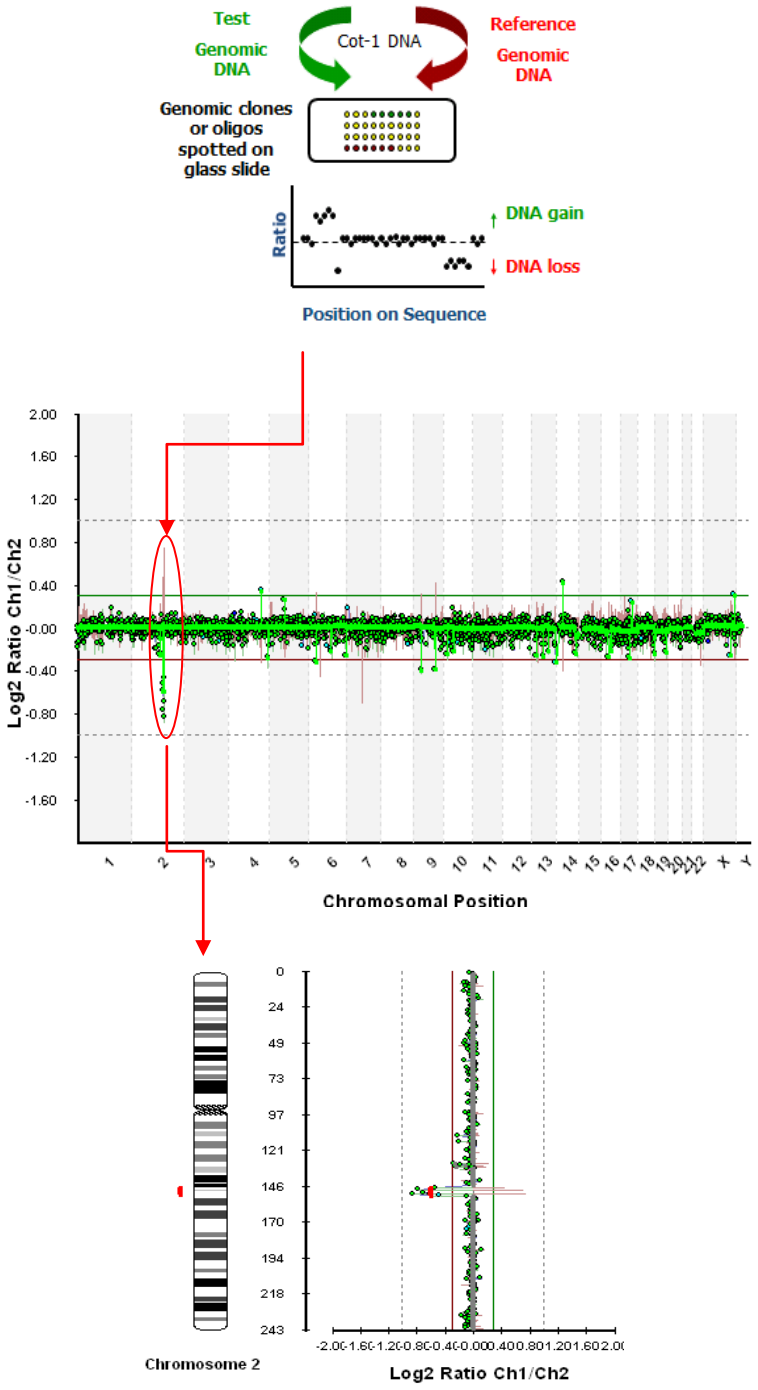
FISH and QFPCR are ‘targeted’ approaches capable only of assessing a limited number of loci and so require the referring clinician to suspect a specific chromosomal aberration from the fetal phenotype.

From FISH analysis comparative genomic hybridisation (CGH) was derived, providing a way of looking for chromosomal changes genome-wide. CGH used a test and a control genome and labelled with fluorescent dyes hybridized them to metaphase chromosomes (7). Unlike its precursors which relied on examination of a single target and prior knowledge of a chromosome region that should be studied, CGH could search the whole genome for imbalances. However its resolution was still often limited to 5-10Mb (8).

The next development was array Comparative Genomic Hybridisation (aCGH) or as it will be referred to hereafter Chromosomal Microarray (CMA). CMA combined the CGH technique with the use of microarray (9). Originally designed for use in oncology studies, CMA was (and is still) used to develop molecular classification of different tumour types, look at tumour progression and identify therapeutic targets (7;10). Later it was used to identify submicroscopic deletions or duplications in children with undiagnosed learning difficulties and mental retardation (11-14) before being used in the prenatal setting.

Chromosomal microarray involves hybridisation of patient DNA onto predetermined targets representative of the whole genome; BAC clones, or synthetic oligonucleotide probes spotted onto glass slides. The patient or 'test' DNA (in a prenatal context obtained by amniocentesis, chorionic villus sampling (CVS) or cordocentesis) is extracted from the relevant sample, labelled with a fluorochrome, mixed with a reference DNA pool (labelled with a different fluorochrome) and then hybridized on the microarray slide in the presence of cot-1 DNA to suppress repetitive sequences. After hybridisation and laser scanning, fluorescent ratios on each array spot are calculated and normalized so that the median log 2 ratio is 0 (15-17). User defined thresholds are then set for calling copy number changes as loss or gain (Figure 1) (18).

Figure 1 The Basic Principle of chromosomal microarray (18)



Legend Figure 1 (18): The basic principle of chromosomal microarray. Differentially labelled test and reference DNA are co-hybridised to the microarray, spotted with genomic clone or oligonucleotide probes, in the presence of cot-1 DNA which suppresses repetitive sequences. After hybridisation and laser scanning, fluorescent ratios on each array spot are calculated and normalized so that the median log 2 ratio is 0. User defined thresholds are set for calling copy number changes as loss or gain.

Advantages of CMA

CMA has many advantages when compared with the chromosome testing strategy described above. CMA utilises uncultured cells reducing the “turnaround time of the results” (in our laboratory to 5 days, presently) and is amenable to automation and high throughput analysis that provide the potential for positive health economic effects. Microarrays will become quicker to analyse as chromosomal variants detected will be compared to local (within the laboratory), national and international databases. Most importantly it is of “high resolution” allowing detection of submicroscopic deletions or duplications though the specific choice of CMA platform determines the resolution of the test (15-17).

Earlier CMA technology used BAC (Bacterial Artificial Clones 100-200kb in size) and these are targeted to known regions of microdeletion or duplication syndromes. More recently, mostly in the postnatal setting, synthetic oligonucleotide probes of 25-75bp are used. These typically are higher resolution but also allow flexibility in the selection of probes and can therefore be customised by the user i.e. using a lower resolution in the prenatal setting to minimise any results of unknown clinical significance. Single Nucleotide Polymorphism or SNP based arrays have the advantage of detecting Long Continuous Stretches of Homozygosity (LCSH) that can enable the detection of some forms of Uniparental Disomy (Uniparental isodisomy but uniparental heterodisomy is not detected unless the parents are also tested) or

consanguinity[1] (17;19). Such 'high resolution' CMA is increasingly utilised in prenatal diagnosis.

Disadvantages of CMA

CMA technology does have limitations. CMA is only able to potentially detect unbalanced chromosomal changes. De-novo (non-inherited) balanced chromosomal rearrangements (such as reciprocal translocations or insertions) may disrupt genes and lead to phenotypic disease without detectable gains or losses at breakpoints (20). However, in practice, many apparently balanced rearrangements detected by G-banding are not truly balanced at a DNA level, and microarray testing can be used to detect small regions of DNA loss or gain and so clarify the exact nature of the rearrangement (21). Furthermore, array CGH may not detect triploidy, unless SNP based arrays are used. However, this limitation, certainly in the UK, is avoided as QFPCR is likely to continue to be used prior to array analysis as a rapid and cost effective screen for common aneuploidy and triploidy. In addition, low level mosaicism may not be detected by CMA technology (15;17).

The most significant current potential disadvantage of CMA is the identification of novel, previously unreported, variants of unknown significance (VOUS) that, particularly in the prenatal setting, can cause difficulties in clinical management because of the uncertainty of the relationship between the VOUS and likely clinical

[1] UPD occurs when a fetus inherits both two copies of a chromosome, or part of a chromosome, from one parent (e.g. both chromosome 15 are inherited from the mother and no chromosome 15 is inherited from the father). In isodisomy there are two copies of the same chromosome and in heterodisomy the two chromosomes are different. UPD may cause developmental defects if the chromosome involved contains imprinted genes or, in the case of isodomy, the involved chromosome carries a mutation for an autosomal recesively inherited disorder).

effect. In order to facilitate more accurate assessment and interpretation of VOUS there is an ongoing major effort to catalogue and collate genomic and clinical information. The development of copy number variation (CNV) databases, such as the International Standards for Cytogenomic Array Consortium (ISCA) from within the dbGaP (Database of Genotype and Phenotype at NCBI www.ncbi.nlm.nih.gov/gap) and the Database of Genomic Variants (DGV http://projects.tcag.ca/variation) will facilitate the resolution of VOUS into benign or pathogenic variants. Postnatally detected pathogenic cases are also recorded in DECIPHER (Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources www.ensembl.org/index.html) (15-17). The detection of VOUS may increase the emotional burden on the parents. If prenatal diagnostic tests have been utilised in the presence of a suspected congenital malformation there is the possibility that such information, however 'non-informative', may increase the possibility of termination of pregnancy. Hence the aim when using such technology in prenatal diagnosis is to identify known pathogenic variants but not to increase parental anxiety by identifying variants of uncertain or no clinical significance in a prenatal diagnostic situation. In addition, the identification of copy number variations (CNVs) of uncertain significance that then require the analysis of parental DNA for further interpretation has financial consequences and delays the availability of definitive results.

Copy number variation (CNVs) and Classification of CNVs

Development and application of these microarrays led to the emergence of multiple whole genome CNV studies in normal populations. Until this point single nucleotide

polymorphisms or SNPs were thought to be the predominant form of genetic variation (A SNP is a base substitution involving a single nucleotide). The publication of two cohorts reporting widespread copy number variation in normal individuals (22;23) began to change this thinking and it is now recognised that CNVs accounts for more (estimate range from 4Mb-24Mb of variation between individuals) nucleotide variation than SNPs (approximately 2.5Mb) (24). CNVs are segments of DNA of 1 kb or larger present at a variable copy number in comparison with a reference genome (25).

CNVs lead to disease or alter phenotype through different mechanisms involving influencing gene dosage and expression. Figure 2 below (Feuk et al) shows these different mechanisms (25).

Redon et al determined that approximately 12% of the human genome exhibits copy number variation (27). With such widespread benign CNV density application and interpretation in a clinical setting can be challenging in terms of classifying variants as pathogenic, benign or novel variants of uncertain clinical significance (VOUS)(15;17).

Figure 2 How CNVs affect gene expression and phenotypic traits (25)

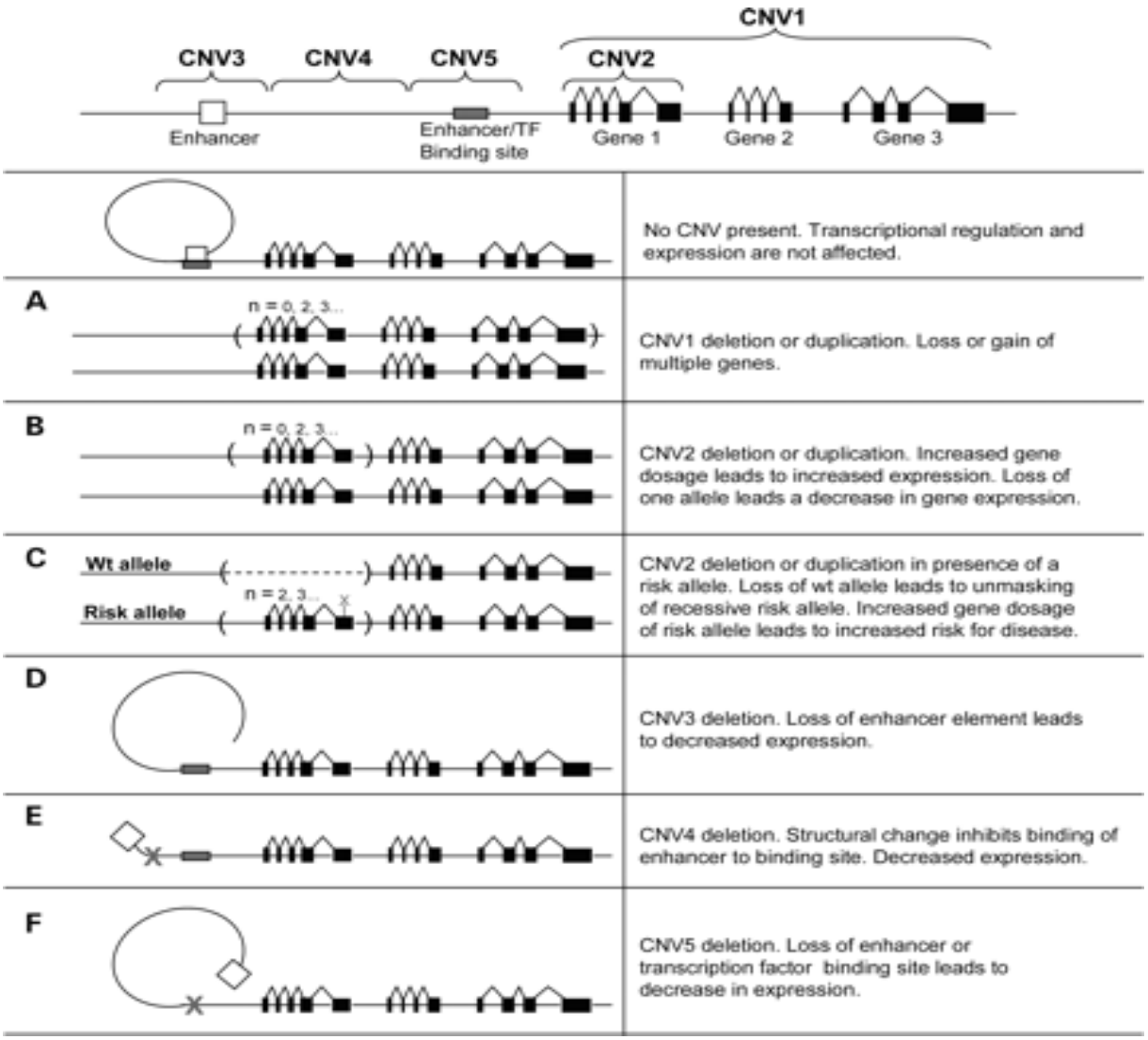


Figure 2 Legend (25): A) Involves the deletion or duplication of a region resulting in loss or gain of multiple genes and a direct correlation between genotype and phenotype, resulting in a microdeletion or duplication syndrome. B) Shows that when a gene is located within a region that varies in copy number there can be increased gene dosage leading to increased gene expression. C) Shows a CNV involving a deletion or duplication that can lead to an unmasking of a recessive risk allele. The subsequent decreased gene dosage of the wild-type allele can lead to disease. The opposite can occur with increased numbers of copies harbouring a risk allele, which may result in increased disease susceptibility. D and F) Show how a CNV can lead to inhibition of a DNA enhancer interaction, the chromatin structure or access to transcription factors to their binding sites as in E).

There are general principles that can be applied to the analysis of CNVs in order to establish their pathogenicity (Table 1) (28). In general terms a CNV is more likely to be pathogenic if (a) it is inherited from an affected parent or affected relative, if it overlaps an imbalance in the database of affected individuals, DECIPHER, (b) it is particularly gene “rich” and these genes are classified as causing morbidity by OMIM (online Mendelian Inheritance in Man http://www.ncbi.nlm.nih.gov/omim). Minor criteria for a pathogenic CNV is that a deletion is more likely to be pathogenic than duplication and the larger it is the more likely it is to be pathogenic. A CNV is more likely to be benign if inherited from an unaffected parent or relative, overlaps with the database of health individuals (database of genomic variation), be gene “poor” and that genes in the region are not known to cause morbidity. CNVs are also more likely to be benign if small in size, a duplication and devoid of regulatory elements(15-17).

Table 1 Factors influencing the risk assessment of a CNV[a] (28)

Major criteria		Characteristic of pathogenic CNVs	Characteristic of benign CNVs
1.	a. CNV is inherited from a healthy parent		×
	b. CNV is inherited from an affected parent	×	
2.	a. CNV is similar to a CNV in a healthy relative		×
	b. CNV is similar to a CNV in an affected relative	×	
3.	a. CNV overlaps a genomic imbalance in a CNV database for healthy individuals (for example, Database of Genomic Variants)		×
	b. CNV overlaps a genomic imbalance in a CNV database for affected individuals (for example, DECIPHER)	×	
4.	CNV contains morbid OMIM genes	×	
5.	a. CNV is gene rich	×	
	b. CNV is gene poor		×
Minor criteria		**Characteristic of pathogenic CNVs**	**Characteristic of benign CNVs**
1.	a. CNV is a deletion	×	
	b. CNV is a homozygous deletion	×	
2.	a. CNV is a duplication		×
	b. CNV is an amplification (gain of more than one copy)	×	
3.	CNV is >3 Mb in size	×	
4.	CNV is devoid of known regulatory elements		×

The resolution of arrays

The sensitivity of CMA is determined by the number and density of the probes and their resolution. In the clinical setting, in order that CMA has an improved detection over G-band karyotyping, it must accurately detect imbalances smaller than 5Mb (the smallest resolution visible by light microscopy with G-band preparations) (15-17). Array platforms in common clinical use have a typical resolution of 10kb in targeted disease specific regions of the genome and 200kb in the genome backbone. Although most known pathogenic CNVs are 400kb or larger, as array resolution increases smaller recurrent imbalances will be detected. More recent information available using high resolution arrays show that 95% of benign CNVs may be less than 100kb in size (29). A table contrasting and comparing commonly used CMA platforms and their resolution is presented below (Table 2) (30).

Table 2 Comparison of array design strategies.

Adapted from a table taken from the National Genetic Reference Laboratory technology assessment "Comparison for cytogenetics array platforms hardware and software for use in identifying copy number aberration in constitutional disorders."

	Affymetrix 2.7M	**ISCA 4x180K (Agilent)**	**ISCA 8x60K (OGT/Agilent)**	**NGRL 4x44K (Agilent)**	**1Mb constitutional targeted array (Bluegnome)**
Backbone coverage	1kb/probe	25kb/probe	60kb/probe	75kb/probe	1Mb/probe
Target region coverage	690bp/probe	5kb/probe or 20 probes per targeted region(average 50 probes per targeted region)	Average 40 probes per targeted region	2 to 5 extra probes per targeted region	1Mb/probe but in triplicate
Backbone resolution	Depend on markers, size and confidence	100kb (4 probes to make a call)	240kb (4 probes to make a call)	225 kb (3 probes to make a call)	2Mb (2 probes to make a call)
Target region resolution	If set as 20 markers resolution will be 20x690bp for the targeted region	20kb (4 probes to make a call)	48kb (4 probes to make a call)	60kb (3 probes to make call)	200kb (2 probes to make a call)
Probe types	2.3million non polymorphic markers for CNV, 400,000 SNP markers for LOH and UPD	Non-polymorphic markers	Non-polymorphic markers	Non-polymorphic markers	Non-polymorphic markers
Probe size	49mer	60mer	60mer	60mer	150-200kb
Number of included targeted regions	Coverage (%) using filter 20 markers minimum size 50kb cancer genes (318)(100%) HPI (548)(98%) X chromosome genes (786)(98.1%) OMIM genes (12,242)(99.2%)	501 targeted regions	498 targeted regions	155 targeted regions	143 targeted regions

CNV= Copy Number Variant LOH=Loss Of Heterozygosity OMIM=Online Mendelian Inheritance in Man UPD=Uniparental Disomy
HPI= Haploinsufficiency OGT= Oxford Gene Technology SNP= Single Nucleotide Polymorphism

THE USE OF CMA TESTING PRENATALLY AND THE LITERATURE UP UNTIL THE END OF 2009

Prenatal fetal karyotyping can be offered during a pregnancy because a screening test has indicated a 'high' risk that the fetus may have aneuploidy; because of a structural anomaly on ultrasound examination; a family history of chromosomal abnormality; or because of parental choice. Although I aimed to answer the question of prenatal CMA testing when a structural anomaly was seen on scan much of the literature from other parts of Europe and the USA include other referral indications.

In 2009 when this research was in its infancy the literature looking at CMA use in a prenatal context was limited, but I took the decision to review it comprehensively by way of a systematic review and meta analysis (31). My aim was to look at the amount of extra information provided by CMA over conventional G–band karyotyping in the existing literature for any referral indication for testing, and when the indication for testing was structural abnormalities on ultrasound scan.

Methods

My systematic review followed a prospective protocol developed using widely recommended and comprehensive methodology(32).

Data sources

The search focused only on prenatal studies using microarray technology. A search strategy was developed based on existing advice for prevalence searches(33;34). MEDLINE (1970–Dec 2009), EMBASE (1980–Dec 2009), and CINAHL (1982–Dec 2009) were searched electronically. The search of MEDLINE and EMBASE captured citations containing the relevant MeSH keywords and word variants for “microarray” and “prenatal”. The following terms were used to describe CMA; array, Comparative Genomic Hybridisation, microarray and oligonucleotide array. Similarly, antenatal diagnostics, fetal diagnostics, prenatal and fetal were used to capture “prenatal”. Bibliographies of relevant articles were manually searched to identify papers not captured by the electronic searches. Web of science (1996-2009) was used to capture any grey literature. Experts were also contacted for completeness of the search (the authors of the papers by Coppinger J et al (35) and Van den Veyver et al (36)). There were no language restrictions in the search or selection of papers.

Eligibility criteria for selecting studies

Studies were selected in a two-stage process. Initially, all abstracts or titles in the electronic searches were scrutinised by two reviewers (SH and SP-a fetal medicine Consultant) and full manuscripts of potentially eligible citations were obtained. Differences were resolved by discussion. Unresolved disagreements were resolved by a third reviewer (AC Professor of Gynaecology). Studies were selected if chromosomal microarray or microarray had been used on prenatal specimens (either analysed during pregnancy or after delivery). I also selected reports if the same technology had been used on postnatal specimens following termination of pregnancy for structural abnormalities detected on ultrasound scan. Papers were excluded if the testing was performed postnatally and the indication for running the CMA had not been determined prenatally. Papers were also excluded if the array was performed on children, or adults, or it was used for pre-implantation genetic diagnosis, or the diagnostic investigation of recurrent miscarriages. Finally papers were excluded if they used comparative genomic hybridisation technique and not array-comparative genomic hybridisation/chromosomal microarray. Non-English studies were assessed by people with the command of the relevant language if the title or abstract appeared to fit the criteria. Only papers that allowed generation of a 2x2 table (comparing karyotyping to array) were included. In two instances in order to construct a 2x2 table the authors were contacted. In the case of Coppinger J et al (35) direct discussion/correspondence allowed extra information to be obtained and us to include the paper. In the second case (Van den Veyver et al (36)), I was still unable to include the paper as the authors were unable to provide us with enough information to complete a 2x2 table.

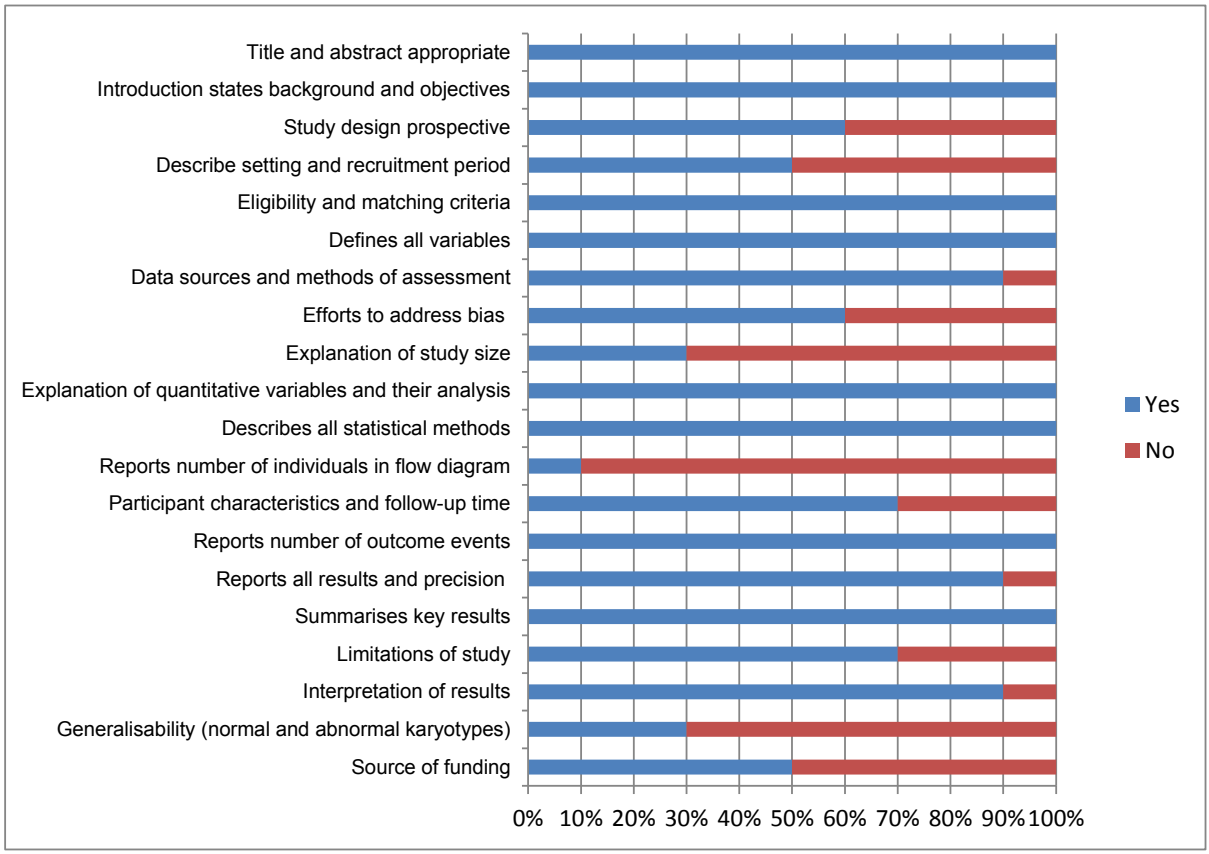
Data extraction

Data were extracted by two reviewers (myself and SP-a fetal medicine consultant). For each of the outcomes, data were extracted into tables, giving descriptive and numerical information for each study. Data were extracted on study characteristics and data quality. Data were used to construct 2x2 tables of test accuracy comparing normal and abnormal karyotype results against normal and abnormal microarray results. Case studies $\leq$5 cases were excluded from the meta- analysis.

Quality assessment

All articles meeting the selection criteria were assessed for quality using the validated tool STROBE (37) (Figure 3). A study was considered to be of good quality if it used a prospective design, if it used a representative population (it used array technology on all samples not just those with that demonstrated array in exclusively normal or abnormal conventional karyotype), it performed array testing on parents to aid interpretation of CNVs and it used a validated assessment tool (i.e. an identifiable, reproducible array). Expert opinion from cytogeneticists at the West Midlands Regional Genetics Department was sought to determine the validity of the array used (DM-Head of Cytogenetics at the WMGL).

Figure 3 Quality assessment using STROBE



Legend: STROBE is used to assess the quality of the publications included within the systematic review (37). Many publications were retrospective and did not have a set period of recruitment and did not account for the cohort size.

Data synthesis

The analysis was performed in two steps depending on the way in which the CNVs were grouped. The first analysis grouped pathogenic, unknown and benign CNVs as array detectable variants. The second analysis moved benign CNVs into the normal group leaving the abnormal CNVs being those that were pathogenic plus those that were of unknown significance and potentially pathogenic. Analysis was performed for samples undergoing both karyotyping and array regardless of the clinical indication (maternal anxiety, high risk on serum Downs screening, structural abnormality on

ultrasound scan). Analysis was then performed for those undergoing chromosomal analysis for structural abnormality on scan.

I explored the possibility of separating the data further by attempting to compare different samples types (amniocentesis and chorionic villous samples) and the effect that this may have on the CMA results. However this was not possible as the studies did not record which sample type the DNA was extracted from when an abnormal chromosomal result was found (with the exception of one paper) (35).

Using 2x2 tables, I computed and pooled the percentage agreement between the two technologies (with 95% confidence intervals) for the articles overall. The calculated percentage of extra cases identified by array in those with a normal karyotype (both overall and by referral indication) with 95% confidence intervals was calculated and pooled (Appendix J). Finally, I calculated and pooled the percentage of cases in which a result of “unknown significance” was reported (Appendix J). Heterogeneity in rates was examined graphically and statistically. Statistical heterogeneity manifests itself in the observed intervention effects being more different from each other than one would expect due to random error (chance) alone. The Chi-Squared test was performed for a statistical assessment of heterogeneity (Appendix J). For graphical assessment, “Forest plots” of point estimate of rates and their 95% CIs were used. For exploration of reasons for heterogeneity, stratified analysis was performed according to the features of the population (indication for referral). For the meta-analysis log rates were pooled, weighting each study by the inverse of its variance and the summary estimates exponentiated (Appendix J). A random effects model was used in the light of heterogeneity. All statistical analyses were performed using Stata 8.0 statistical software (Stata Corp., College Station, Texas, USA).

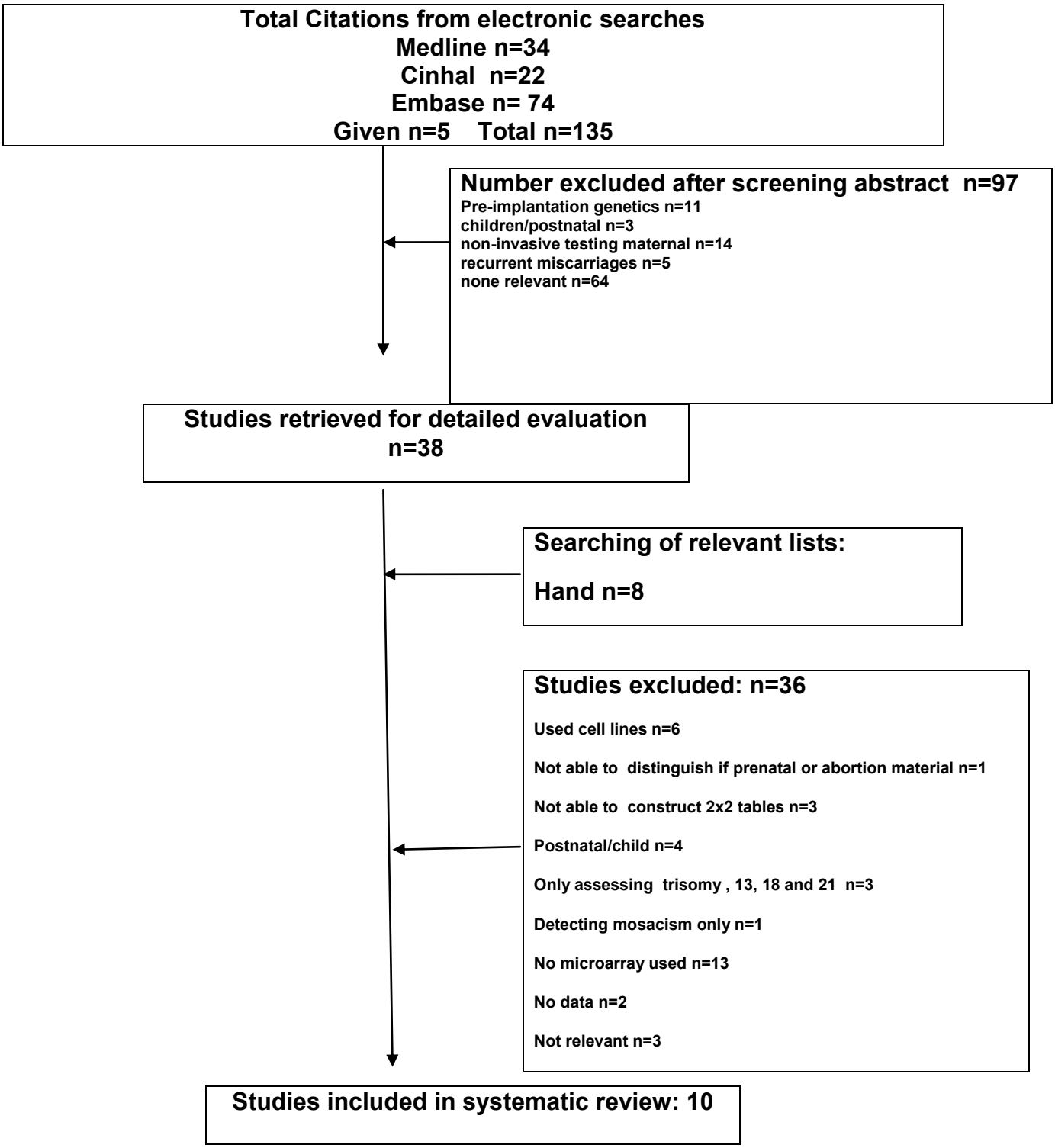
Results

Literature identification and selection

I summarised the process of literature identification and selection in Figure 4. There were 10 primary articles identified as meeting the selection criteria (21;35;38-45).

Of the original articles, 97 were excluded as they did not meet the selection criteria. The remaining 38 articles were obtained and reviewed and a further 8 articles were requested after review of the reference lists. Of these 46 articles, 36 were then excluded as they did not meet selection criteria. The ten primary studies, containing 798 participants met the inclusion criteria to be included in the systematic review (21;35;38-45). Eight of these studies were included in the meta-analysis (35;38-40;42-45), two were excluded from the meta-analysis as array CGH was only performed when an abnormal karyotype had been detected (21;41).

Figure 4 Study selection algorithm



Legend: The flow diagram above accounts for all the papers reviewed during the process of a systematic review.

Table 3 Characteristics of 10 included studies

	Author Year	Design	Array type	Probes	Whole genome (WG) /targeted genome (TG)	Sample Type	Indication for array	Sample Size
1	Tyreman M et al.(43) 2009	retrospective	Genechip SNP 6.0 array (Affymetrrix) Commercial	946,000 probes (CNVs)	WG	Amniotic fluid =87 Chorionic Villus Sampling =15 Placenta biopsy following termination of pregnancy =4	Ultrasound anomaly: (Cardiac = 34 Multisystem = 24 Large NT/hydrops/CH = 18 CNS 16 Skeletal = 6 Abdominal wall = 2 Others = 6)	106
2	Bi W et al.(39) 2008	prospective	BCM V6 oligonucleotide array (V6 Oligo) (Agilent) Commercial	44,000 oligo probes	WG and TG	Amniotic fluid	Maternal age =6 Anomaly on US= 5 Family History abnormality =2 multiple miscarriages=1	14 pregnancies 15 fetuses
3	Shaffer LG et al. (40)2008	prospective	Prenatal BAC array version (signature) Commercial	2,100 BAC probes	TG	Prenatal cultured Amniotic Fluid or Chorionic Villus Sampling Postnatal blood	Family history = 19 Maternal age = 2 Parental anxiety = 20 Anomaly US = 110: (Abnormal genitalia=6 CNS = 19 CH/NF/NT/hydrops =22 GI = 9 Dysmorphic = 4 Cardiac = 17 GD = 2 Multiple = 3 Micrognathia = 2 Midline defect = 8 Renal = 2 Skeletal = 11 SUA = 1 Others= 4)	151 prenatal
4	De Gregori M et.al. (21) 2007	retrospective	60-mer oligonucleotide microarray Commercial	60 mer oligo probes	WG	Does not state	Reciprocal translocations=14 Maternal age =3	17
5	Rickman L et al.(41) 2006	retrospective	BAC/PAC resolution 10Mb common microdeletion syndrome own array	600 BAC probes	TG	Cultured amniocytes or Chorionic Villus Sampling	Previously known karyotypes All known unbalanced rearrangements	30
6	Sahoo T et al.(38) 2006	prospective	BCM v4.0 Baylor Commercial	366 BAC probes	TG	Amniotic Fluid =56 (26 uncultured) Chorionic Villus Sampling =42 (32uncultured)	Increase maternal age Increased serum screen Family history Abnormality on US	98 samples total

7	Le Caignec C et al. (42)2005	retrospective	Genosensor BAC array 300 (Vysis/Abbott) commercial	287 BAC probes	TG	Frozen fetal tissue	All normal karyotype All had malformations All had at least 3 anomalies in CVS/urogenital/ digestive/CNS	49
8	Vialard F et al. (45)2009	prospective	Genosensor BAC/PAC array 300 (Vysis/Abbott) commercial	287 BAC probes	TG	Muscle biopsy =15 Lung biopsy=13 thymus = 4 skin = 3 liver = 3 bladder = 1	2 or more abnormalities cardiovascual/urogenital/skeletal/digestive/CNS	39
9	Coppinger J et al.(35) 2009	prospective	Signature prenatal chip V 4.0 Commercial	>2100 BACs	TG	Amniotic fluid = 40 CVS = 22	Anxiety= 6 Family history= 19 Advanced maternal age= 3 Abnormal maternal serum screen =1 Abnormal ultrasound =33: (CNS= 3 Skeletal =1 CH/NT/hydrops =18 GI =1 IUGR= 1 MCA =7 Unspecified =7) All normal Karyotype	62
			Signature Chip Whole Genome commercial	4670 BACS	WG	Amniotic fluid=149 CVS=30 Unspecified prenatal cell type =3	Anxiety =3 Family history =17 Advanced maternal age=5 Abnormal maternal serum screen =2 Abnormal ultrasound =155: (CNS= 16 Musculoskeletal =8 Cleft lip =6 CH/NT/hydrops =24 GI= 4 Renal/ambiguous genitalia =4 Cardiac =13 IUGR =7 Multiple =63 Unspecified =10)	182
10	Kleeman L et al. (44) 2009	prospective	Signature prenatal chip V 4.0 26 patients Signature whole genome chip commercial	1887 BACs 4685 BACs	TG WG	Amniotic fluid = 47 CVS = 3	Abnormal ultrasound scan and normal karyotyping: (Cardiac =24 CNS = 6 Skeletal = 6 Urogenital = 4 Cleft lip/palate = 2 CH/NT/hydrops = 3 GI = 2 Multiple = 17 Growth disorder =3)	50

BAC=Bacterial Artificial Clone
BCM=Baylor College of Medicine
CNS=Central Nervous System
CNV=Copy Number Variant
GI=Gastrointestinal
IUGR=Intrauterine growth retardation
NT=Nuchal Translucency
SNP= Single nucleotide Polymorphism
SUA=Single Umbilical artery
TG= Targeted Array
WG=Whole Genome array

Study Characteristics

Table 3 summarises the characteristics of the publications used including the design of the study (retrospective or prospective), the array type, sample type, if the array was targeted or covered the whole genome, the indication for the array (divided into different structural abnormalities where known) and the sample size. Study quality assessment showed deficiencies in many areas of methods (Figure 3). Only 3 papers meet all four quality criteria; being prospective in design, using array and karyotyping on a representative population (i.e. population did not have all known abnormal/normal karyotype), investigating parents to aid interpretation of CNVs and using a validated assessment tool (38;39;45).

Agreement between Tests

The overall agreement between karyotype and array results was 88.2% (95% CI 79.2-98.2-100%). When benign CNVs were removed from the abnormal array group and treated as normal array results (as described above in the second analysis) the agreement was increased; 95.6% (95% CI 86-100%). The data were homogeneous (Chi^2 p=0.99, p=0.97 respectively). Four out of ten papers were used to review the overall agreement between karyotype results and array results (n=333). Six out of ten papers could not be included as the data set was not complete (i.e. sample population was skewed by only using CMA on those samples with all normal or all abnormal karyotypes, papers (21; 40-44)).

Results: in cases where testing was performed for any referral indication

CMA detected overall 12% (95% CI 8.8 -16.4%) more chromosomal imbalances when karyotyping was “normal” (Figure 5a) when the array was performed for any clinical indication. When benign CNVs were recognised, removed and treated as normal results, the detection rate decreased to 3.6% (95% CI 1.5 - 8.5%) (Figure 6a). This 3.6% included all CNVs known to be pathogenic and those of unknown significance with the potential to be pathogenic. I therefore calculated how often a result of unknown significance would be found when CMA was performed prenatally for any clinical indication. Results of “unknown significance” were found in 1.1% (0.4 - 2.7%) of cases. Eight out of ten papers were used for these meta-analyses (35;38-40;42-45) (n=751). Two were excluded as they did not contain data for chromosomal anomaly detection rate by array when a normal karyotype was reported ((21;41). These data were heterogeneous (Chi^2 p=0.00).

One of the papers appeared to contribute disproportionately to the heterogeneity of the data (Tyreman et al (43)). This paper used a higher resolution array (Affymetrix SNP 6) and did not use parental testing for clarification of CNVs of unknown significance. It therefore had a high detection rate of all CNVs; pathogenic, unknown significance and benign. Because of this I performed a sensitivity analysis by excluding the results from this paper. With this paper excluded array detected 10.7% (95% CI 9.1-12.6%) more chromosomal imbalances when karyotyping was normal compared with 12% (95% CI 8.8 -16.4%) with the paper included. With exclusion of the paper by Tyreman et al and when benign CNVs were removed and treated as normal results, the detection rate of chromosomal imbalances by CMA decreased

from 3.6% to 2.9% (95% CI 1.3-6.3%) when karyotyping was normal. Exclusion of this paper did not significantly reduce the CMA detection rate; in addition the data were still heterogeneous. Taking this into account, and given that the paper by Tyreman et al is an important paper with one of the largest cohorts of patients, it was decided that the inclusion of this paper was important to present the totality of evidence.

Conventional karyotyping did not detect any chromosomal imbalances that were not detected by array CGH in the same 8 papers used in the above meta-analysis. Two papers included in the systematic review (21;41) but not in the meta-analysis used CMA only when an abnormal karyotype had been found. They did find that CMA was not able to detect one case of triploidy and 14 cases of balanced translocation. These two papers were not included in the meta-analysis as they only looked at cases with abnormal karyotype results and therefore a 2x2 table of their results could not be constructed.

Results: in cases when a structural abnormality was noted on ultrasound scan

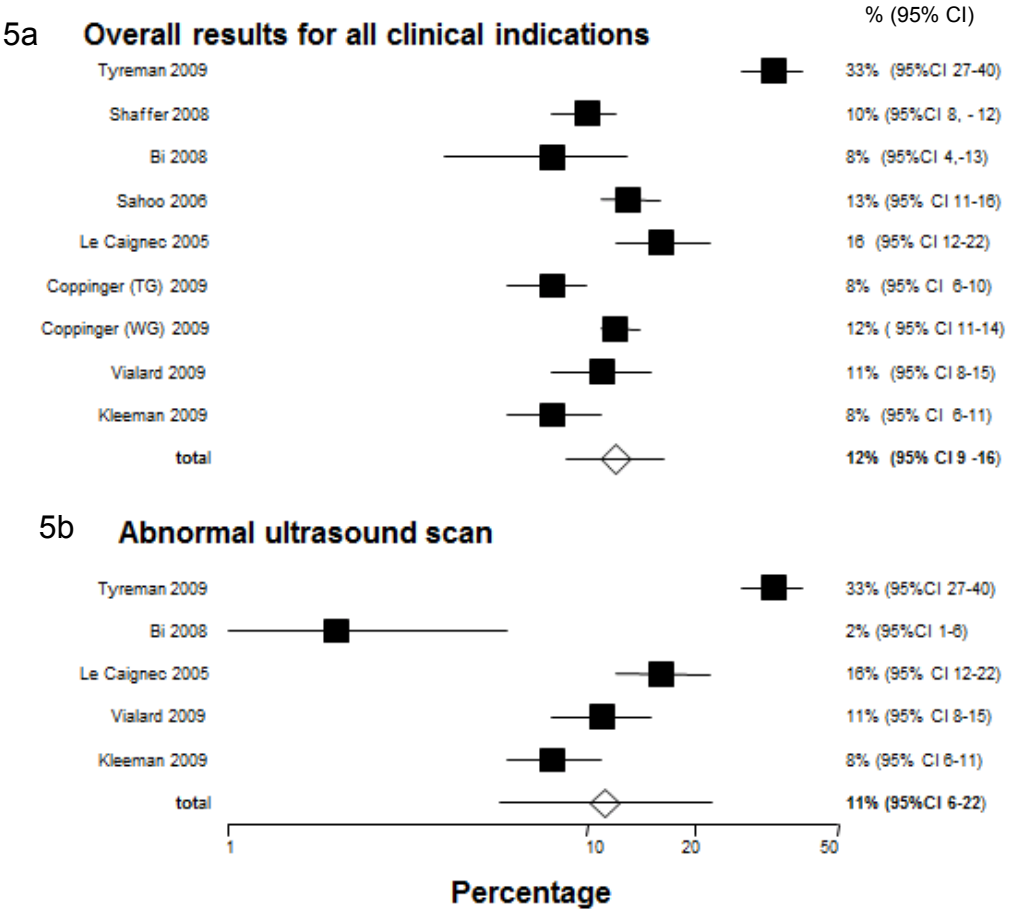
Array technology detected overall 11.2% (95% CI 5.7 - 22.1%) more chromosomal imbalances above that of conventional karyotyping (Figure 5b). When “benign CNVs” were removed from the analysis and placed with the normal array results, the detection rate of chromosomal abnormalities decreased to 5.2% (95% CI 1.9 - 13.9%). This included results that are known to be pathogenic and those of unknown significance with the potential to be pathogenic (Figure 6b). Six papers were used

(39;40;42-45). These papers all contained results on patients that had undergone karyotyping, and array tests as they had pregnancies where a structural fetal malformation was suspected on ultrasound scan (n=359). The size of these chromosomal imbalances ranged depending on the resolution of the CMA used from 60kb(18) to 60Mb(17). In 1.9% (0.4 - 9.5%) of cases where the patient was referred with a fetal anomaly on ultrasound and conventional karyotyping was “normal”, a result of “unknown significance” was reported. These data were heterogeneous (Chi ^{2} p=0.000).

The actual numbers of different structural abnormalities were recorded in 4 out of the 6 papers (35;40;43;44). Where possible the different structural abnormalities are recorded in table 3. The pooled data from the 4 papers show that the largest numbers of patients had cardiac abnormalities (n=88), increased nuchal translucencies, cystic hygromata or hydrops (n=82) or central nervous system abnormalities (n=60). It is not possible to divide the structural abnormalities into groups to perform separate analysis for CMA testing as all four papers include groups with either “multiple congenital abnormalities”, which was not broken down further, or “other” groups where the abnormality is not specified. None of the papers include single “soft” markers although the paper by Tyreman et al includes multiple soft markers (43). The vast majority are major abnormalities. The papers also do not always provide data post delivery/post mortem so it is not possible in most cases to say if the abnormality was confirmed or, in the case of increased nuchal translucency, if it was linked to other structural abnormalities.

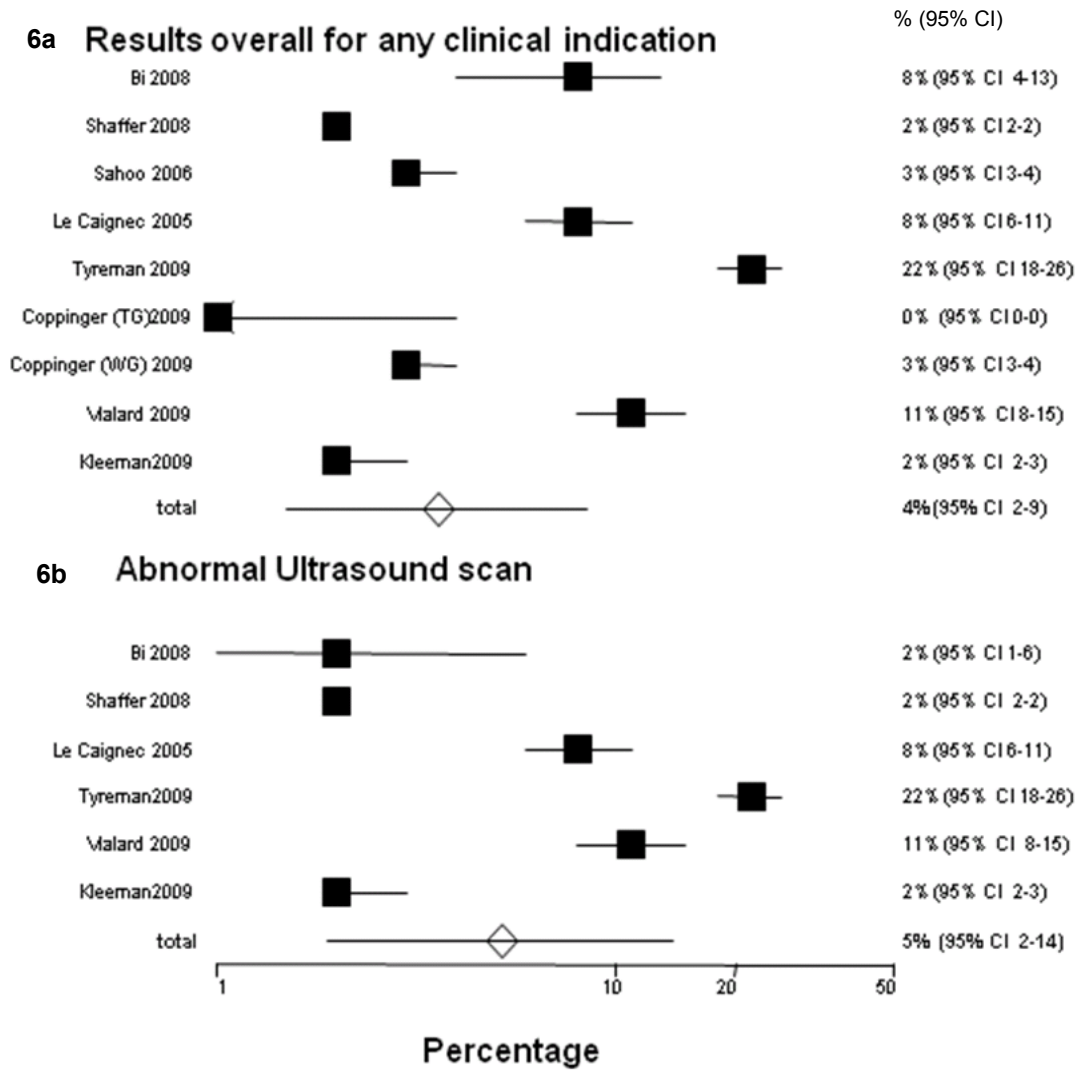
Conventional karyotyping did not detect any chromosomal imbalances that were not detected also by CMA.

Figure 5 Detection of chromosomal imbalances by CMA when Karyotype is normal (including benign CNVs)



Legend Figure 5: Forest Plot showing the percentage of chromosomal imbalances detected by CMA over conventional G-band karyotyping when the analysis is performed including pathogenic CNVs, VOUS and benign CNVs. 5a) Results when testing is performed for any clinical indication, 5b) Results when testing performed for abnormal fetal ultrasound findings.

Figure 6 Detection of chromosomal imbalances by CMA when Karyotype is normal (excluding benign CNVs)

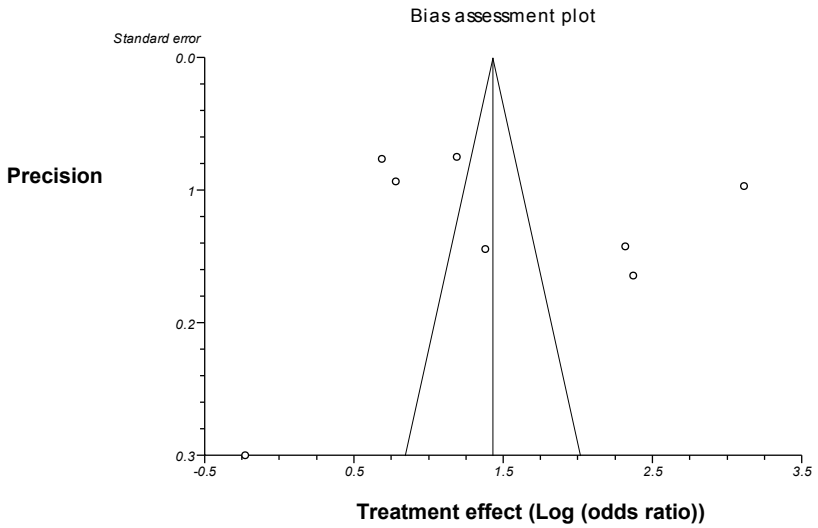


Legend Figure 6: Forest Plot showing the percentage of chromosomal imbalances detected by CMA over conventional G-band karyotyping when the analysis is performed including pathogenic CNVs, VOUS but excluding benign CNVs. 6a) Results when testing is performed for any clinical indication, 6b) Results when testing performed for abnormal fetal ultrasound findings.

Publication Bias

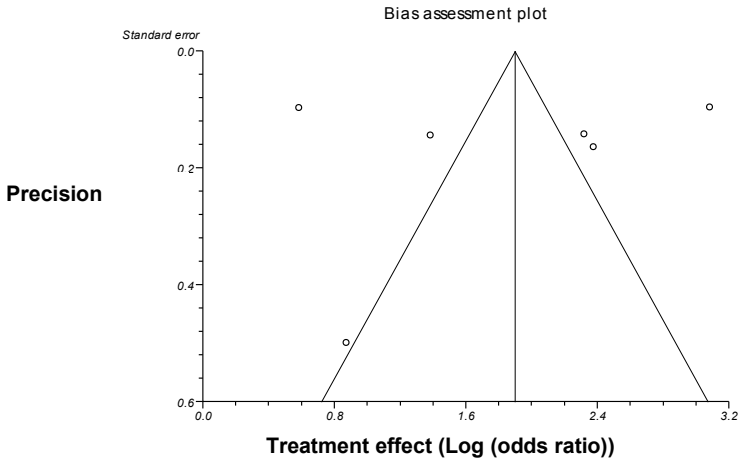
Publication Bias was assessed by performing Eggers Test (based on a linear regression method where the standard normal deviate is plotted against precision Appendix J) and by use of funnel plots. When array detection over karyotype was meta-analysed there was no significant publication bias for either analysis (Eggers testing performed for any indication p=0.667 when testing performed for abnormal scan findings p=0.9406). This can be shown in the funnel plots below (Figure 7 and 8).

Figure 7 funnel plot of publication bias when testing performed for all indications



Legend Figure 7: Funnel plots graphically represent the papers precision (or number of participants in the study) against the treatment effect (from a negative effect on the far left to a positive effect on the right). We can see graphically if there is any publication bias if small negative studies (those on the bottom left) have been excluded. The funnel plot above does not show evidence of publication bias.

Figure 8 Funnel plot of Publication bias when testing performed for abnormal ultrasound findings.



Discussion

Overall agreement between prenatal array technology results and conventional cytogenetic karyotyping from data in the literature until 2009 was good. This increased further with removal of “benign” CNVs. It is to be expected that the percentage agreement between technologies would increase with removal of “benign” CNVs as the remaining microarray results are more likely to be pathogenic.

CMA detected notably more chromosomal imbalances over conventional karyotyping both when it was performed prenatally for any clinical indication and when it was performed for a structural abnormality on scan. As well as results of a known pathogenic nature this does, however, include results of unknown pathogenic significance.

The frequency of CNV of unknown significance is increased if parental samples are not available, as analysis will reveal whether the variant detected in the fetus is

familial or *de novo*. However, because of incomplete/variable penetrance, familial variants are not always benign (28). Variants of unknown significance (or VOUS) results are of concern as they may both increase parental anxiety and lead to problems in prenatal counselling. I was aware that it would be important to define the VOUS rate in my work going forward, but in addition to the rate it would also be important to ask women and their partners how they thought or felt about these results.

Karyotype did not detect any chromosomal imbalances when CMA was reported as normal in those papers used in the meta-analysis (35;38-40;42-45). This was reassuring to us as it suggested that CMA alone will not miss many significant results, and that areas in which microarray technology is weak are not common in a typical referral population (balanced translocations, inversions and a lower sensitivity for triploidy).

The strength of the study lies in the rigor of the methodology. It met quality criteria laid down in the MOOSE(46) statement. But the meta-analysis contained a relatively small sample size of 751 participants for overall analysis and 409 participants with fetal anomalies identified using ultrasound. Many cohorts were retrospective with case selection rather than consecutive cases. Papers were heterogeneous with both prospective and retrospective methodology, different indication for referral and different CMA used. This accounts for the large confidence intervals in my analysis.

The inclusion of some published studies that include participants with a known abnormal karyotype may have influenced the results as they are not necessarily a representative population. This was particularly so when they were being used to illuminate the weaknesses with CMA technology such as detection of mosaicism, or when cases of known inversions or translocations were tested by CMA to see if the rearrangement was balanced.

Array technologies have increased in resolution over the time these studies were conducted, but they have also become more commercially available allowing greater validation of results between different published studies. I could not allow for ascertainment bias towards cases that clinicians may have felt would have yielded an abnormal result from array and therefore included them in their results (i.e. choosing a case with multiple structural abnormalities).

In 2009 this systematic review provided evidence of the relative advantage of using array testing in prenatal diagnosis even when karyotype is normal. But many of the cohorts were small and retrospective using custom designed arrays, and cases were selected by clinicians/scientists. Many questions at this point in time were still left unanswered. What would be the added information provided by CMA over convention G-band karyotyping in a prospective large cohort where consecutive women were recruited? The indication for referral with the largest detection rate by CMA appeared to be fetuses with abnormal scan findings. What would be the true detection rate by CMA in this cohort? Would it be reasonable to suggest CMA as frontline testing in this group? Would CMA represent a cost effective option in the

NHS? Would variants of unknown significance be commonly encountered and how would women and their partners view results that had an element of uncertainty?

Up to 12% of any individual's genome is likely to exhibit normal copy number variation (CNV) and there is emerging evidence of a huge degree of structural complexity within these chromosomal regions (27). In addition, limited data exists on the prevalence of CNVs between different ethnic populations (47). In 2009 These concerns led to recommendations from the ACOG that conventional G-band karyotyping should remain the principal cytogenetic tool in prenatal diagnosis. They also suggested that targeted arrays can be offered as an adjunct in prenatal cases with abnormal anatomical findings and a normal conventional karyotype (48).

This systematic review and meta-analysis (prior to the undertaking of my own cohort) revealed an increased detection rate of chromosomal abnormalities with CMA, both for prenatal indications overall and when congenital malformations were noted on ultrasound scan. But large confidence intervals included in the analysis show that more work must be done before I could answer the question of absolute detection rate over conventional karyotyping. Prospective research was needed in this area with a large cohort that undergo both karyotyping and a commercially reproducible array. The optimum resolution array to be used in a prenatal setting had not yet been decided, and a targeted array would be suitable in its ability to identify CNVs in known disease specific loci of the human genome, but risks missing a pathogenic CNV not in these particular genomic regions. A high resolution array will have the ability to detect more CNVs but risks having more results of unknown certainty which

need additional time for interpretation and provoke additional uncertainty. I concluded that perhaps while this technology was being investigated targeted arrays were more suitable when they were to be used in the clinical setting and results fed back to patients.

It was concluded that a Health Economic assessment of microarrays was of importance when evaluating the implementation of this prenatal diagnostic test into routine practice. Within the UK NHS setting almost all prenatally obtained samples are screened using QFPCR to exclude common trisomies. A proportion of centres also continue to offer full karyotyping and depending upon the type of structural malformation noted, target testing for specific chromosome anomalies are performed (such as FISH for Di-George syndrome when a cardiac anomaly is detected).

Finally it was concluded that there was an urgent requirement for patient satisfaction and qualitative research into the emotional response in parents with the implementation of such prenatal diagnostic tests.

Published or work in press from Chapter 1

Published: Additional information from array comparative genomic hybridization technology over conventional karyotyping in prenatal diagnosis: a systematic review and meta-analysis. Hillman SC, Pretlove S, Coomarasamy A, McMullan DJ, Davison EV, Maher ER, Kilby MD.Ultrasound Obstet Gynecol. 2011 Jan;37(1):6-14

Published: Microarray comparative genomic hybridization in prenatal diagnosis: a review. Hillman SC, McMullan DJ, Williams D, Maher ER, Kilby MD. Ultrasound Obstet Gynecol. 2012 Oct;40(4):385-91

Published: The use of Chromosomal Microarray (CMA) in prenatal diagnosis Hillman SC, McMullan DJ, Maher ER, and Kilby MD The Obstetrician & Gynaecologist. 2013 April; 15(2):80-84

Published: Prenatal microarray technology for the identification of chromosomal anomalies. Hillman SC, McMullan DJ, Maher ER, and Kilby MD. Current progress in Obstetrics and gynaecology 1. Chapter 10: 167-181

AIMS

1) To define the detection rate by CMA over conventional G-band karyotyping when there is a structural anomaly on ultrasound scan using my chosen CMA platform. I would define this by recruiting a prospective consecutive cohort of women that had accepted chromosomal testing because of structural abnormalities on fetal ultrasound scan.

2) In a subset of the cohort to run (postnatally) a higher resolution (60K) array to evaluate the effect of pathogenic chromosomal detection rates and also variant of unknown significant detection rate.

3) To perform a health economic analysis looking at the amount the NHS would have to be willing to pay for a case to be detected by CMA.

4) To perform qualitative work to evaluate how women and their partners feel about the use of CMA testing, particularly when a result has uncertainty attached.

5) To define the ethical dilemmas presented by prenatal CMA testing and suggest potential solutions.

CHAPTER 2 PROSPECTIVE PRENATAL MICROARRAY COHORT

“THE BIRMINGHAM BAC ARRAY COHORT”

Introduction

The introduction to CMA testing and the literature until 2009 have been outlined in Chapter 1. In order to attempt to answer aim 1 (To define the detection rate by CMA over conventional G-band karyotyping when a congenital structural anomaly is detected on ultrasound scan using my chosen CMA platform.) a prospective cohort study was designed (49). Pregnant women whose babies were noted to have a congenital malformation on ultrasound examination were offered fetal karyotyping and after consent would undergo both full, "conventional" G-band karyotyping and CMA testing. Other "sub aims of this study" included:

1) to define the Variant of Unknown Significance (VOUS) rate in this case cohort as this would be of extreme importance when and if translated into routine clinical care.

2) to determine if the tissue sample type (amniocentesis, Chorionic Villus Sampling, fetal blood sampling) made a difference to quality of DNA extracted and the failure rate of the CMA technique.

I decided, after initial discussion, that a "targeted", relatively conservative, constitutional Bacterial Artificial Chromosome (BAC) array should be used for my study. Although of lower resolution (and therefore potentially would miss pathogenic CNVs) there were advantages to using this array:

a) The West Midlands Genetics laboratory (WMGL) that would be performing the microarray testing were experienced with the use of the array in postnatal testing and preliminary analysis indicated that it worked with prenatal DNA

(which is often of lower quality and quantity when compared with postnatal) samples.

b) The “turnaround time” for results was <14 days which would be important in a prenatal cohort.

c) I felt that using this BAC array would limit the CNVs that were judged to be of unknown significance.

Limiting Variants of Unknown Significance (VOUS) is of the upmost importance in the prenatal period, particularly if the VOUS is unrelated to the phenotype seen on scan, as a more detailed phenotype is unavailable prenatally (i.e. autism). There is currently no national or international guidance on the reporting of VOUS and I felt that I should consider each case on a “case-by-case” basis but if a VOUS did arise that had potential clinical significance it would be discussed with a consultant clinical geneticist who would feed back the information to the patient alongside a fetal medicine consultant.

Methods

Recruitment

A protocol was written (Appendix a) after discussion with the fetal medicine consultant lead at Birmingham Women's Foundation Trust, a consultant clinical geneticist of the West Midlands Regional Clinical Genetics Service and also the Head of Cytogenetics in the West Midlands Genetics Laboratory (WMGL). Patient information sheets were also written after discussion with Dr Peter Farndon, Director of the National Genetics Education and Development Centre in Birmingham (Appendix b). Two information sheets were created; one for on-going pregnancies and one for when a decision had been made to terminate the pregnancy. Consent forms were produced in line with guidance from the National Research and Ethics Service (Appendix c).

Pregnant women were approached at Birmingham Women's Foundation Trust within the tertiary referral centre for Fetal Medicine, as a fetal anomaly was suspected. After a detailed ultrasound scan had confirmed the fetal anomaly (or redefined the fetal anomaly they had been referred with) women were counselled by a fetal medicine consultant (and if appropriate other specialists). Part of this counselling involved discussion relating to the possibility of a chromosomal anomaly being associated with the abnormal ultrasound scan findings. Prenatal testing for common autosomal aneuploidies in the form of QFPCR (for Trisomy 13, 18 and 21) as well as sex chromosome aneuploidy was offered. In our centre, all fetal karyotypes are routinely further investigated using conventional G-band karyotyping. If accepted, women were approached to have CMA testing, within the Birmingham BAC Array

study. Women were formally counselled regarding the increased resolution of the test over G-banding but were also informed of the disadvantages, namely detection of VOUS. The fact that CMA would miss balanced rearrangements was not discussed as G-band karyotyping was being performed in parallel which would detect this. Written information was given to the patient.

Inclusion and exclusion criteria

Inclusion criteria for the study included if a congenital malformation on ultrasound scan or a nuchal translucency measuring over 3.5mm (between 11-13+6 weeks) was noted. Single, so-called “soft markers” (echogenic bowel, choroid plexus cyst, echogenic cardiac foci, and single umbilical artery) were not indications for inclusion, although “multiple ultrasound markers” were included. Women were also excluded from consent if they were under 18 years old or it was felt by the counselling consultant that the patient lacked the capacity for providing informed consent of the test.

Details of the Cohort

I prospectively recruited 317 (97%) prenatal women seen at the Fetal Medicine Centre at Birmingham Women’s Foundation Trust. Six women (1.5%) were recruited by University Hospital Coventry and Warwickshire NHS trust and five (1.5%) were recruited from City and Sandwell NHS trust. Recruitment took place between November 2009 and April 2012. Ethical approval for the study was granted by the Staffordshire Research and Ethics Committee (reference no. 09/H1203/74). The

fetal sample that was taken was either amniotic fluid by amniocentesis (typically 20mls) n=154 (63.5%), Chorionic Villus Sampling (typically 15-25mgs) n=53 (21.5%), fetal blood (typically 1-1.5mls) n=29 (12%), fetal tissue taken at post-mortem (n=6; 2.5%) and in one case (0.5%) a pulmonary effusion sample (containing a high density of fetal lymphocytes).

Parental (maternal and paternal) venous blood samples were obtained at the same time as the invasive fetal sample to exclude maternal cell contamination and to establish inheritance of CNVs where necessary. Samples types and the need for culturing to increase the DNA quantity are described in detail in Table 4.

Table 4 Sample type used in Birmingham BAC cohort

Sample type	Sample number and percentage
Uncultured amniocentesis	146 (60%)
Cultured amniocentesis	8 (3.5%)
Uncultured Chorionic Villus Sampling	50 (20.5%)
Cultured Chorionic Villus Sampling	3 (1%)
Fetal Blood Sample	29 (12%)
Fetal Tissue	6 (2.5%)
Pulmonary effusion sample	1 (0.5%)

.

CMA testing was not initiated until the results of the quantitative fluorescent polymerase chain reaction (QFPCR) were available and not processed further if Trisomy 13, 18, 21 or Monosomy X were detected. If QFPCR was normal then karyotyping and microarray testing were run in parallel.

Laboratory methodology

Differently fluorescently labelled test and reference DNAs of the same gender were competitively hybridised to whole genome BAC (Bacterial Artificial Chromosome) microarrays, (CytoChip Focus constitutional, BlueGnome Cambridge, UK®). The methods for which are outlined in appendix D. These arrays have BAC data points at every 1Mb in the genome as a whole and tiled, overlapping BACs in regions associated with recurrent constitutional syndromes and within subtelomeric and centromeric regions. Each data point is replicated within the array design (triplicate in the genome backbone and quadruplicate in targeted regions) negating the need for a dye-swap experiment. This relatively “focused” microarray was chosen to minimise detection of VOUS. In essence I used a >2 x BAC threshold for calling which equates to a working resolution of >2Mb genomic backbone and >200kb in targeted regions. The laboratory methods were performed by staff in the WMGL due to the number of samples processed and the rapid turnaround time required.

CMA Analysis

Detected copy number gains or losses were compared with known CNVs in publicly available databases (Database of Genomic Variants http://www.ncbi.nlm.nih.gov/dbvar/, Decipher, http://decipher.sanger.ac.uk/, International Standards for Cytogenomic Arrays Consortium database, https://www.iscaconsortium.org/index.php/search) and against our own internal database to ascertain the clinical significance of the variation. Those CNVs of clinical significance or unknown significance were confirmed by fluorescence in situ hybridisation (FISH) on metaphase spreads using one or more BAC clones within

the abnormal region and in some cases were repeated on higher resolution microarray (Affymetrix 2.7M Cytogenetics Research array or ISCA 60K CytoChip oligonucleotide array). The same BAC microarray was also performed on parental samples to evaluate if the CNV was inherited or had occurred de novo.

Classification of CNVs and blinding

CNVs were classified as benign, VOUS or pathogenic in accordance with the American College of Medical Genetics (ACMG) guidelines (50). Pathogenic CNV are documented as clinically significant in multiple peer-reviewed publications. Penetrance and expressivity of the CNV is well defined, even if known to be variable. They may include large CNVs not described in the medical literature at the size observed in the fetus, but overlap a smaller interval with clearly established clinical significance.

A benign CNV will have been reported in multiple peer-reviewed publications or curated databases as being a benign variant, particularly if the nature of the copy number variation has been well characterised, and will typically represent a common polymorphism (documented in greater than 1% of the population).

VOUS will have included findings that are later demonstrated to be either clearly pathogenic or clearly benign, but insufficient evidence was available for unequivocal determination of clinical significance at the time of reporting (50).

Results were fed back to patients when they were pathogenic or a VOUS. On average the turnaround time for CMA was 10 days. When a potentially pathogenic difference was detected on CMA I was contacted by the WMGL and I then made an appointment for the patient and her family to be seen by a consultant clinical geneticist, normally the next working day.

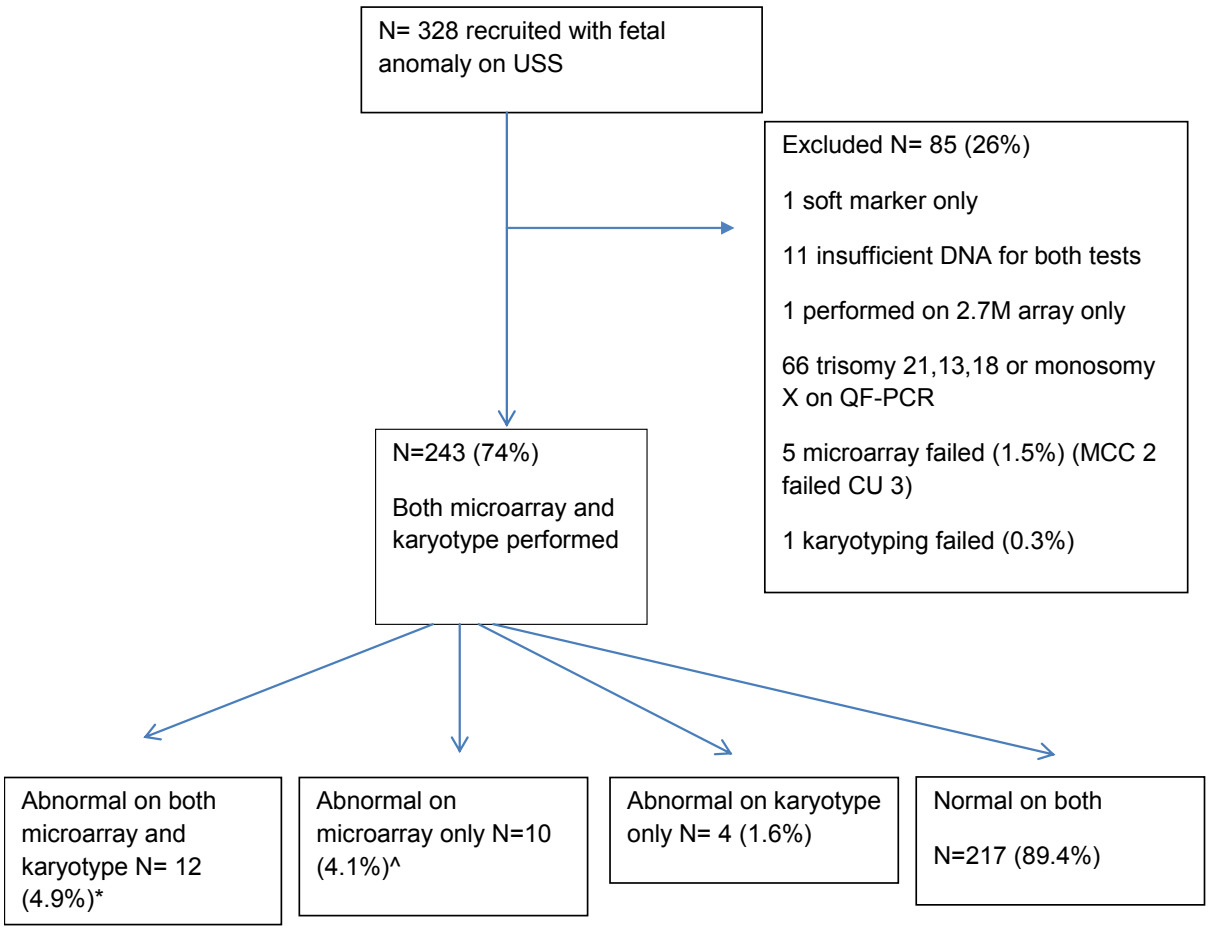
Analysis of the CMA took place at the WMGL where conventional G-band karyotyping results for the sample were also being interpreted. The analysts were not strictly blinded from one another and therefore it was a possibility that a positive result by CMA could alter how the conventional G-banding karyotyping was interpreted. Therefore, to exclude that possibility, slides were sent to a second laboratory for G band karyotyping (where the results of the microarray were unknown) for prospective and independent interpretation (South East Scotland Cytogenetics Service, Western General Hospital, Edinburgh). Analysis was included into the workflow of this second laboratory to ensure that the analysis was blinded.

Results

328 women were prospectively recruited to have both CMA and full, conventional G-band karyotyping performed. Uptake was 90% of those approached. One patient was excluded, as at recruitment, there was only a single soft marker visualised on ultrasound scan. Eleven subjects were excluded as there was insufficient DNA to perform both tests. One was only performed on a very high resolution Affymetrix 2.7M array and was therefore excluded. 66 samples were excluded as QFPCR demonstrated either Trisomy 21,13,18 or Monosomy X (20%). The BAC microarray failed in 5 cases (1.5%), two of which were due to maternal cell contamination and three due to failure of the DNA quality. Two cases were fetal blood samples, two were amniocentesis samples and one was a CVS sample. There was no association between different invasive sample types and failure of the CMA test. One case of conventional G-band karyotyping failed (0.3%). Therefore BAC CMA was performed on 243 samples (from 243 patients) and compared to G-band karyotyping in the same samples (Figure 9).

A total of 228 (94%) samples from fetuses with congenital abnormalities in a single 'anatomical' system were included and 15 (6%) samples from fetuses with abnormalities within two or more systems as seen on scan. The range and type of fetal anomalies are described in Table 5 and were classified according to the Human Phenotype Ontology Website (www.human-phenotype-ontology.org/).

Figure 9 Flow diagram of samples for the Birmingham BAC chromosomal microarray Cohort



QF-PCR = Quantitative Fluorescent PCR

MCC = Maternal cell contamination

CU = clean up

*Blinded results

^Including 9 pathological and 1 VOUS reported to patients

Legend: The flow diagram above account for all cases that were included in the BAC array cohort

Table 5 Structural anomaly classified using the Human Phenotype Ontology (HPO) System

Structural anomaly	N=	Percentage
Single	**228**	**94%**
Central nervous system	51	21%
Cardiovascular system	41	17%
Increased Nuchal Translucency>3.5mm /Cystic hygroma	40	16.5%
Musculoskeletal system	25	10.5%
Genitourinary system	20	8.5%
Congenital diaphragmatic hernia	15	6%
Abdominal wall defect	11	4.5%
Head/face/neck	7	3%
Respiratory system	5	2%
Spina Bifida/ encephalocele	5	2%
Hydrops	4	1.5%
Gastrointestinal tract	3	1%
Tracheal/oesophageal fistulae	1	0.5%
Multiple systems	15	6%

In 2 fetal samples the original chromosomal abnormality seen at the WMRGL was not noted on the 'blinded' review at a second laboratory where the cyto-geneticists were unaware of the microarray findings (Table 6 fetal samples 16-17). These included a mosaic duplication of 12p13.33-p11.1 consistent with Pallister Killian syndrome (case 16) and a de novo 1.7-3 Mb deletion between 1p36.33 and 1p36.32 consistent with 1p36 microdeletion syndrome. Both would have been causative of the findings on scan (case 17). The results presented are those of the "blinded" data series.

243 BAC microarrays were processed; 156 (64%) had no CNVs and 87 had at least one CNV (36%). In total 121 CNVs were found within these 87 samples and these were divided into benign, VOUS and pathogenic CNVs. 90/121 (74%) were common benign CNVs, 2 (1.6%) were uncommon benign CNVs requiring further testing with FISH (in 2 separate samples) and these results were not fed-back to patients.

Three VOUS were detected in 3 separate samples (2%) (Table 6 fetal samples 1-3) although this was reduced to just n=1 (fetal sample 1) (0.8%) after further testing using higher resolution microarray (two VOUS were reclassified as either artefact or uncommon benign CNV). The single "true VOUS" result (case 1) was fed back to the patient and presented a difficult counselling scenario. A 0.5-1Mb duplication between Xp22.32 and Xp22.31 was detected in a male fetus with a cardiac anomaly (truncus arteriosis). This had led to partial duplication and possible disruption of the gene NLGN4. The gene has no known cardiac link but has been linked to neurodevelopmental delay and autism. This duplication was maternally inherited (the

mother having no learning difficulties). Only one, phenotypically normal, male relative was available for testing, but he did not have the CNV. X-inactivation studies carried out on maternal DNA were inconclusive.

Seventeen CNVs (14% of the CNVs) were classified as pathogenic and were also seen by karyotyping (in 12 separate fetal samples, 4.9% of the cohort Table 6; 4-15). These included 4 cases of aneuploidy (2 cases of Triple X also detected by QFPCR and 2 cases of Trisomy 9). In seven cases there was a large structural chromosome anomaly present visible on both G-band and microarray (cases 8-14). In one case the structural chromosome abnormality (a de novo ~3.5Mb deletion within 17p11.2 consistent resulting in Smith-Magenis syndrome) was at the limit of cytogenetic resolution and was only visible due to high quality chromosome preparation.

Nine CNVs (7.4%) in 9 fetuses were considered pathogenic and were not detectable by karyotyping (Table 6; 16-24). These included a finding of a mosaic isochromosome 12p (consistent with Pallister Killian syndrome), 1p36 microdeletion, 4 cases of 22q11.2 microdeletion (Di George syndrome) and deletion of PMP22 (associated with hereditary neuropathy with liability to pressure palsies). One fetus (Table 6 Fetal sample 17) had a de novo 6-8Mb duplication between 11q24.2 and 11q25 including ~50 HGNC genes and 17 OMIM genes. Finally one fetus (Table 6 fetal sample 24) had a ~3.2 Mb deletion at 5q35.3. More detailed analysis using an ISCA 60K oligoarray further defined the deletion as being ~1.9Mb in size with additional ~1.1Mb duplication at 17q25.3, both of which had been inherited from the mother who had an unbalanced translocation between 5q and 17q. The mother has

dyspraxia and mild facial dysmorphia. The unbalanced translocation was inherited from the proband's maternal grandmother who had the translocation in a balanced form and does not have any learning difficulties. Prenatal ultrasound findings demonstrated absent corpus callosum and a meningocele. As the mother shows a phenotype consistent with the chromosomal abnormality it was deemed likely to be pathogenic.

In five fetal samples (2% of the cohort) karyotyping detected a chromosomal anomaly when microarray was reported as normal; one was a false positive due to maternal cell contamination, and three were balanced inherited inversions unlikely to have a phenotypic effect on the fetus. The fifth fetus was mosaic for monosomy X on an amniocentesis sample and triple X on CVS (undetected by QFPCR), in a fetus with a univentricular heart and hydrops fetalis. Postnatal blood taken from the baby after birth showed mosaicism for Monosomy X and triple X (47,XXX[45]/45,X[15]). This chromosomal anomaly is consistent with the phenotype on scan (Table 6 25-28).

In the cohort those samples showing Trisomy 13,18 and 21 and monosomy X did not go on to have CMA testing (n=66 had Trisomy 13,18, 21 and monosomy X and n=46 (70%) had an increased nuchal translucency over 3.5mm). Of the 22 pathogenic chromosomal and one true VOUS identified by either CMA and/or conventional karyotyping it is worth noting that 7 cases (30% of pathogenic findings) had an increased nuchal translucency >3.5mm as either part or the sole reason for testing.

Table 6 Positive chromosomal abnormalities detected by CMA and/or G-band karyotyping

No.	Ultrasound scan findings	G-banding results centre 1	G-banding results centre 2	Chromosomal Microarray	Classification	Pregnancy outcome
1	**Truncus Arteriosis**	Normal	Agree centre 1	arr cgh Xp22.32p22.31(RP11-60N3->RP11-769N24)x2, Xp22.3 (RP11-44F2)x2 mat possible disruption NLGN4	VOUS Confirmed on 2.7M array	LB Had cardiac surgery postnatally. Normal development at 7 months of age
2	**Arms and hand in fixed flexion Feet severe equinous deformity Hydrothorax Possible micrognathia Small cerebellum**	Normal	Agree centre 1	arr cgh 9q34.11(RP11-339B21-RP11-409K20)x3 dn	VOUS BAC array. Repeated on fetal tissue 2.7M array most likely artefact report amended	TOP PM confirmed micrognathia abnormal posture and pleural effusions Cerebellum appeared normal
3	**Increased nuchal translucency >3.5mm and bladder outflow obstruction**	Normal	Agree centre 1	arr cgh 6q27 (RP1-19N21-RP11-114B3) x1 mat	VOUS BAC array; FISH and 60k ISCA array likely uncommon benign	LB no follow up data available

4	**Increased nuchal translucency >3.5mm**	Trisomy 9	Agree centre 1	Agree	Pathogenic	TOP PM report demonstrated hydrocephaly. Severe dysmorphism abnormal lung lobation. Abnormal GI tract. Bilateral renal cystic disease
5	**Increased nuchal translucency >3.5mm**	Trisomy 9	Agree centre 1	Agree	Pathogenic	TOP No Pm report available
6	**Scoliosis hemivertebrae small kidney**	Trisomy X	Agree centre 1	Agree	Pathogenic	LB absent left kidney and hemi-vertebral anomalies. Seen at 4 months old. Also noted flattened occiput with abnormal epicanthic folds and inverted nipples.
7	**Ventriculomeg-aly**	Trisomy X	Agree centre 1	Agree	Pathogenic	LB no follow up data available
8	**Holoprosencep-haly**	46,XX,der(2)del(2)(p16.2p22.3)del(2)(q14.1q22.1)dn,t(5;6)(q22;p11)dn	Agree centre 1[1]	arr cgh 2p22.1p21(RP11-173C1->RP11-110G2)x1, 2q14.2q22.1(RP11-6906->RP11-279M2)x1 dn	Pathogenic	TOP PM confirmed alobar holoprosencephaly. Unilateral cleft lip and palate. Interrupted aortic arch and abnormal lung lobation

9	**Short long bones, skeletal dysplasia, IUGR, enlarged heart**	45,X,idic(Y)(q11.2)[27]/45,X[7]	Agree centre 1	agree	Pathogenic	SB PM confirmed skeletal dysplasia. IUGR. Dilated heart and small thymus
10	**Increased Nuchal translucency >3.5mm**	46,XX,del(6)(q11.1q16.1)dn	Agree centre 1	arr cgh 6q11.1q16.1(RP1-91N13->RP11-538A16)x1 dn	Pathogenic	TOP No Pm report available
11	**Increased nuchal translucency >3.5mm**	47,XX,+r(8)(p10p21.2)[12]/46,XX[8]	Agree centre 1	arr cgh 8p21.2p12(RP11-138J2-RP11->197P20)x2~3 dn, 8p12p11.1(RP11-479P21-RP11->65O11)x1~2 dn	Pathogenic	TOP No Pm report available
12	**Holoprosencep-haly**	46,XX,del(7)(q32)dn	Agree centre 1	arr cgh 7q32q36.3(RP11-36B6->RP11-867L5)x1 dn	Pathogenic	TOP No Pm report available
13	**Dandy-Walker malformation**	46,XX,del(6)(q25.1)dn	Agree centre 1	arr cgh 6q25.1q27(RP1-297M16->RP11-114B3)x1 dn	Pathogenic	TOP No Pm report available
14	**Hypoplastic left heart, borderline ventriculomegaly**	46,XX,der(8)del(8)(p23)invdup(8)(p23.1p11.2)dn	Agree centre 1	arr cgh 8p23.3 (RP11-1029B10->RP11-479M8)x1 dn 8p22 (RP11-145015->RP11-65011)x3 dn	Pathogenic	TOP PM report confirmed Hypoplastic left heart syndrome and bilateral mild ventriculomegaly. Also hypoplastic aortic arch, ventricular septal defect and agenesis of corpus callosum

15	**Nuchal translucency > 3.5mm**	46,XY,del(17)(p11.2p11.2)dn	Agree centre 1	arr cgh 17p11.2(RP11-219A15-RP11-121A13)x1 dn Smith Magenis syndrome	Pathogenic	TOP No Pm report available
16	**Increased Nuchal translucency >3.5mm, diaphragmatic hernia, short femur**	47,XX,+der(12)(pter-p11::p13.3-p11::pter)dn[10]/46,XX[5]Pallister Killian syndrome	Disagree centre 1[2]	arr cgh 12p13.33p11.1(RP11-519B13->RP11-460N10)x3 dn Pallister Killian syndrome	Pathogenic Confirmed using FISH analysis	NND
17	**Bilateral ventriculomeg-aly**	46,XY,del(1)(p36.3)dn	Disagree centre 1	arr cgh 1p36.33p36.32(RP5-857k21->RP11-333E3)x1 dn 1p36 microdeletion syndrome	Pathogenic	TOP No Pm report available
18	**Nuchal translucency > 3.5mm developed cardiac defect**	Normal	Agree Centre 1	arr cgh 11q24.2q25(RP11-10N17->RP11-358H4)x3 dn	Pathogenic	TOP No Pm report available
19	**VSD aortic coarctation**	Normal	Agree centre 1	arr cgh 22q11.2(RP11-800B02->RP11-330P17)x1 dn Di George syndrome	Pathogenic	Miscarriage. No PM.

20	**Double outlet right ventricle**	Normal	Agree centre 1	arr cgh 22q11.21(RP11-800B02->RP11-330P17)x1 dn Di George syndrome	Pathogenic	TOP No Pm report available
21	**Tetralogy of fallot**	Normal	Agree centre 1	arr cgh 22q11.21(RP11-800B02->RP11-330P17)x1 dn Di George syndrome	Pathogenic	TOP No Pm report available
22	**Truncus arteriosis**	Normal	Agree centre 1	arr cgh 22q11.21(RP11-800B02->RP11-330P17)x1 mat Di George syndrome	Pathogenic	TOP PM confirmed truncus arteriosus type 1
23	**Bladder outflow obstruction**	Normal	Agree centre 1	arr cgh17p12(RP1-27J12-RP11-385D13)x1 pat Including gene PMP22: hereditary neuropathy with liability to pressure palsies (HNPP)	Pathogenic Likely incidental finding	TOP Before result of microarray known. No PM.
24	**Absent corpus callosum and meningocele**	Normal	Agree centre 1	arr cgh 5q35.3(RP11-281O15->RP11-99H18) x1 mat	Pathogenic	LB Admitted to SCBU following delivery. Anomalies confirmed.

25	**Univentricular heart, hydrops**	45,X Amniocentesis 47,XXX CVS[3]	Agree centre 1	Normal	Pathogenic	LB admitted to SCBU following delivery. Anomalies confirmed.
26	**Lower limb abnormality**	46,Y,inv(X)(p11 q13)mat	Agree centre 1	Normal	Benign	TOP PM confirmed absent fibulae and short angular tibiae with three toes on each foot
27	**Borderline ventriculomegaly**	46,XX,inv(10)(q 22q23)pat	Agree centre 1	Normal	Benign	LB no follow up data available
28	**Transposition great arteries Double outlet right ventricle Ventricular septal defect**	46,XY,inv(19)(p 13.1p13.3)mat	Agree centre 1	Normal	Benign	LB Admitted to SCBU following delivery. Anomalies confirmed.

IUGR= intrauterine growth retardation
LB = live birth
NND= neonatal death
PM = post-mortem
SB = still birth
SCBU special care baby unit
SHH= sonic hedge hog
TOP = Termination of pregnancy

[1] Blind laboratory :Deletion 2q and translocation detected but missed 2p deletion
[2]Abnormality not present on the 6 cells examined
[3]Postnatal blood sample 47XXX[45]/45X[15]

Legend: The table represents positive findings by either CMA and/or G-band karyotyping in my prospective cohort. It also shows the concordance between the two centres performing G-band karyotyping (centre 1 is the West midlands Genetics Laboratory, centre 2 is South East Scotland Cytogenetics Service). The classification of the chromosomal variant and the pregnancy outcome are also represented.

Discussion

In my cohort I found that chromosomal microarray had an increased detection rate over conventional karyotyping in 4.1% of cases (10/243). Compared with other cohorts this seems appears a conservative increase over karyotyping (51;52). However the reason for this is that the choice of CMA was targeted and purposefully conservative to limit the amount of parental follow up required and VOUS detected. I found CMA to be a robust and accurate method and (in the vast majority of samples) without culturing cells. The turnaround time in the WMGL is 10 days, longer than previously reported (52). The main reason for this was that samples were kept on hold and then analysed in one “batch” at the beginning of the week. With more dedicated staff this could be reduced to 5 days, significantly reducing the wait for couples.

The VOUS rate was just 0.4% and with sensitive but accurate counselling by a clinical genetics consultant the patient did not find the result concerning, was grateful for the information and continued the pregnancy. However had the result not been managed well this may have led to increased anxiety both in the antenatal period and postnatally if the pregnancy continued. More qualitative work on the effect of VOUS on women and the partners is available in chapter 5.

When the G-banding analysts were blinded from the microarray results they did not detect three of the chromosomal differences that were detected when analysis was taking place in the same laboratory. The blind results are probably more

representative of what is routinely seen or missed by conventional cytogenetics if CMA was not to be performed. However the blinded analysts did not have as much clinical information regarding the pregnancy and problems seen on scan which may have affected their analysis of particular chromosomal regions.

In my cohort increased nuchal translucency >3.5mm had a high rate of pathogenic chromosomal differences even when common autosomal or sex aneuplodies were excluded.

Published work from chapter 2

This work has been published:

S.C.Hillman, D.J.McMullan, G. Hall, F.S. Togneri, N. James, E.J Maher, C.H. Meller, D. Williams, R.J. Wapner, E.R. Maher and M.D.Kilby. Use of Prenatal chromosomal microarray use: a prospective cohort of fetuses and a systematic review and meta-analysis. USOG 2013 Jun;41(6):610-20

CHAPTER 3 HIGH RESOLUTION OLIGONUCLEOTIDE 60K MICROARRAY PLATFORM

Introduction

The potential advantages and disadvantages of CMA testing in the prenatal setting have been discussed in previous chapters. However the correct resolution of the CMA platform to use is debatable. As the resolution of the microarray platform increases, generally the number of detectable VOUS will increase in addition to an increase in pathogenic findings (see chapter 4) Spearman's rank correlation coefficient (Rho) = 0.809108 (P = 0.0012) (49). If VOUS are to be reported to women and their partners this would mean additional counselling, and depending on how the information is viewed/handled additional anxiety and emotional stress. This anxiety has the potential in some instances to cause women to consider termination of the pregnancy, for a “CNV” that may in time be redefined as uncommon but benign.

Few studies have used different resolutions of CMA (35;44;53) and none have directly compared differing resolutions on the *same* prenatal samples. I sought to examine a sub-set of cases where a 1Mb targeted BAC array had been performed prenatally and, in addition, perform a whole genome cytochip oligonucleotide 60K CMA (BlueGnome Cambridge) once the pregnancy was complete (54). The aim would be to determine the “extra” CNV pathogenic information gained from the higher resolution array and the amount of uncertainty obtained from the raised resolution of the CMA.

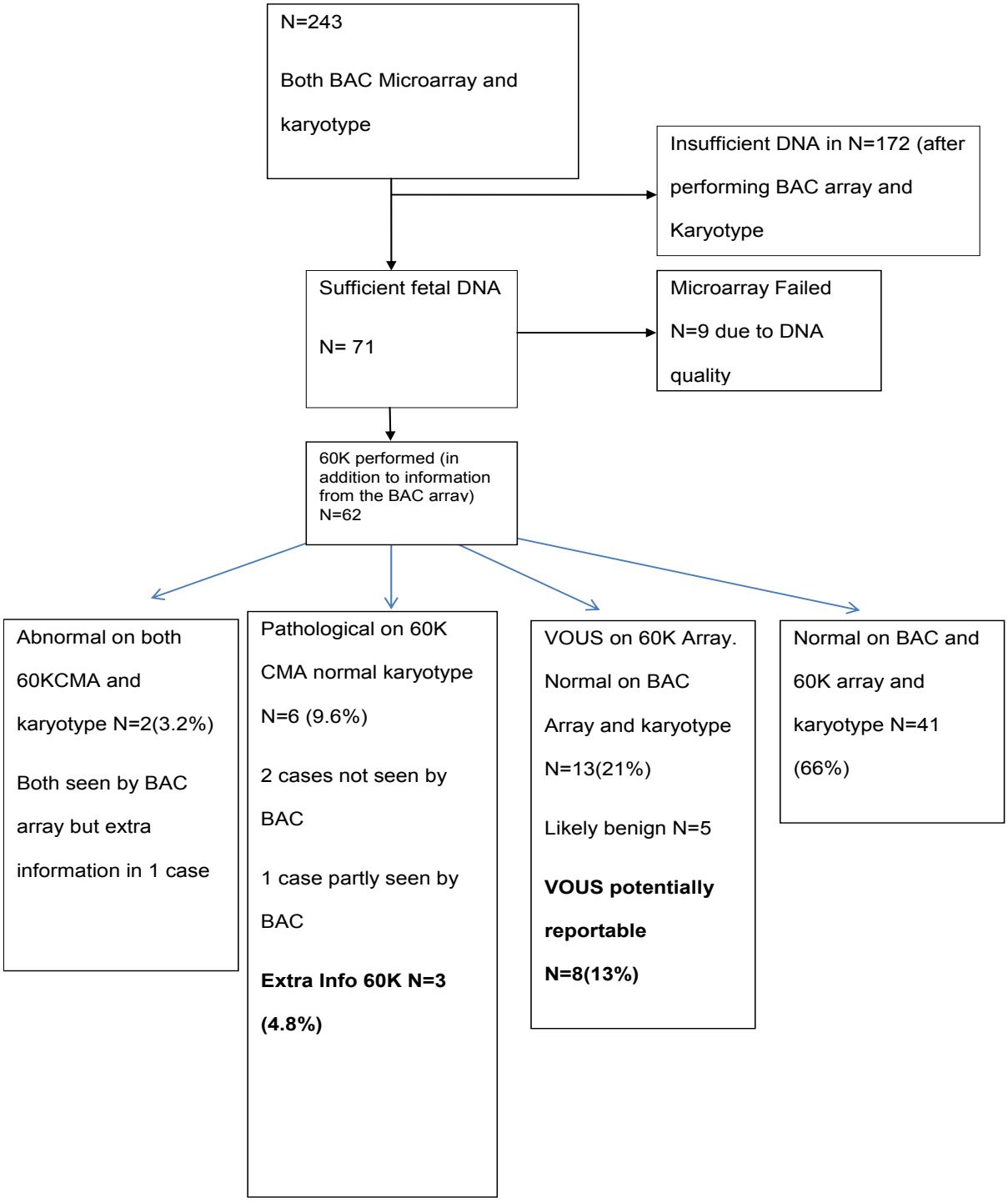
Methods

At the time of written consent for the 1Mb BAC array study, parents were asked if they would give consent for a higher resolution array test to be performed when the pregnancy was complete (either after a live birth, miscarriage or termination of pregnancy). As the VOUS rate had not been determined when using the higher resolution array, women were told that they would only be informed of the 60K CMA outcome if it showed a “pathogenic CNV”.

In total, 243 fetal cases underwent a 1Mb BAC array test (49). Of these there was enough DNA available to process 71 cases for an additional 60K oligonucelotide array postnatally (see Figure 10). In the main this was because the prenatal sample was cultured or an additional sample was taken, prior to termination of pregnancy at feticide (Table 8). In 172 cases, all available DNA had been utilised following QFPCR, conventional karyotyping and 1Mb BAC array. The 60K oligonucleotide microarray used was the International Standard for Cytogenomic Array (ISCA) design v2.0 (https://iscaconsortium.org/) and data was analysed using BlueFuse Multi v3.0 such that theoretically the majority of CNVs >15kb in 500 disease gene/telomeric regions (including all well characterized microdeletion and microduplication syndromes) and CNVs >180kb in the genomic backbone would be detectable. The laboratory methodology for the CMAs was conducted by me in ~50% of cases and by WMRGL staff in ~50% of cases.

The majority of the fetuses had a single structural anomaly (n=66/93%). Of these the most commonly represented malformations were; central nervous system anomalies (22.5%), cardiovascular system (predominantly heart anomalies) (18%), musculoskeletal (14%), and genitourinary system (12.5%) abnormalities (see Table 7). Of the 71 cases (where sufficient DNA was available) to process the 60K array, the majority were umbilical cord blood samples at delivery (n=14/20%); fetal blood samples (at late termination of pregnancy) (n=18/25%) or fetal tissue following miscarriage or termination of pregnancy (n=22/31%) (see Table 8). Few were taken from CVS or amniocentesis samples (cultured or uncultured) and in all 3 cases where CVS samples were used the 60K array failed. The reason for few cases being taken from CVS and amniocentesis samples is the low quantity of DNA remaining after preceding primary tests (full karyotype, QFPCR and BAC array).

Figure 10 Flow diagram of cases available for high resolution 60K CMA testing



Legend: The above flow diagram account for all participants included in the 60K CMA cohort

Table 7 Structural anomaly using the Human Phenotype Ontology system

Structural anomaly	N=	Percentage
Single	**66**	**93%**
Central nervous system	16	22.5%
Cardiovascular system	13	18%
Increased Nuchal Translucency>3.5mm Or Cystic hygroma	7	10%
Musculoskeletal system	10	14%
Genitourinary system	9	12.5%
Abdominal wall defect	5	7%
Head/face/neck	2	3%
Respiratory system	1	2%
Spina Bifida &/or encephalocele	3	4%
Multiple systems	5	7%

Table 8 Sample type of those cases that had 60K CMA performed

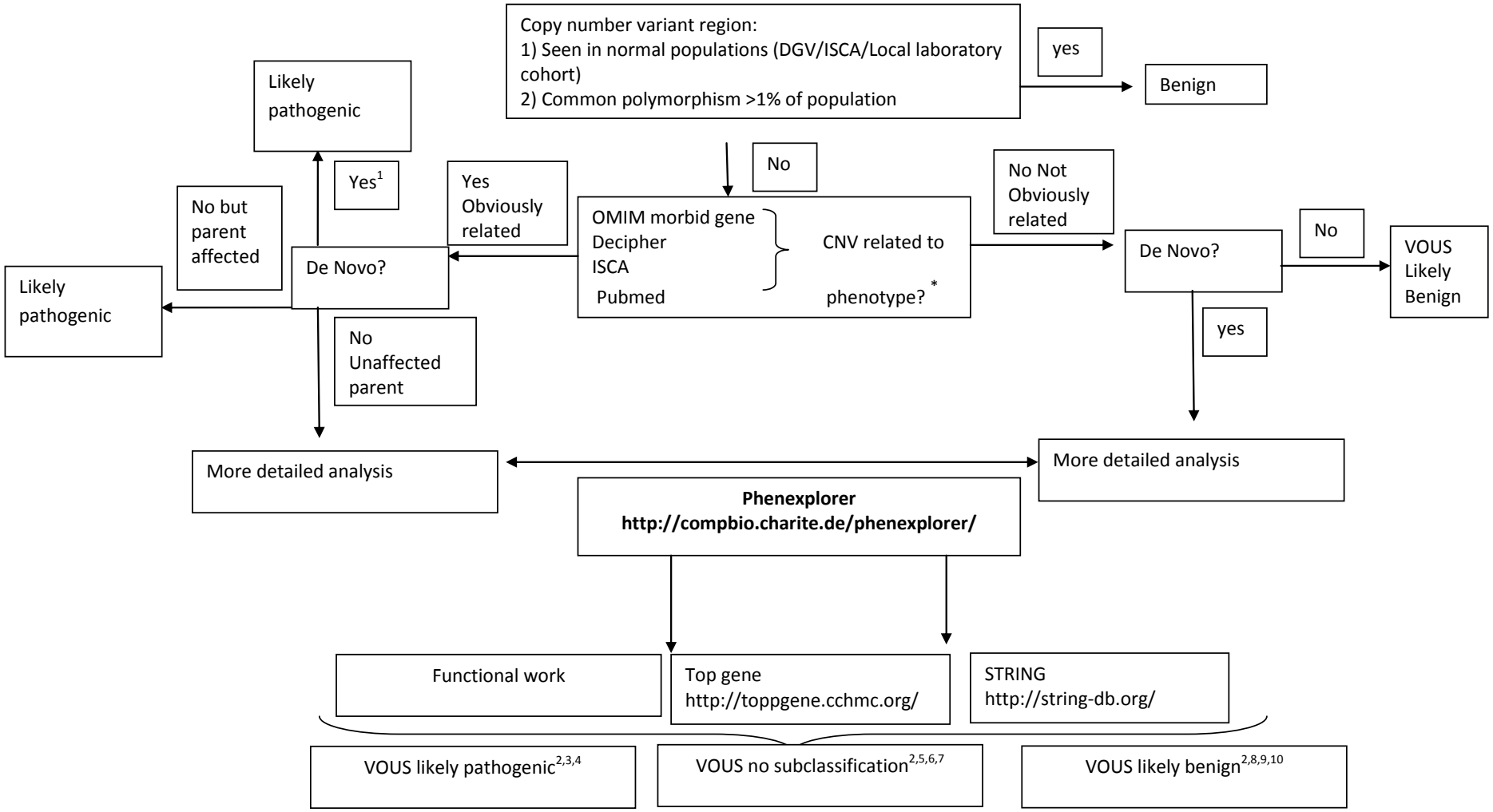
Sample type	Sample number and percentage (n=71)	Samples where CMA failed (%of those that failed n=9)
Uncultured amniocentesis	6 (8.5%)	-
Cultured amniocentesis	7(10%)	1(11%)
Uncultured Chorionic Villus Sampling	3 (4%)	3 (34%)
Cultured Chorionic Villus Sampling	1 (1.5%)	-
Fetal Blood Sample (Pre-N)	18 (25%)	2 (22%)
Fetal Tissue	22 (31%)	2 (22%)
Umbilical Cord blood (Post-N)	14 (20%)	1 (11%)

Pre-N=Prenatal.

Post-N=Postnatal.

Of the 71 prenatal samples obtained, in 9 the microarray failed due to the quality of the DNA remaining following the fore-mentioned tests. 62 samples therefore had both a postnatal 60K Oligonucleotide array as well as a the prenatal1Mb BAC array.

Figure 11 Tools to assess the pathogenicity of a copy number variant



Legend: The above figure is a flow diagram showing the process and tools available to assess the pathogenicity of a CNV. If seen in normal populations or is a common variant with no phenotypic effect the CNV is defined as benign. If not it needs more detailed evaluation including Decipher, ISCA, OMIM and PubMed and may require parental evaluation. Further analysis uses the online tools Phenexplorer, Topgene and STRING to link phenotypes and genes included in the CNV.

[1]Penetrance and Expressivity of CNV is documented and well understood

[2]Each CNV will have unique considerations requiring clinical judgment

[3]CNV is described in a single case report but phenotype relevant to scan findings

[4]Gene within the CNV has compelling gene function relevant to the phenotype

[5]CNV contains genes, but genes are dosage sensitive or Haploinsufficiency scores <30%

[6]CNV genes with some evidence for biological links to phenotype

[7]CNV is contradictory in publications/databases, conclusions regarding significance are not established

[8]CNV is described in a small number databases of variation but is not a common polymorphism

[9]CNV contains genes but no firm biological links to phenotype

[10]The CNV is a duplication and contains unremarkable genes which have Haploinsufficiency scores of>30%

[*]X chromosome imbalances should be treated as a separate entity because of differing dosage tolerance. X chromosome imbalances are also underrepresented in the database of genomic variation and internal data maybe more useful. Smaller imbalances are expected to have a more significant effect than their autosomal counterparts, and may be tolerated in female carriers due to X inactivation.

Results

This chapter summarises a case-cohort of 62 “fetal” DNA samples that underwent both prenatal targeted BAC array and postnatal 60K array. My study noted that the higher resolution 60K array was able to detect all pathogenic CNVs detected by the targeted BAC array. This included three previously published resultsm(49) including a case of 22q11.2 deletion (Di George syndrome), a deletion of 6q25.1q27 (46,XX,del(6)(q25.1)dn) visible on both the BAC array and conventional G-band karyotyping and a rearrangement of chromosome 2 (46,XX,der(2)del(2) (p16.2p22.3) del(2)(q14.1q22.1)dn,t(5;6)(q22;p11)dn) again large enough to be seen by conventional G-band karyotyping. All three of these findings were pathogenic CNVs (pCNVs) and associated with the findings on fetal ultrasound scan.

In addition to the chromosomal anomalies detectable by the Birmingham BAC array study (prenatally), the 60K array performed after the end of the pregnancy, also detected CNVs defined as either pathogenic or VOUS in a further 16 cases. Five of these were re-defined as VOUS (likely benign) (Table 9 cases 1,2,3,4,5). The decision was made not to report these findings by a multidisciplinary discussion and review of the cases (between senior cytogeneticists, clinical geneticists and fetal medicine subspecialists).

In three cases pathogenic CNVs were detected by the 60K array (not previously noted with the prenatal microarray). These included:

i) Case 6: A de novo 47kb deletion of Xp22.2 in a female fetus with ventriculomegaly and agenesis of the corpus callosum. This deletion included the gene OFD1 (orofaciodigital syndrome). Cases have been described with central nervous system abnormality and agenesis of the corpus callosum (which was also noted on post-mortem examination of this case (55) (Table 9; case 6).

ii) **Case** 7: A *de novo* 118kb duplication of Xp22.33 including the PQBP1 region causing Renpenning syndrome(56;57). This syndrome is linked to neurodevelopmental morbidity (mental retardation) and cardiac anomalies (Table 9; Case 7).

iii) Case 8: A 1.1Mb duplication on chromosome 17q25.3, found in addition to the deletion on 5q35.3 detected by the prenatal BAC array (and confirmed and further delineated by 60K array). This was from a maternally inherited unbalanced translocation. Given the size and gene content of this deletion/duplication and the fact that the mother suffered from dyspraxia and had mild facial dysmorphism, it was determined that this was a pathogenic finding, associative of the findings on scan (Table 9; Case 8).

A one sided Fisher's exact test showed a non-significant difference in detection rate of pCNVs detected by the 60K platform compared with the BAC array (p=0.0605)(Appendix J). There is a chance this may be attributed to a small cohort undergoing the higher resolution array.

However, the 60K array detected a further 8 cases in which the CNVs were classified as a VOUS and could not be redefined as either probable benign or pathogenic (Table 9; Cases 9-16). This represents 13% of the 60K cohort and there was a significantly increased frequency of VOUS detection with the 60K platform compared with the BAC array (One sided Fisher's exact test p=0.0001)(Appendix J).

VOUS detected included:

i. Case 9). A maternally inherited duplication 15q25.2 in a fetus with USS findings of exomphalos. No obvious link to the phenotype could be found but it could be a reciprocal variant of the 15q25.2 deletion cases with links to congenital diaphragmatic hernia and cognitive defects (58).

ii. Case 10). A paternally inherited deletion 9q22.32 in a fetus with an absent femur on USS. Possible disruption of the homeobox gene BARX1 (expressed in proximal fore and hindlimbs) may have been implicated in the phenotype findings (59).

iii. Case 11). This case shows of the difficulty in interpretation when parental DNA is not available to interpret the CNV. This duplication on 22q11.22 in a fetus with USS findings of Dandy Walker syndrome is a VOUS without knowledge of parental inheritance.

iv. Case 12). Shows a paternally inherited duplication of 3q21.1 in a fetus with findings of fetal akinesia syndrome. Here there were no clear cut gene associations but the gene ADCY5 is associated with fetal growth abnormalities (60).

v. Case 13). Here a 20Mb deletion 6q25.1-6q27 had been detected by conventional cytogenetics, the BAC array and the 60K array, and was interpreted to be pathogenic and causal of the Dandy Walker syndrome found on USS. In addition the 60K array found a 2Mb deletion at 8p21.1-p12 containing many genes and may have contributed to the USS findings.

vi. Case 14). A maternally inherited duplication at 1q21.1 in a fetus with USS findings of LUTO was found. This duplication covered the Thrombocytopenia and absent radius susceptibility region. Although this fetus did not have any skeletal anomalies on postmortem examination and the CNV is not likely to have been causative of the phenotype.

vii. Case 15). A de novo duplication at Xq21.1 in a male fetus that had USS findings of an enlarged thickened bladder and lower urethral tract obstruction. This duplication included the gene ATRX (Alpha-thalassemia/MR syndrome) (61). This is an example of an X linked imbalance with disease associated gene but not related to the phenotype.

viii. Case 16). A maternally inherited duplication of Xq13.3 in a male fetus with a lower limb abnormality. This duplication involved the gene KIAA2022 (linked to MR)(62) and again shows the difficulty in interpretation of an X linked imbalance with disease associated gene but unrelated to the phenotype.

Table 9 Results of the 60K CMA cohort

ID	Chromosome	Del /dup	Phenotype on ultrasound	Sample type	Inheritance	Outcome of pregnancy	Comments	VOUS Class.
1	arr 11q25(133,244,652-133,842,923)x3 pat	Dup	VSD Hypoplastic left heart	CB	Paternal	Live Birth	No obvious genes related to phenotype; may disrupt OPCML (Ovarian Cancer gene)(63)	bCNV
2	arr Xq21.31(89,444,893-89,517,088) x1 mat male fetus	Del	Exomphalos Increased NT Enlarged lateral ventricles Hypoplastic cerebellum Enlarged cistern magna	Tissue	Maternal	TOP PM available: Bilateral talipes Large omphalocele Thoracic hemivertebrae Anus atresia Cardiac hypoplasia Small bowel atresia Bent femur/tibia and humerus Renal agenesis	No significant genes; inherited and seen in local cohort of postnatal cases	bCNV
3	arr 6p22.3(20,740,983-21,162,532) x1 mat	Del	Skeletal dysplasia	Tissue	Maternal	TOP PM available: Lethal skeletal dysplasia, short stature , large head and severe rhizomelic shortening of the limbs. Consistent with thanatophoric dysplasia type 1	Deletion in CDKAL1; associated with diabetes. No evidence for association with structural anomalies (64)	bCNV
4	arr 15q11.1 (20,787,768-23,146,102)x1 pat	Del	Increased NT	CB	Paternal Note code	Live Birth	Common CNV associated with susceptibility to neurological conditions	bCNV
5	arr 4q35.1 (186,826,674-187,139,541)x3 mat	Dup	Truncus arteriosis	FBS	Maternal	TOP PM unavailable	Seen in low levels in DGV but also in local cohort.	bCNV

6	arr Xp22.2 (13,769,563-13,817,279)x1 dn female fetus	Del	Ventriculomegaly	FBS	De novo	TOP PM available Confirmed hydrocephalus. Absent corpus callosum Absence of left uterine horn and fallopian tube	OFD1 and GPM6B Female fetus OFD1 (Orofaciodigital syndrome) related to phenotype cases described with CNS abnormalities including agenesis of corpus callosum.(55)	pCNV
7	1)Xp11.23 2)Xp22.33 arr Xp11.23(48,699,033-48,818,269)x3 mat arr Xp22.33(492,278-877,581)x3 dn Male fetus	Dup And Dup	Univentricular heart	FBS	Maternal	TOP PM unavailable	1)SHOX duplication 2)PQBP1 region (TIMM17B, PQBP1, SLC35A2). Inherited but not a common CNV. Mutation of PQBP1 causes Renpenning syndrome(56;57) Linked to MR and sometimes cardiac anomalies	1) bCNV 2) pCNV
8	1)arr 5q35.3(178,791,009-180696,777)x1 mat and 2)arr 17q25.3(79,953,997-81,044,524)x3mat	Del and dup	EncephaloceleAbsent corpus callosum	Amnio	Maternal	Live Birth	17q contains 32 genes (missed by BAC array) 5q contains 41 genes (seen by BAC array) Gene list attached	pCNV
9	arr 15q25.2(83,597,474-84,946,159)x3 mat	Dup	Exomphalos	CA	Maternal	Live Birth	No Obvious link to phenotype found. Maybe a reciprocal variant of the 15q25.2 deletion cases with links to congenital diaphragmatic hernia and cognitive defects VOUS: Uncertain(58)	uCNV

10	arr 9q22.32(96,698,759-96,946,777)x1 pat	Del	Absent femur	CB	Paternal	Live Birth	Deletion of PTPDC1 no known interactions or associations. Possible disruption of BARX1 (homeobox gene). Mouse Barx 1 is strongly expressed in areas of head and neck mesenchyme and in the wall of the developing stomach and is expressed at weaker levels in the proximal fore- and hindlimbs.(59)	uCNV
11	arr 22q11.22 (22,905,038-23,720,200)x3 ?dn	Dup	Dandy Walker Agenesis corpus callosum meningeocoele	FBS	? de novo (maternal sample failed QC)	TOP PM available: Posterior fossa Dandy Walker Cyst Hydrocephalus Corpus callosum present Small defect in upper cervical spine	22q11.22: ~50% overlap with benign CNV in DGV ~50% more unique (includes BCR gene and little DGV here).	uCNV without parental samples
12	arr 3q21.1(122,505,327-123,152,363)x3 pat	Dup	Micrognathia Hemivertebrae Hydrothorax Clindodactyl	Tissue	Paternal	TOP PM available Fetal hydrops Findings consistent with fetal akinesia most likely muscle in origin	Not a common variant; no clear cut gene associations. ADCY5 associated with fetal growth (60)	uCNV
13	arr 8p21.1p12(27,579,264-29,765,976)x3 ?dn?pat	Del	Dandy Walker	FBS	? de novo No paternal samples	TOP PM unavailable	~2Mb deletion including many genes Phenotype likely caused by concurrent 20Mb deletion 6q25.1-6q27 detected also by BAC array	uCNV

14	arr 1q21.1 (143,700,200-146,507,547)x3 mat	Dup	LUTO Dilated renal pelvis Anyhydramnios Fluid in abdominal cavity	Tissue	Maternal	TOP PM Available: Findings of Wenstrup syndrome-cloacamembrane abnormality Rectum agenesis, recto-vesicular fistula, bicornate uterus Indifferent external genitalia Oesophagus atresia and trachea-oesophageal fistule No skeletal anomalies.	Dup of the 1q21.2/TAR susceptibility region. Not likely causative of phenotype though.	uCNV
15	arr Xq21.1(76,921,053-76,934,913)x3 dn Male fetus	Dup	Enlarged thicken bladder ? lower urethral tract obstruction	Amnio	De novo	Live Birth	Including gene ATRX (Alpha-thalassemia/MR syndrome)(61) Example of X imbalance with disease associated gene but not related to phenotype	uCNV
16	arr Xq13.3(73,802,202-73,878,653)x3 mat Male fetus	Dup	Lower limb abnormality	Amnio	Maternal	TOP PM available: Missing fibula Short bowed tibia Three toes bialterally	Partial duplication involving gene KIAA2022 (linked to MR) (62)	uCNV

bCNV= likely benign VOUS
Del=deletion
Dup=Duplication
MR = mental retardation
NT= nuchal translucency
pCNV= likely pathogenic VOUS
PM = Post Mortem
TAR = Thrombocytopenia with absent radius
TOP = Termination of pregnancy
uCNV=uncertain VOUS

Discussion

To summarise, this prenatal cohort of 62 samples are the only reported series to have undergone two different platform of arrays of different resolutions on the same proband. This has allowed us to directly compare the effects of increasing array resolution on both the pathogenic detection rates but also the rate of VOUS.

It is conceded that a degree of non-specific selection bias occurred as cases were selected because of the availability of fetal DNA (26% of total prenatal cohort). However, this sub-cohort of babies with congenital malformations appeared representative of the whole cohort. My study noted that the higher resolution 60K array was able to detect all pathogenic CNVs detected by the targeted BAC array. The BAC cohort study (Chapter 2) suggests that using this "targeted" array platform in a prenatal population of fetuses with congenital malformations on USS would give a pathogenic CNV rate over and above conventional G-band karyotyping of 4.1% (95%CI 2.1-7.6)% (n=10 cohort size 243). However this array would only give a VOUS rate of 0.4% (95% CI 0.07-2.2) (n=1 cohort size 243) (49). Other studies in the literature, most notably the NICHD study (65), using a 44K array platform, noted that CMA analysis revealed clinically relevant deletions or duplications in 6.0% with a structural anomaly noted, higher than the prospective Birmingham BAC array cohort. However, VOUS rates were also correspondingly high.

In this comparative cohort study, when I used the higher resolution 60K array platform on 62 of the same fetal samples, a higher detection rate of *additional* pathogenic CNVs (4.8%(95% CI 1.6-13.3 n=3) was detected over and above BAC array and 9.6%(95%CI 4.5-19.5% n=6) over and above conventional G-band karyotyping. This was, however, not a significant increase (One-sided Fisher's exact test p=0.06). However, in line with previous literature, VOUS rates were significantly higher at 21% (95%CI 12.7-32.6) (n=13 cohort size 62) (49). When systematic analysis of the VOUS occurred, this was lowered to 13% (95%CI 6.7-23.4) (n=8 cohort size 62), with 5 of the VOUS being reclassified as VOUS "likely benign", but still showed a highly significant increase in VOUS from the BAC array (One-sided Fisher's exact test p=0.0001).

These observations demonstrate that higher resolution microarray platforms are associated with higher detection rates of true pathologic CNVs, but also the higher the rate of VOUS. These observations are relevant as qualitative evidence from the NICHD study has shown that reporting of VOUS in the antenatal period can be considered "toxic knowledge", and that it raises women's anxiety levels and potentially psychological morbidity in pregnancy, and that this continues after their child is born (66).

Currently there is no guidance either nationally or internationally regarding the reporting of VOUS to women and their partners in the antenatal period. One may have the view that the reporting of VOUS in the antenatal period should be at the very least 'guarded' and many would argue that this information should not be

divulged. This is further complicated as classification of CNVs is often not straightforward and a spectrum of “uncertainty” can be attributed to particular CNVs. There will therefore be likely disagreements between clinicians, scientists and patients themselves as to what constitutes enough evidence to make reporting of the CNV in the best interests of the woman and her partner. The decision as to report should ideally be taken by multidisciplinary consensus rather than decision being made by one group alone.

Submitted manuscript from Chapter 3

The following manuscript from chapter 3 has been published:

Hillman SC, McMullan DJ, Silcock L, Maher ER, Kilby MD.

How does altering the resolution of chromosomal microarray analysis in the prenatal setting affect the rates of pathological and uncertain findings?

J Matern Fetal Neonatal Med 2013; Aug 19.

CHAPTER 4 UPDATED SYSTEMATIC REVIEW AND META-ANALYSIS

Introduction

In Chapter 1 the literature up until 2009 was systematically reviewed and meta-analysed where possible. Between December 2009 and December 2012, and over the period of the Birmingham BAC and 60 K cohorts, other large prospective cohorts have been published adding to the literature. In this chapter I sought to bring the literature at the end of the study up to date and to also include data taken from my own Birmingham BAC Cohort and then more contemporary cohorts using a 60k or high resolution 'platform', as recent additions to the literature. This provides the most complete and up to date critical appraisal of the literature looking at the use of prenatal CMA use firstly for any indication and secondly when the indication is an abnormal scan finding (with a congenital malformation). It also provides the most complete analysis on VOUS rates and how they vary between indications and types of 'platform' for CMA (49).

Method

The systematic review followed a prospective protocol developed using widely recommended and comprehensive methodology (32). This was done by framing the question for review in this case "In the prenatal setting how much extra information, regarding chromosomal anomalies, does CMA provide over traditional karyotyping in cases where both tests are performed?". And in addition to this question "In a prenatal setting what is the detection rate of Variants of Unknown Significance when CMA is performed?". Secondly the relevant literature was identified as described below, thirdly that the quality of the literature was assessed using recognised tools

as described below. Fourthly that the evidence was summarised and meta-analysed if appropriate. Finally that the findings were interpreted and discussed.

Data sources

The search focused on prenatal studies using microarray technology. A research strategy was developed based on existing advice for prevalence searches (67). Medline (January 1970 to December 2012), Embase (January 1980 to December 2012) Cinhal (January 1982 to December 2012) and clinicaltrials.gov databases were searched electronically. The search of MEDLINE and EMBASE captured citations containing the relevant MeSH keywords and word variants for “microarray” and “prenatal”. The following terms were used to describe microarrays: microarray, DNA microarrays, array comparative genomic hybridisation, array CGH. Similarly, antenatal diagnosis, prenatal and fetal, were used to capture “prenatal”. Bibliographies of relevant articles were manually searched to identify papers not captured by electronic searches. Experts were also contacted for completeness (36;51;68). There were no language restrictions in the search or selection of papers.

Eligibility criteria for selecting studies

Studies were selected in a two-stage process. Initially all abstracts or titles were scrutinised by two reviewers (myself and Dr César Meller, a visiting Fetal Medicine Sub-speciality Fellow) and full manuscripts of potentially eligible citations were obtained. Studies were included if chromosomal microarray had been used on prenatal specimens (analysed during pregnancy or after delivery). The CMA may

have been performed for any referral indication not just because there were abnormal findings on fetal ultrasound scan. Papers were excluded if microarray was not performed on prenatal acquired samples, if they were performed for pre implantation diagnostics or if they were performed for recurrent miscarriage. Finally papers were excluded if they used the CGH technique (the precursor to CMA) and not array CGH/chromosomal microarray. Non-English studies were assessed by someone with a command of the relevant language if the title or abstract appeared to fit the criteria. Only papers that allowed generation of a 2x2 table comparing the outcomes of CMA against the outcomes of conventional karyotyping were included.

Data was extracted by two reviewers (myself and Dr César Meller). Differences were resolved by discussion with a third reviewer (Professor Mark Kilby). The author Dr Ron Wapner (RJW) also gave us information on the National Institute of Health's (NIH) cohort study prior to the data being published in the public domain, and also gave an accompanying explanation (65). For each of the outcomes data were extracted into tables giving descriptive and numerical information for each study. Data were extracted on study characteristics and data quality. Data were used to construct 2x2 tables of test accuracy comparing normal and abnormal microarray results. Case studies of fewer than 10 cases were excluded from the meta-analysis.

Quality assessment and data synthesis

Study quality was assessed using STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) (37). Microarray results were considered to be

“positive” if they had VOUS (and therefore potentially pathogenic) or pathogenic results. Benign results were included in the CMA “negative” group as they are not clinically relevant and would not be reported to clinicians or patients.

These data were further broken down to repeat the same analysis but when the clinical indication for CMA was a structural abnormality seen on ultrasound scan. Using 2x2 tables, I computed and pooled the percentage agreement between the two technologies (both for any clinical indication and for abnormal ultrasound scan) with 95% confidence intervals. The calculated percentage of extra cases identified by microarray in those with a negative karyotype (both for any clinical indication and for abnormal ultrasound scan) with 95% confidence intervals was calculated and pooled (Appendix J). Conversely, I then calculated and pooled the percentage of extra cases identified by karyotyping in those with a negative microarray result (both for any clinical indication and for abnormal ultrasound scan) with 95% confidence intervals. Finally I calculated and pooled the percentage of cases in which a result of VOUS was reported. Heterogeneity in rates was examined graphically and statistically (Chi-squared test) (Appendix J). For graphical assessment, “Forest plots” of point estimate of rates and their 95% CI’s were used. For exploration of reasons for heterogeneity, stratified analysis was performed according to the year of publication. A random effects model was used in the light of heterogeneity. All statistical analyses were performed using Stata 11.0 statistical software (Stata Corp., College Station, Texas, USA).

Results

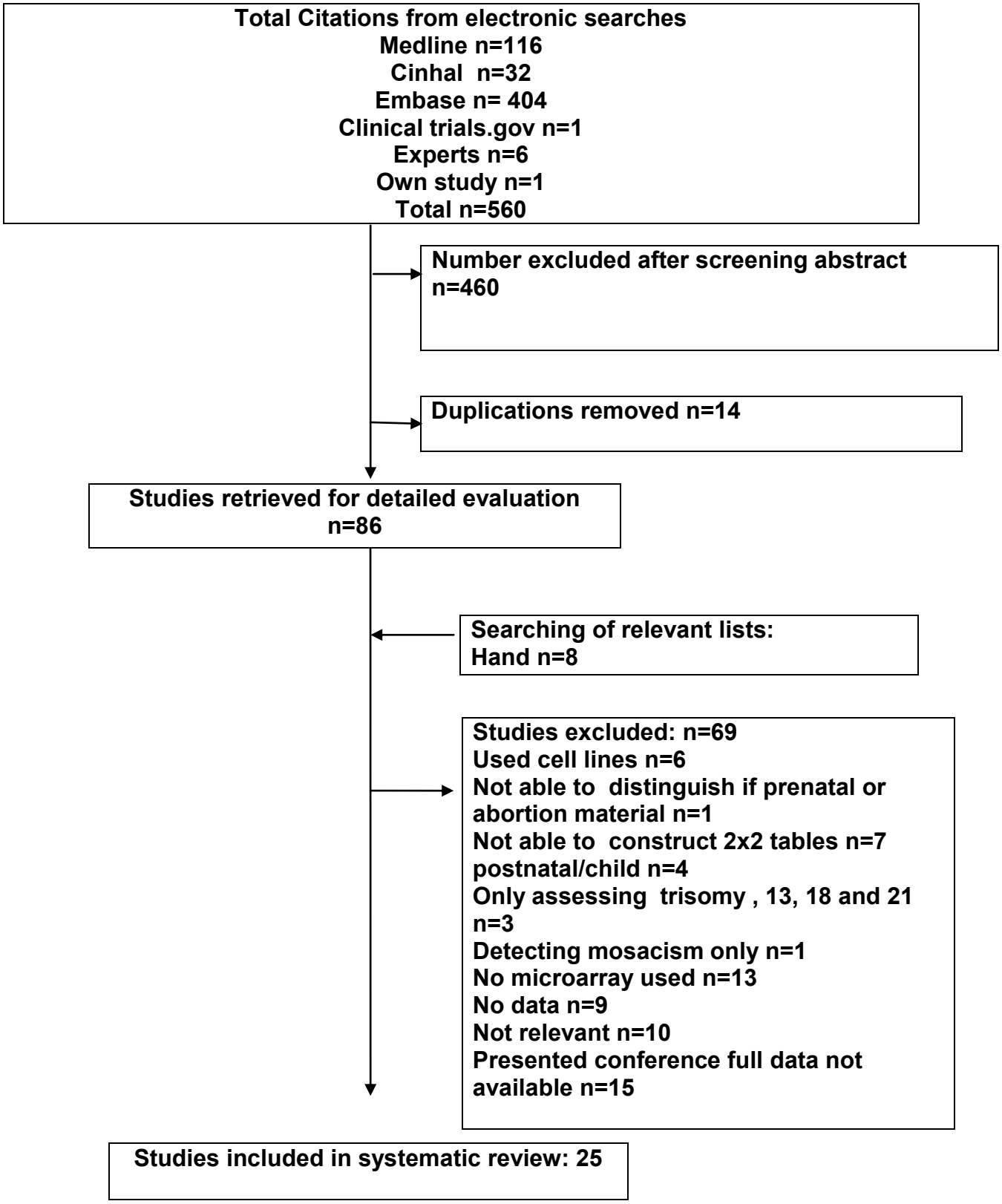
Literature identification and selection

The process of literature identification and selection is summarised in Figure 12. In addition to these data from my cohort, there were 25 primary articles that met the selection criteria (21;38;39;41-45;51-53;65;68-80) (16 not included in my previous 2009 meta-analysis, prior to embarking upon my Birmingham BAC study (51-53;65;68-80)). 22 cohorts (including my own cohort study) were included in the meta-analysis and four were excluded as either microarray was only performed when an abnormal karyotype had been detected (21;41) or because I could not extract data required for a 2x2 table (36,70). In all 25 cohorts the collective number of samples analysed was 17,113. The meta-analysis was then performed using the same cohorts but additionally adding data taken from my 60K cohort (26 cohorts in total, collective number of samples analysed is 17,175). In the case of Fiorentino *et al* the paper published in 2012 (69) contains data from their publication in 2011(52). I was able to extract data from the 2012 paper to look at CMA detection over full karyotype. However the 2011 publication is used to look at overall agreement between the two tests, VOUS rate and karyotype over CMA rate as this was not extractable from the 2012 publication.

Study Characteristics

Table 10 summarises the study characteristics including the study design, microarray type, sample type, indication for sampling and sample size. Figure 13 shows the quality assessment of papers included.

Figure 12 study selection process for updated systematic review



Legend: The above flow diagram accounts for all journal articles reviewed during the process of the systematic review

Table 10 Study Characteristics of studies included in the systematic review

	Author	Year	Design	Array type	Whole genome (WG) /targeted genome (TG)	Sample Type	Indication for array	Sample Size
1	Le Caignec C et al (42)	2005	Retrospective	Genosensor BAC/PAC array 300 (Vysis/Abbott)	TG	Frozen fetal tissue	All normal karyotype All had malformations All had at least 3 anomalies in CVS/urogenital/ digestive/CNS	49
2	Rickman L et al (41)	2006	Retrospective	BAC/PAC resolution 10Mb common microdeletion syndrome	TG	Cultured amniocytes or Chorionic Villus Sampling	Previously known karyotypes All known unbalanced rearrangements	30
3	Sahoo T et al (38)	2006	Prospective	BCM v4.0 Baylor	TG	Amniotic Fluid =56 (26 uncultured) Chorionic Villus Sampling =42 (32 uncultured)	Increase maternal age Increased serum screen Family history Abnormality on US	98 samples total

4	De Gregori M et al (21)	2007	Retrospective	60-mer oligonucleotide microarray	WG	Does not state	Reciprocal translocations=14 Maternal age =3	17
5	Bi W et al (39)	2008	Prospective	BCM V6 Oligonucleotide array (V6 Oligo) (Agilent)	WG and TG	Amniotic fluid	Maternal age =6 Anomaly on US= 5 Family History abnormality =2 multiple miscarriages=1	14 pregnancies 15 fetuses
6	Vialard F et al (45)	2009	Prospective	Genosensor BAC/PAC array 300 (Vysis/Abbott)	TG	Muscle biopsy =15 Lung Biopsy=13 Thymus = 4 Skin = 3 Liver = 3 Bladder = 1	2 or more abnormalities cardiovascular/urogenital/skeletal/digestive/CNS	39
7	Tyreman M et al (43)	2009	Retrospective	Genechip SNP 6.0 array (Affymetrrix)	WG	Amniotic fluid =87 Chorionic Villus Sampling =15 Placenta biopsy following termination of pregnancy =4	Ultrasound anomaly: (Cardiac = 34 Multisystem = 24 Large NT/hydrops/CH = 18 CNS 16 Skeletal = 6 Abdominal wall = 2 Others = 6)	106

8	Kleeman L et al (44)	2009	Prospective	Signature prenatal chip V 4.0 26 patients Signature whole genome chip	TG WG	Amniotic fluid = 47 CVS = 3	Abnormal ultrasound scan and normal karyotyping: (Cardiac =24 CNS = 6 Skeletal = 6 Urogenital = 4 Cleft lip/palate = 2 CH/NT/hydrops = 3 GI = 2 Multiple = 17 Growth disorder=3)	50
9	Maya I et al (72)	2010	Retrospective	236 used signature chip whole genome V1.0-2.0/BCM BAC chromosomal microarray V5 or 6 19 samples used oligo 105K whole genome array or BAC V5.0-6.0	WG	Amniocentesis = 243 CVS = 16 Unknown=10	AMA=61 AUS=102 FIS=43 AFK=15 PA=46 MSS=2	269
10	Faas BH et al (73)	2010	Retrospective	Affymetrix Genechip 250k SNP array	WG	Amniocentesis = 28 CVS=3 Blood=5 Fibroblasts=2	AUD =32 AFK=4 FIS=1 Karyotype not possible =1	35 3 excluded as no karyotyping performed

11	Park JH et al (80)	2010	Prospective	Customised M-chip MacArray	TG	Amniocentesis =94	AMA=42 MSS=38 AUS=12 FIS=2 ICSI=6 Rubella=1 Other=3	93 1 excluded as no karyotype
12	Evangelidou P et al (75)	2010	Prospective	Cytochip focused constitutional (Blue Gnome Cambridge)	WG	Amniotic fluid=16 Chorionic villi=9	AUS=15 AFK=10	25
13	Valduga M et al (76)	2010	Retrospective	Agilent 44k oligonucleotide array	WG	FBS=26 Tissue=21 Cultured amniocytes=2 Cultured chorionic villi=2	Fetal anomaly	50
14	Fiorentino F et al (52)	2011	Prospective	Cytochip focused constitutional (Blue Gnome Cambridge)	WG	Amniotic fluid=919 CVS =99 Cultured cells in AF =15 Uncultured cells in AF= 4	AMA=444 MSS=13 AUS=48 AFK=8 FIS=11 PA=484 Cell culture failure =4	1029 Although 1037 arrays performed only 1030 karyotypes performed and 1 case of false positive excluded *Used in meta-*

								analysis For abnormal karyotype over array and VOUS
15	Park SJ et al (74)	2011	Not stated	Customised MacArray Karyo 1440 BAC-chip	WG	Amniotic fluid =4033 Chorionic villus = 40	FIS AMA AUS MSS PA	4073 prenatal (postnatal excluded)
16	Leung TY et al (77)	2011	Retrospective	Fetal DNA chip(44K) Agilent technologies	TG	Chorionic villi	Increased NT>3.5	47 (1 excluded as result not validated)
17	D'Amours G et al (53)	2011	Prospective	Signature chip WG v1.0.1 Nimblegen CGX-12 Signaturechip oligo solution v1.1 Signaturechip oligo solution v2.0	WG	Amniocentesis =30 Chorionic villus=4 Cord blood=2 Placenta =2 Tissue =11	AUS	49
18	Schmid M et al (71)	2012	Prospective	Affymetrix SNP array 6.0	WG	Amniocentesis =11 Chorionic villius=1	Cardiac anomaly	12
19	Lee CN et al (51)	2012	Prospective	1MB BAC=2497 followed up by 105K oligonucleotide 60K oligonucleotide	WG	Chorionic villi =16 Cultured chorionic villi=66 Uncultured	AMA=1911 PA=989 AUS=194 AFK=51 MSS=25	194

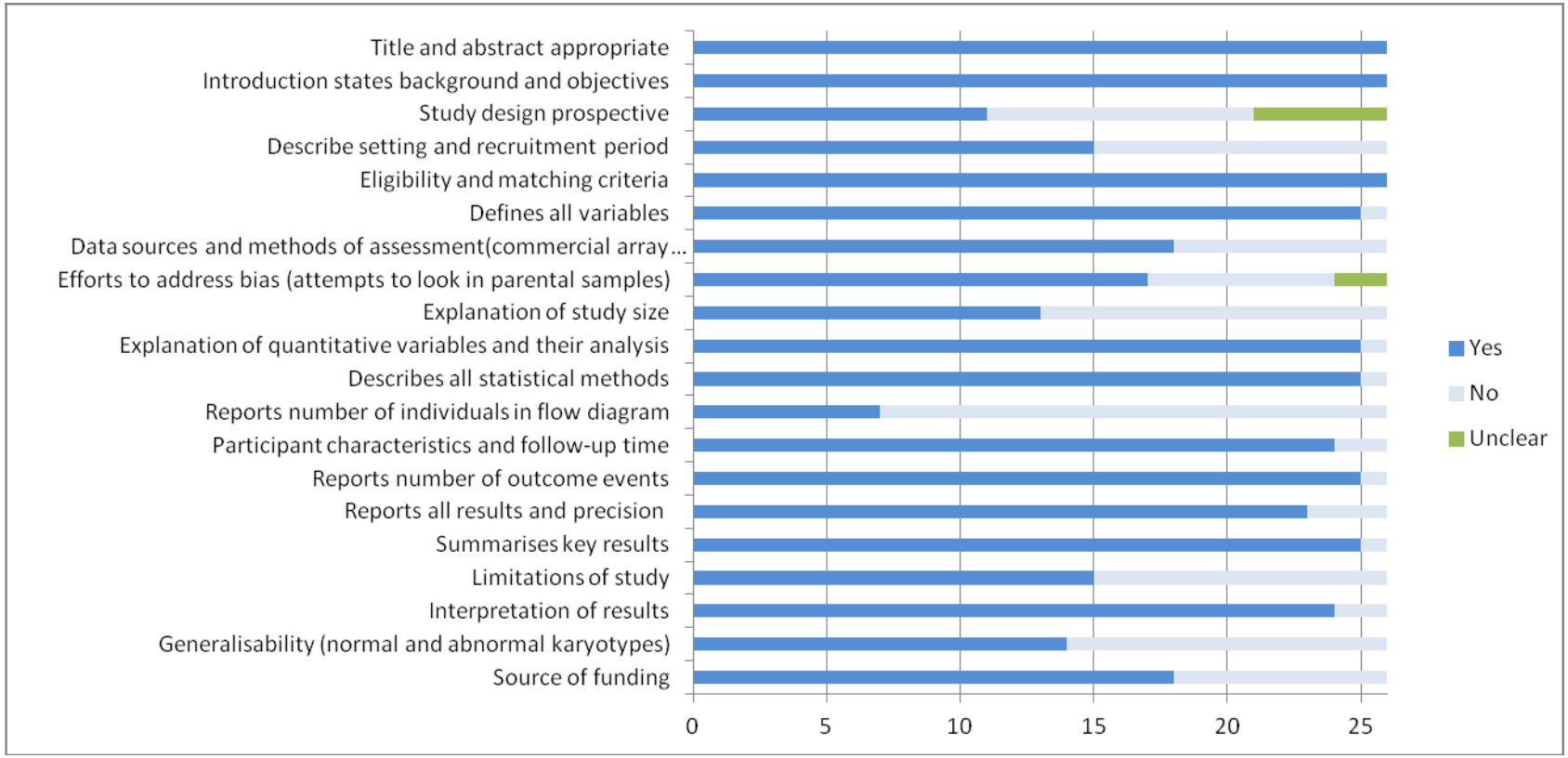
						amniotic fluid =19 Cultured amniotic fluid =2977 Cord blood=93		
20	Breman A et al (70)	2012	Retrospective	BCM V4, V%, V6 BAC arrays n=282 All others client customised oligonucleotide array	TG/WG	Amniotic fluid =840 Chorionic villus =273 Cystic hygroma fluid n=1 Fetal blood = 1	AUS =410 AMA= 394 FIS=137 AFK=61 MSS=37 PA=61 Other=4 Not provided =12	1116 Unable to draw 2x2 table not included in meta-analysis
21	Srebniak MI et al (78)	2012	Prospective	HumanCytoSNP-12Array Illumina	WG	Not stated	AUS	207
22	Armengol L et al (79)	2012	Prospective	BAC	TG	Chorionic villus=164 Amniocentesis = 728 Fetal blood=14	AUS=173 MSS=235 HIS=145 AMA=273 PA=60 Other = 20	906
23	Fiorentino F et al (69)	2012	Prospective	Cytochip focused constitutional (Blue Gnome Cambridge)	TG	Amniocentesis =2660 Chorionic villus = 308 Cultured amniocytes = 32	AUS=95 AMA=1118 PA=1675 AFK=25 MSS=29 FIS=25 Cell culture failure =33	3000 Used in meta analysis for abnormal karyotype over array in

								abnormal scan results
24	Wapner R et al (65)	2012	Prospective	44k oligonucleotide array customised Affymetrix SNP 6.0 masked to be equivalent of 44K	WG	Chorionic villus sampling =2209 Amniocentesis =2073	AMA=1085 MSS/AUS/FIS/PA Mosaicism excluded	4282
25	Shaffer LG et al (68)	2012	Retrospective	Signature prenatal chip V 4.0 Signature Chip Whole Genome	TG WG	Cultured amniocytes = 3269 Amniocytes = 343 Cultured chorionic villus = 854 Chorionic villus = 63 Fetal blood = 25 Products of conception = 432 DNA unspecified sources = 17	AFK=648 FIS=549 Fetal demise = 417 AUS=2858 MSS=77 AMA=346 PA=95 Not specified = 13 Included in my study only when karyotyping done and soft markers excluded 2041 AUS 38 other indications	2079
26	Hillman SC et al (49)	2013	Prospective	Cytochip focused constitutional (Blue Gnome Cambridge)	WG	Chorionic villus=50 Cultured chorionic	AUS	243

						villus=3 Amniocentesis =146 Cultured amniocentesis =8 Fetal blood=29 Fetal tissue=6 Pulmonary effusion=1		

AFK=Abnormal fetal karyotype
AMA= Advanced maternal age
AUS=Abnormal UltraSound findings
BAC=Bacterial Artificial Chromosome
BCM=Baylor College of Medicine
CH = Cystic hygroma
CNS= Central Nervous System
CVS=Chorionic Villus Sampling
FIS=Family history of genetic condition
GI = Gastrointestinal
ICSI= Intracytoplasmic sperm injection
IUGR = Intrauterine growth restriction
MCA = Multiple congenital abnormalities
MSS=Abnormal maternal serum screening
NT = Increased Nuchal translucency
NF = Nuchal Fold
PA = Parental anxiety
SUA = Single Umbilical Artery
TG=Targeted array
WG=Whole Genome array

Figure 13 STROBE quality assessment of included figures



Legend: STROBE is used to assess the quality of the papers included in the systematic review

Results

Chromosomal testing for any clinical indication

When microarray and conventional karyotyping were performed for any clinical indication (details in Table 10) the overall agreement between the two tests was good 93.4% (95% CI 90.4-96.5), these data were heterogeneous (Chi^2 p=0.017). 21 cohorts out of the 23 included in the meta-analysis were used here. Two were excluded as the information could not be used to extract a 2x2 table (51;69). In the Birmingham BAC cohort study the results used were where the G-banding karyotype analysts had been blinded from the microarray results.

I attempted to meta-analyse the rate of CMA detection over karyotyping. However when the referral indication was taken into account (various) the results were highly heterogeneous (Chi^2 p=0.00) with CMA detection over karyotyping ranging from 0.4%-50%. Four papers seemed to contribute disproportionately to the 'high' heterogeneity of these data. In three cases this can be explained by small samples size (D'Amours et al n=49, Schmid et al n=12 Faas et al n=35) and is likely not to be representative of the true detection rates by CMA (53;71;73). The fourth paper used a high resolution CMA (Affymetrix SNP 6.0) but did not use parental samples to follow up results of unknown significance therefore presenting a unusually high detection rate by CMA (43).

Even when I performed a sensitivity analysis by removing these four papers the detection rate of chromosomal imbalances detected by CMAs over karyotyping was still unexplained (Chi^2 p=0.00). I therefore felt a pooled result would be misleading. Although heterogeneous all cohorts showed a positive result with CMA finding chromosomal abnormalities over and above standard G-band karyotyping.

Conventional karyotyping detected an extra 0.6% (95%CI 0.2-1.6%) abnormality rate when microarrays were normal. These data are also heterogeneous (Chi^2 p=0.00). Eight papers were excluded from this meta-analysis (42-44;53;69;71;76;77) as microarray analysis was only performed on samples that had a known normal karyotype by G-band analysis.

The rate of VOUS was 1.4% (95%CI 0.5-3.7%) when samples were analysed for any indication. The meta-analysis was performed using VOUS rates from 17 cohorts. In the cohort from Wapner *et al* the reclassified VOUS rate from 2012 was meta-analysed (65). In 4 papers the VOUS rate could not be extracted (69;74;75;80). The Tyreman *et al* paper was excluded from this analysis as parental samples were not tested in order to reclassify results as benign or pathogenic, leaving a disproportionate proportion of VOUS [25.] I excluded the Hillman et al cohort as by using a targeted array the VOUS rate was low (0.4%) in comparison to other cohorts but it did not change this meta-analysis significantly; 1.5% (95% CI 0.5-4.2%).

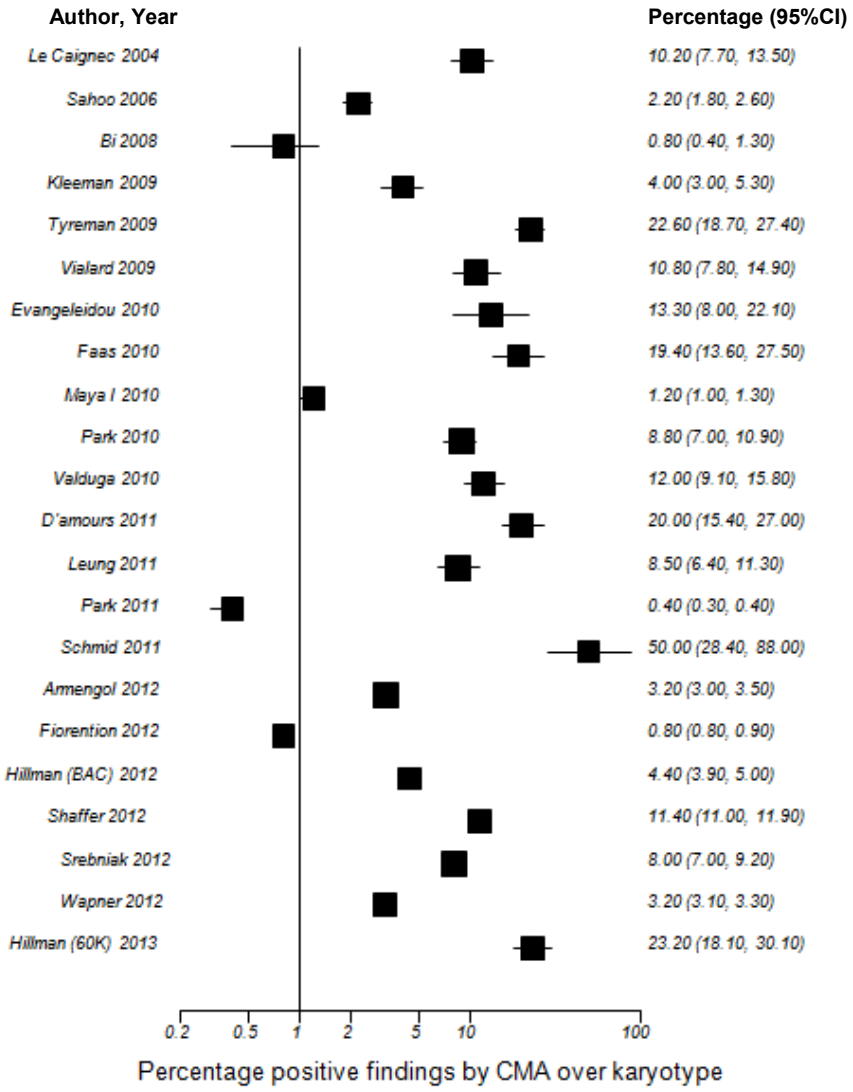
Sub group analysis by date of publication for any clinical indication

In order to see if I could account for the heterogeneity within the meta-analysis, publications were subdivided into those published in 2009 or before and those published after 2009. Publication from 2009 or before had a lower detection rate by microarray over karyotyping compared with 2010-2012 publications but both analyses were still heterogeneous (Chi^2 p=0.00).

Addition of 60K data

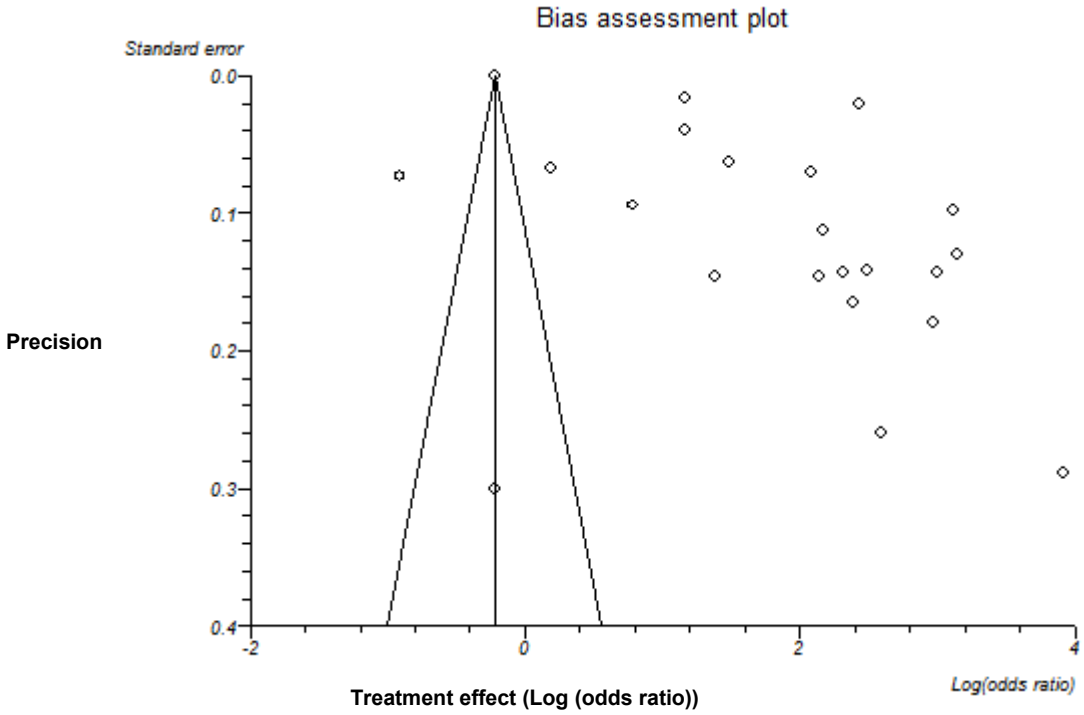
The analysis examining CMA detection rates over full, conventional, karyotyping (when testing performed for any clinical indication) was then repeated adding data from the 60K cohort This is the cohort presented in Chapter 3 and contains 62 samples (19%) of the original Birmingham BAC cohort presented in Chapter 2. The data was still heterogeneous so a pooled meta-analysed percentage is not presented but the additional cohort within the forest plot is presented in Figure 14.

Figure 14 Forest plot of percentage of chromosomal anomalies found by CMA over karyotyping including 60K cohort all indications for testing



Legend Figure 14: Forest plot showing the amount of extra information (in terms of chromosomal abnormalities) detected by CMA over karyotyping for ant referral indication. In theis case the data was too heterogenic to perform a meta-analysis

Figure 15 Funnel Plot looking at publication bias for CMA detection over karyotyping when referral was for any indication



There was no significant publication bias present confirmed by Egger test P = 0.6646 However Funnal plot represented by Figure 15 above does show a tendency to publication bias as there is a lack of small trials with a small treatment effect.

I noted an increase in the number of VOUS as the detection rate by CMA increases (i.e. as the resolution of the array increases). For all papers published from 2010 onwards I performed a Spearman's rank correlation looking at the association between the overall detection rate by CMA and VOUS rate. For these 12 publications ((53;65;68;71-73;76-79)) Spearman's rank correlation coefficient (Rho) = 0.809108 (P = 0.0012 (H_1: positive correlation). This shows a significant positive relationship between the increase in VOUS rate and the overall detection rate by CMA.

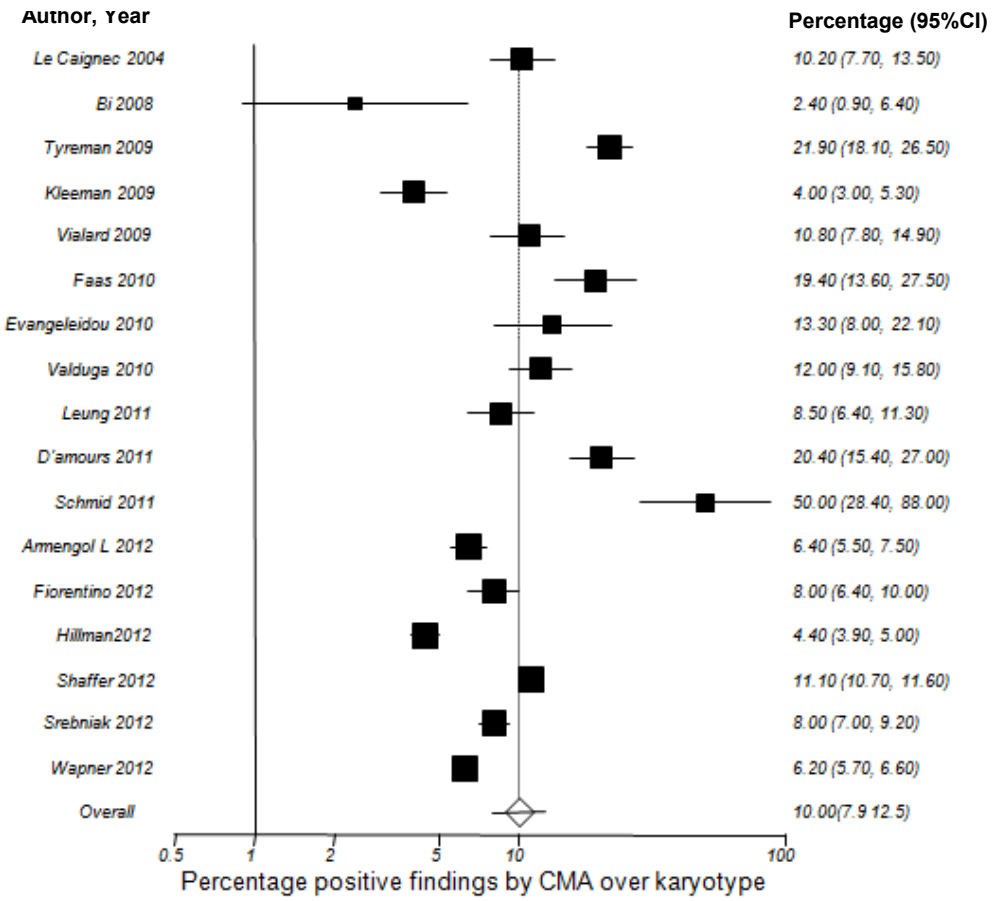
Abnormal ultrasound scan findings

Data from 16 cohorts plus my own were used to compare conventional karyotyping to microarray when the indication was the detection of a suspected structural abnormality on ultrasound scan (39;42-45;52;53;65;68;69;71;73;75-79). Here the rate of detection by CMA over full karyotyping is 10% (95%CI 8-13) (Figure 16) (Chi^2 p=0.00). Again I excluded the Hillman et al cohort to see if it would have a significant effect on the meta-analysis rate which it did not at 10.5% (95%CI 8.4-13.1%). This means that the results from the Birmingham BAC cohort were not an "outlier" and are in keeping with other recently published cohorts.

Conventional full karyotype detected only 0.8% (95%CI 0.2-2.4%) more chromosomal abnormalities than microarray using data from 9 cohorts plus my own (39;45;52;65;68;73;75;78;79), the main examples being balanced rearrangements and triploidy. Both analyses were heterogeneous (Chi^2 p=0.00).

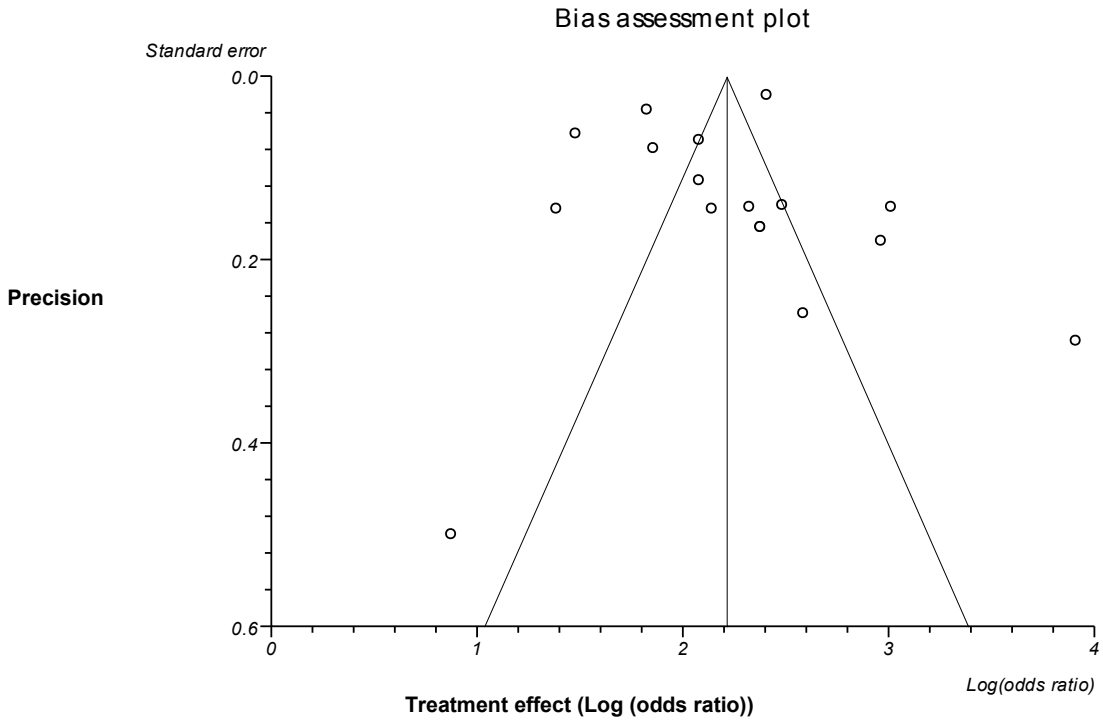
The VOUS rate was meta-analysed using data from 16 cohorts (39;42-45;53;65;68;71;73;75-79). When indication for testing is an "abnormal scan" the VOUS rate is 2.1% (95%CI 1.3-3.3), higher than the meta-analysed rate of 1.4% for any indication. Exclusion of the Hillman *et al* cohort did not change this meta-analysis significantly at 2.3% (95%CI 1.5-3.5%).

Figure 16 Forest plot showing percentage of chromosomal anomalies detected by CMA over karyotyping when indication is abnormal ultrasound scan



Legend: Forest plot showing the extra information (in terms of chromosomal abnormalities) detected by CMA over karyotypingwhen the indication is abnormal ultrasound scan. Here the meta-analysed rate is 10%(95% CI 7.9-12.5).

Figure 17 Funnel plot for publication bias when referral for testing was for abnormal findings on fetal ultrasound.



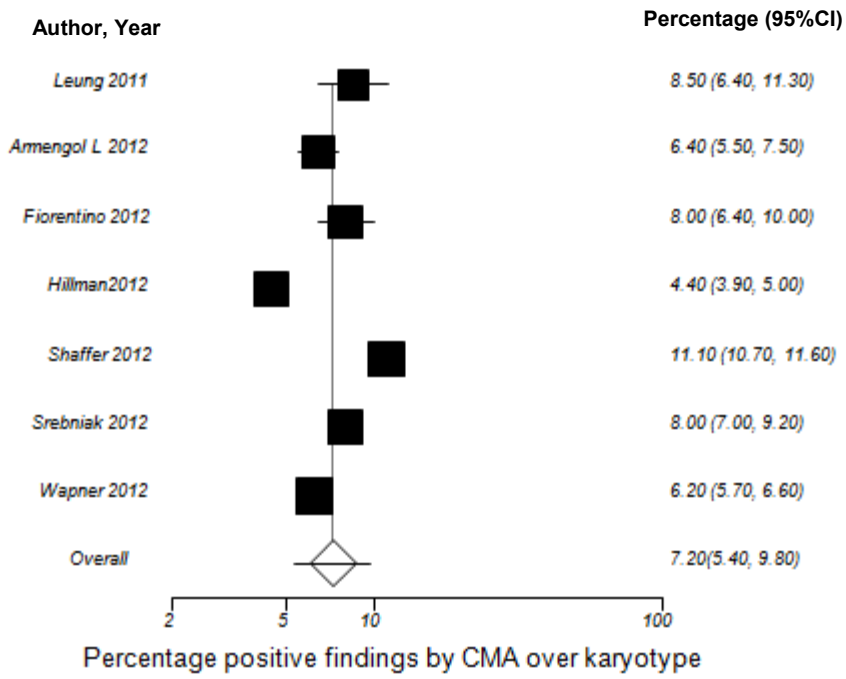
There was no significant publication bias as confirmed by Egger test 0.7221 (Forest plot represented by Figure 17). The funnel plot above also shows no evidence of publication bias.

Sub-group analysis by date of publication for abnormal ultrasound scan findings

Given the increasing resolution of CMA over time and the heterogeneity present in the meta-analysis, I performed sub-group analysis by date of publication when the indication for CMA was abnormal USS findings. 2011-2012 saw the publication of many larger cohorts of fetuses having CMA for abnormal USS findings (52;65;68;69;77-79). In this sub analysis two papers were removed. Schmid *et al* (78) had a 50% detection rate by CMA over Karyotyping, partly due to a very small sample size (n=12), a high resolution array (SNP 6.0 Affymetrix) and no evidence that parental samples were analysed to see if the CNV was de novo. This paper was therefore excluded. The second paper to be excluded was D'Amours (53). It also was an outlier having a detection rate by CMA of 20.4% over full karyotyping. This was in part due to a smaller cohort size (n=49) and the use of 4 custom-designed arrays.

Sub-analysis of the remaining 7 cohorts (included Hillman *et al, 2013*) still gave a heterogeneous outcome statistically (Chi^2 p=0.00) but the graphical representation (Figure 18) shows the analysis to appear more homogeneous than previously discussed (Figure 16). This sub-analysis using cohorts published between 2011 and 2012 demonstrates that the detection rate by CMA for an "abnormal ultrasound" scan finding appears to be 7% (95% CI 5 -10) over conventional karyotyping. I believe this to be a more accurate detection rate when performing CMA for abnormal scans.

Figure 18 Forest plot of percentage detection of chromosomal anomalies by CMA over karyotyping when the indication is an abnormal ultrasound scan 2011-2012

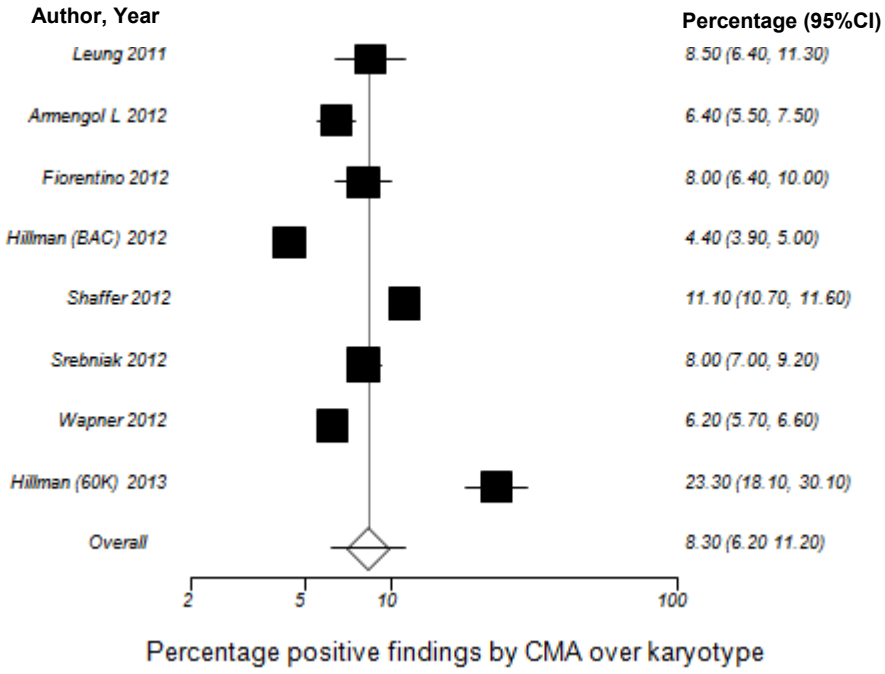


Legend: Forest plot showing the amount of extra information gained by CMA over karyotyping when the indication is an structural anomaly found on prenatal ultrasound. Studies included are those published between 2011-2012. CMA detected 7.2% (95% CI 5.4-9.8) over karyotyping.

Addition of 60K array

The analysis looking at CMA detection rates over karyotyping when testing for abnormal fetal ultrasound findings was then repeated, adding data from the 60K cohort. (Figure 19). This increase the detection rate by CMA over karyotyping to 8.3% (95%CI 6.2-11.2).

Figure 19 Forest plot percentage of chromosomal anomalies detected by CMA over karyotyping 2011-2012 including 60K cohort



Legend: Forest plot showing the amount of extra information gained by CMA over karyotyping when the indication is an structural anomaly found on prenatal ultrasound. Studies included are those published between 2011-2012 including our 60K. CMA detected 8.3% (95% CI 6.2-11.2) over karyotyping.

Targeted FISH

Using eight cohorts (seven other cohorts plus my own) it was possible to analyse the detection rate of 22q microdeletion (Di George) syndrome, if FISH were used instead of CMA for samples that had been sent for testing due to a fetal cardiac anomaly (43;44;51;68;71;73;78). Meta-analysis revealed a 4% (95%CI 1-10) detection rate of a 22q microdeletion in those samples requesting testing due to a fetal cardiac anomaly. This would imply a similar level of detection by microarray (for any clinical indication) across the genome compared with clinician request for specific FISH probes based on the prenatal phenotype.

Discussion

Results from my prospective, case cohort BAC study found that chromosomal microarray had an increased detection rate over conventional karyotyping in 4.1% of cases (10/243) (Chapter 2) when a fetal abnormality was suspected on ultrasound scan. This is lower than the reported literature to date, with a meta-analysed rate of 10%. Our group chose to use a purposefully conservative microarray design for prenatal diagnosis at the time of inception of the project. This was to limit, in theory, the amount of parental follow up required and VOUS detected.

Our referring region within the United Kingdom also has a high number of consanguineous families. According to the UK Census 2001, 13.5% of Birmingham residents are Asian (66,085 UK Pakistanis) compared to 3.5% in the whole of the UK. The prospective Birmingham Births study by Professor Bundey and colleagues (81;82) established that the high neonatal and childhood mortality observed in West Midlands families of Pakistani origin is mostly attributable to recessive inherited disease. Approximately 75% of Pakistani marriages in the West Midlands were consanguineous (in many cases the degree of consanguinity is higher than first cousin equivalents) and there was a 1 in 10 risk of serious genetic disease in the children of these consanguineous unions. This may have lowered the detection rate by CMA as the actual cause of the scan findings may be autozygosity associated with a single gene autosomal recessive condition, undetectable by CMA platforms not containing SNP probes.

The meta-analysis rate of 10% may also have been raised by inclusion of some studies with artificially elevated detection rates (due to small cohort numbers, not testing parents for inherited CNVs and high VOUS rates) (43;53;71). The sub-analysis using papers between 2011and 2012 showed a detection rate by CMA of 7% which I believe is closer to the actual rate detected by CMA over full karyotyping using up to date microarray platforms, although addition of the 60K data increased this to 8.3%, in part due to the increased VOUS detected in this cohort (see Chapter 3).

The Birmingham BAC Study's lower detection rate cannot be solely attributed to the targeted nature of the BAC array as other studies using the same array (Fiorentino and Lee (51;69) reported detection rates above karyotyping of 6.3% and 8.2% respectively) have shown higher detection rates. However both of these studies report chromosomal differences >10Mb in size missed by CMA. I conclude that the high quality preparations for full G-band analysis available in our laboratory have also contributed to the lower detection rate by CMA above conventional karyotyping.

This meta-analysed result further strengthens the evidence for the use of microarray technology for this particular prenatal indication. When microarray is performed for an abnormal scan (suspected congenital malformation) the VOUS rate is higher (2.1%) than when performed for any indication (1.4%). This is likely to be due to the increased chromosomal pathology (pathogenic CNVs) within this sub-cohort. With

time some of the VOUS detected will be redefined as benign but some will also be reclassified as pathogenic variants.

The systematic review and meta-analysis of outcomes was performed in order to answer important questions regarding overall detection rates by CMA over conventional, full karyotyping, the rate of VOUS and how this has changed over time**.** The concern with increasing resolution of CMA is the potential subsequent increase in VOUS rate. Certainly in papers published from 2010 onwards a higher detection rate is positively correlated with a higher VOUS rate as was seen by the positive Spearman rank correlation. It is therefore going to become fundamentally important that as CMA platforms increase in resolution that there is national and international guidance regarding reporting of VOUS in the prenatal setting. When performed for any indication, conventional karyotyping has an additional abnormality detection rate of 0.6% over CMA. However, as I and others have shown, the majority of this can be explained by balanced inversions and translocations. These are unlikely to cause a phenotype in the absence of a phenotype in the parent, and when accompanied by a normal microarray result provide reassurance that the rearrangement is balanced. Triploidy can also be detected by conventional karyotyping and missed by microarray analysis. However it is likely to be “picked up” by other tests such as studies for maternal cell contamination or QFPCR for common Trisomies.

There would have been a 4% detection rate by targeted FISH for 22q microdeletion (Di George) Syndrome (if the clinical indication was a suspected cardiac anomaly on

echocardiogram scan). This is interesting as it shows that “intelligent targeting” of FISH probes according to the phenotype on ultrasound still only yields the same detection rate as microarray when performed for any indication (4%). I only had the data available to look for the potential detection rate of DiGeorge Syndrome, but the same analysis could be performed to look at potential detection rates of Miller Dieker when a neuronal migration order was detected, or Pallister Killian Syndrome when Congenital Diaphragmatic hernia is identified on fetal ultrasound. In addition FISH only examines a single chromosomal locus whereas CMA interrogates thousands or even millions of loci simultaneously, without requiring prior knowledge by the clinician to request tests for particular chromosomal anomalies.

This systematic review contained data on 17,113 pregnancies, substantially larger than the 751 pregnancies included in the first systematic review (Chapter 1) (31) and is likely to be more representative of the true detection rate of microarray over karyotyping. These data are, however, still heterogeneous. This is possibly due to smaller cohorts with an artificially high detection rate, and the different platforms used in the different chromosomal microarray studies. The time in which these articles were published ranges from 2004-2012. During this period, although microarray design has changed and increased in resolution, subgroup analysis by time of publication did not account for the heterogeneity.

Statements issued by the American College of Obstetrics and Gynecology (ACOG) (48) and Italian Society of Human Genetics (SIGU) (83) have recommended that

karyotyping should remain the principal cytogenetic tool in prenatal diagnosis and microarray used as an adjunct when a structural anomaly is seen on scan. This presents collective evidence for the higher detection rate by CMA not just for abnormal scan referrals but for other indications for invasive testing.

Submitted manuscript from chapter 4

The following manuscript from Chapter 4 has been published

Hillman SC, McMullan DJ, Hall G, Togneri FS, James N, Maher EJ, Meller CH, Williams D, Wapner RJ, Maher ER, Kilby MD. Use of prenatal chromosomal microarray: prospective cohort study and systematic review and meta-analysis. Ultrasound Obstet Gynecol. 2013 Jun; 41(6):610-20.

CHAPTER 5 QUALITATIVE ANALYSIS

Introduction

"*The challenge... during these times of sophisticated genetics and technological manoeuvres will be not to distance ourselves from the individuals we are caring for" (*84).

As prenatal diagnostic tests that examine fetal chromosomes increase in resolution their ability to detect significant as well as potentially insignificant chromosomal or genetic anomalies increase also. It is important that prenatal diagnostic tests are employed because it is the right and accepted thing for families to know if their baby has differences and to have the choice to decide on pregnancy outcome and not just because the technology is available.

As medicine is becoming increasingly technologically advanced, it is at the same time becoming less paternalistic (85). Though we endeavour to offer patients a choice about prenatal testing in pregnancy, the inevitable stress which is evermore apparent when an abnormality has been found on scan, may be increased by the complex and ambiguous results which the technology offers.

The situation is compounded by the biological timetable of pregnancy, particularly as many structural anomalies are not detected until the 18-21 week anomaly scan and the UK law dictates that 24 weeks is the legal limit for termination of pregnancy under the Abortion Act clause 1a. It is further compounded by the scheduling of testing and

the greater clinical and emotional difficulties of late abortion, all of which create a sense of time pressure (86).

It has been argued that, when events are out of the ordinary, people need a sense of what the expected response is (a “social ritual”). In this case, with the newness of the technology, no ritual exists: but this “cultural lag” offers an opportunity to understand more fully what people actually feel, and would therefore be an appropriate time to perform qualitative work (87).

It has been argued that “doctor-patient communication” is a transaction between the biomedical world of the doctor and the life-world of the patient (88). The patient’s primary orientation is a social domain where the meaning of health and illness are firmly situated within their life history, social and environmental situations (89). The doctor is concerned with the technical aspects of predicting and controlling health conditions (85). Studies in prenatal genetic decision-making often concentrate on the biomedical perspective (90).

It is vital that we seek to understand the effect this new technology (and the uncertainty it can sometimes create) is having on couples. If not, we risk incorrectly assuming those women and their partners are making properly informed decisions. In order to achieve aim 5 “To perform qualitative work to evaluate how women and their partners feel about the use of CMA testing, particularly when a result has

uncertainty attached" I chose to undertake a qualitative research method as it gives access to the thoughts of those most concerned and enabled me to discover aspects of reality that I could not necessarily anticipate (91;92).

Methods

As described in Chapter 2, from December 2009 - April 2012 at Birmingham Women's Hospital I prospectively recruited women (with a fetal structural anomaly on USS) for microarray testing in addition to karyotyping. The women then received QFPCR results (for Trisomy 13, 18 and 21) followed by results of the CMA alongside the results of the karyotype.

Interviews were conducted by myself (n=22) or Miss Amie Wilson (n=3) (a research midwife) and were 20min - 60min in length. 25 interviews were conducted in total, 16 with women who had a normal microarray report and 9 with women who had an abnormal report. In 23 cases the woman had received all the results of the testing but in two cases (4b and 9b) results were inconclusive and further tests were required. In 12 cases the interview was conducted with just the woman, in 12 cases the partner was also present and in 1 case the woman's father was present.

Interviews were conducted using a semi-structured interview format (appendix F). This interview schedule was developed by myself, Professor Elaine Denny of Birmingham City University and Miss Elizabeth Quinlan Jones (research midwife).

Professor Elaine Denny and Elizabeth Quinlan Jones have previous experience of prenatal interviews (93). Demographic data on patient's age, ethnicity, parity and anomaly on USS were collected. Information on religion and education was not routinely requested (unless voluntarily given during the course of an interview) so as not to bias the interviewer.

Analysis

Interviews were transcribed verbatim. Data was analysed using Framework Analysis and was performed using previously described methodology (94;95). To gain familiarization of the interviews, transcripts were read and re-read by myself and a sample by Elizabeth Quinlan Jones. Throughout this process key ideas and recurrent themes were noted. A thematic framework was then identified by recognising emerging themes. The framework was refined as transcripts were added. The thematic framework was agreed between two researchers (myself and Elizabeth Quinlan Jones). All text was indexed numerically, with numbers placed in the margin beside the text. The original pieces of data were charted using Excel (Microsoft Word 2010©). Charts were developed using themes and subthemes (charts are presented as appendix G).

Results

At the time of interview the median age of women was 29.6yrs (range 19-40 years). 23 (92%) were British (all were Caucasian), 1 woman was Spanish and 1 woman was Polish. The fetal anomaly for which the testing had taken place is recorded in Table 11.

Table 11 Demographics of women interviewed

Microarray result	Age	Living children	Country of birth	Scan abnormality
1a normal	25	0	UK	Bilateral Talipes
2a normal	40	3	UK	Ascites, echogenic bowel
3a normal	20	0	UK	Cardiac anomaly
4a normal	19	0	Spain	Cystic hygroma
5a normal	31	4	UK	Absent forearm
6a normal	28	3	UK	Abnormal lower limbs
7a normal	31	0	UK	Megacystis
8a normal	25	1	UK	Small cerebellum
9a normal	35	0	UK	Pleural effusion
10a normal	36	5	UK	Cleft lip/palate
11a normal	38	1	UK	Talipes/renal pelvis dilatation
12a normal	31	1	UK	cardiac/polydactyl
13a normal	38	1	UK	Increased Nuchal translucency
14a normal	20	0	UK	Increased Nuchal translucency
15a normal	27	2	UK	cardiac
16a normal	37	1	UK	cardiac
1b abnormal	35	?	UK	Holoprosencephaly
2b abnormal	24	1	UK	Increased Nuchal translucency
3b abnormal	33	1	UK	Dandy Walker malformation
4b abnormal	32	2	UK	Megacystis
5b abnormal	30	1	UK	Cardiac
6b abnormal	19	0	UK	Increased Nuchal translucency
7babnormal	34	2	UK	Cardiac
8b abnormal	32	0	Poland	Increased Nuchal translucency
9b abnormal	26	1	UK	Encephalocele/absent corpus callosum

There were five themes identified. The thematic framework is presented below.

Thematic framework

1: Diagnosis

This theme is concerned with the way the diagnosis was explained and received by the woman, including the differences between her experience at local and tertiary centres

1.1 Explanation of scan findings (the interviewee explains how they were made aware that there was a problems with the baby's scan and what was explained to them).

1.2 Explanation of possible genetic causes (the interviewee explains how they were made aware there could be a genetic problem and how this was explained to them).

1.3 Information forms and sources (hospital/family/others). What forms and sources of information were made available to them, how did they gain further knowledge of the condition.

1.4 Differences between local and tertiary care

1.5 Waiting for a tertiary appointment

2: Genetic testing

This theme is concerned with the way the test was explained and understood by the woman, what influenced her to have the testing and experience of receiving the results.

2.1 Understanding of the genetic test

2.2 Influences to have the genetic test

2.3 Receiving results of the genetic test

2.4 Timing of the genetic results

2.5 Usefulness of having the genetic test

3: Family and support

This theme is concerned with the Implications for the spouse/dependents of the woman and the support available to her.

3.1Impact (on other people apart from woman i.e. spouse/family)

3.2Comparison to other pregnancies/children

3.3Support from friends and family

3.4Support from health care professionals (hospital/Community Midwife/unique)

4: Reflections of the treatment received

This theme is concerned with particularly good or bad communication between health care professionals and the woman and specific improvements to the service suggested by the woman

4.1Communication

4.2improvements

5 Emotions

This theme is concerned with the woman's emotions that permeated the experience

5.1Highs and lows and the effect of time on emotions

5.2Uncertainty

5.3Blame

5.4Hope

Results of themes and Indicative Quotations

1.1 Diagnosis

1.1.1 Explanation of scan findings *(This initial scan was performed at the patient's local hospital)*

Although many interviewees described factually what was seen on the USS others reflected on their experience. One interviewee (2a) described a sense of detachment:

> *"Nobody said anything really they were just busy measuring and I felt just a bit like a patient on the table"*

Another (partner of 4b) describes how the reaction of health care professionals had a direct impact on him:

> *"doctors looked like they were panicking and that panics you"*

In three cases interviewees described how they were taken into a different room to wait for someone to explain the scan findings. Although done for confidentiality, couples remembered this poignantly as if this represented their transition from a normal to abnormal pregnancy.

In four cases interviewees discussed realising that something was wrong as the scan was taking a long time or additional people came into the scan room. This created anxiety, particularly when the HCPs would not allude to why they were concerned.

1.1.2 Explanation of possible genetic causes

In most cases interviewees were satisfied with the way possible chromosomal anomalies were explained. Three recalled being told about the possibility of Trisomy 13, 18 and 21.These being the most commonly found chromosomal anomalies it makes sense that counselling should be, in part, directed towards these findings. However both conventional G-band karyotyping and particularly CMA testing are testing for far more than this and it is concerning that couples did not remember this from their counselling experience.

Only one interviewee (11a) found the explanation insufficiently full:

"They skirted around the edges"

1.1.3 Information forms and sources

Seven women described having written information to take home. This is good as a source of information when couples have had time to reflect on what has happened at their early appointment. It can be read on multiple occasions and also translated for non-English speakers.

Five interviewees spoke about their experiences with the internet. In three cases they were clearly negative, as here (5b)

"I tried to find out on the internet and it scared me"

One interviewee acknowledged the possible skewing of information on the internet (3b)

"*Heart-warming as these stories are they tend to be the better ones"*

Patients experienced an overload of information on the internet and without advice or direction to good websites could easily end up misinformed. This is concerning for patients who may develop a schema stemming from a misunderstanding and HCPs who would then be required to "undo" these incorrect beliefs before imparting correct information.

Only one woman described her experience with the internet as positive (8b)

"I think the internet was a nice source of helping me to survive"

They found that it "bridged a gap" while they were waiting for appointments and was a way of finding a "community" of others that had a similar experience.

1.1.4 Differences between local and tertiary care

Ten people reported a negative experience at their local hospital. Some interviewees concluded that this was due to a lack of knowledge (3b):

"They didn't have a clue"

Others decided it was because the HCPs didn't want to be the bearers of bad news (partner 2b).

"Just to pass it on to someone else"

From the patients point of view this was annoying as they had to wait for an appointment at the tertiary centre before they could be given any definite information, leaving them in a state of "limbo". However from the local hospitals point of view they are not specialists and would not have wanted to misinform patients. It is also the

place often in which the abnormality was first detected and therefore it may be inevitable that the patients will remember their interaction with them negatively.

1.1.5 Waiting for a tertiary appointment

Eight interviewees made reference to the wait between appointments at the local and tertiary centre. In six cases the appointment was the next day, which people reported as "*good*". In two cases the delay was a week and both stated that this wait was "hard".

> "*We were trying to put our thoughts in different things. We bought ourselves a play station and computer games"*

1.2 Genetic testing

1.2.1 Understanding of genetic testing

In five cases the interviewee could not recall detail of the microarray counselling (9a):

> *"I don't remember a lot, you just get told so much and then it's hard for it to go in"*

In these cases it may be that women had just received a new or more complex diagnosis regarding the fetal structural abnormality than expected. It is then hard for further information to be retained, particularly regarding complicated chromosomal testing.

In seven cases the interviewee could recall that the microarray test may pick up something that conventional testing may miss, which is indeed correct.

In three interviews women remembered being told about possible VOUS (11a):

> "*it would perhaps be able to offer more detail, we got the impression that that detail might not necessarily be that helpful because you don't have enough detail to interpret properly but that over a period of years then that information might be useful for somebody"*

Counselling about VOUS and the potential for this testing outcome is important regardless of whether the VOUS is to be reported or not. This should be a particular focus point when counselling for CMA so that women may, with this information, choose not to have the test; or if a VOUS does occur women and their partners are not completely unprepared.

In two interviews the women recalled information incorrectly (14a):

"It (microarray) was looking through a telescope not just the naked eye"

Here it is possible that the patient has incorrectly recalled information about karyotyping being analysed through a microscope.

A second woman (7b) incorrectly recalled that microarrays could:

"pick up anything with the baby, even down to a small skin tag."

It is possible that the woman recalled the increased detail in which microarrays analyse fetal chromosomes, but has translated this into increased detail of the baby's structural abnormalities.

It is inevitable that some misunderstandings may occur particularly when counselling in stressful situations making it particularly important that written information is given and a point of contact for questions to be answered.

1.2.2 Influences to have the genetic test

People had five reasons for undergoing CMA testing. Firstly that it required no further invasive sampling and no extra risk of miscarriage. Secondly was to accept every test in order to find an explanation for the problems on USS. Thirdly was for reassurance. Fourthly that they had "no other choice" and finally, as an act of benevolence, to help in the future through research.

1.2.3 Receiving results of genetic test

Women were asked about receiving the result of the microarray testing.

Women who had normal results described the telephone call as "*clear*", "*good*", "*helpful*". Three women described the relief that they felt with the result (16a)

> *"it was just relief I was just crying, it's one thing off the worry list"*

Those whose results were abnormal reflected that the telephone call was "*complicated*", they "*were stressed*" and they "*thought the worst*".

It may be inevitable that women will view a telephone conversation to give a positive chromosomal result as being poorly conducted compared with one to give a negative result. However HCPs may be able to limit this by having as much information to hand as possible, and importantly a plan of follow up including an appointment to see a clinical genetics specialist as soon as is feasible.

Only one woman felt she was not given enough information about the abnormal result. (8b)

> *"they said they will talk to me later. I thought I want to talk about the results now. They were afraid that it would be too upsetting so they just gave me the minimal information"*

In three interviews women described how after receiving the results of the QFPCR test they then expected the subsequent tests (including the microarray) to be normal. (Partner 3b),

"I certainly walked away thinking great chromosomes not an issue and then to get a phone call a few days later to be told actually it's chromosome 6, it's far worse than we first thought, is slightly misleading because you sort of walk away thinking great this is do-able"

It could be that inadequate counselling had led to the woman and her partner believing that QFPCR was a definitive test. It could be a misunderstanding by the patient or it may be that they were "blindsided" into thinking that the remaining tests would be normal as the most common chromosomal anomalies had been ruled out.

Two people described getting the result of unknown significance (VOUS). *(*Partner 4b)

"You never think a doctor's going to go, phew, don't know what it is"

Uncertainty is a particularly difficult concept as a HCP to relay to patients. A VOUS may be viewed as conveying a lack of information and it should be considered if this is in the best interests of the patient. VOUS need to be considered by a multi-disciplinary team on a case by case basis.

1.2.4 Timing of results

Eight women spoke about the wait for the results following the invasive testing. It was described as "*hard*", "*stressful*", "*hell*", and "*like a weight on your shoulders*". One woman described in detail how her testing had spanned the Christmas holidays and how this meant she would be 24 weeks gestation (the legal limit for termination of pregnancy under the Abortion Act clause 1a).(Woman 11a)

> *"Because of the Christmas holidays that was my 24 weeks - there wasn't going to be any time to have a result and make a decision, so you had to think it through probably further than you would have wanted to."*

CMA is particularly amenable to high throughput, with often the availability for multiple patient samples to be analysed on one array slide. However this can sometimes mean, for economy, that samples are held and then "batched" so as not to waste resources and money.

1.2.5 Usefulness of having the genetic test

Five women reported normal microarray results as reassuring. However normal results may lead to a mistaken idea that there *could not be* a genetic abnormality, when the resolution of the microarray is limited and single gene mutations may be missed.

Five women described microarray as "*useful*" as it had provided, or may have provided, extra information. For one woman this was to "*prepare*" the family.

In two cases women were interviewed when the results of the testing were VOUS. Although people found it hard when HCPs could not fully interpret the findings, all were still pleased they had the testing and did not regret their decision. Although the result was uncertain they still seemed to value the information, which was given to them sensitively and clearly by a clinical genetics consultant (partner 4b):

> *"the more information they can give you the better, if they need to do 4 tests and each test will give us 4 pieces of information then we will have 4 tests"*

Only one woman (whose baby had a pathogenic chromosomal difference identified on both conventional karyotyping and CMA) expressed any regret at having the testing. (6b)

> *"I wish I daren't have the test done cause it's broke me inside"*

However the same woman later reported how the information of an abnormal microarray (and karyotype) helped her make a decision about interrupting the pregnancy (6b)

> *"I don't feel so bad about ending it 'cause I know this child wouldn't of had a life"*

Three women stated that they had not found the microarray testing useful. In all cases this was because the result was normal and had not provided them with any additional information. In one case there was a family history of an undiagnosed genetic condition and the women and her partner were hoping that this technology could have provided long awaited answers.

1.3 Family and support

1.3.1 Impact

In four interviews the woman described the impact of the abnormal USS and testing on her children. Two women described the impact on their partners; one how it was the first time she had seen him cry, while another described how the situation resulted in physical symptoms (4b)

> *"We had to take him to A and E with chest pains and they said "I think it's just basically stress""*

An abnormal genetic result can have implications for the wider family. One woman described the impact of a chromosomal abnormality on the X chromosome (5b)

> *"'cause there are a lot of girls in my family - it's like a bomb. It scares me that she'll (her daughter) have to go through what I've had to go through"*

It is important when counselling for chromosomal testing that that the impact of a diagnosis not only relates to the fetus but back through an entire family, sometimes providing long awaited answers but sometimes providing unwanted information.

1.3.2 Comparison to other pregnancies/children

In three cases people described how different this had been to their previously "normal" pregnancies. Two described how they had undergone something similar previously (12a):

> *"when they confirmed it was just like here we go again"*

One woman compared the possibility of having a disabled child to her healthy two year old (3b):

> "*When you have got a two year old running around the house he owns the house. You do compare"*

Three women compared the outcome of their pregnancy with other people's children, in two cases comparing to "*everyone else healthy babies*" or not being "*like everyone else's*". Comparison may be inevitable, but help maybe gained from directing women to groups and charities (Unique and *Antenatal Results and Choices*) that might put them in touch with families with affected children, so they can draw on similarities.

1.3.3 Support from friends and family

15 women described support from friends and family, and in most cases input was helpful.

In three cases however it was described negatively, with one woman describing how she was defensive discussing her pregnancy. In two cases the feelings of others were perceived as inappropriate (3b)

> ""*It happens" which is a phrase that's been thrown around, it is because nobody knows what to say"*

1.3.4 Support from health care professionals/support groups

14 women mentioned support they had received from health care professionals or official support groups including *Unique* and *Antenatal Results and Choices (ARC).* Women and their partners were complimentary about the support they had received using words such as "*satisfied*", "*excellent*" and "*helpful*". One woman commented that although her family had contacted ARC she was reluctant to: (9b)

> *"I have been a bit too scared because I am quite emotional and I don't really want to cry in front of anybody"*

Only one couple felt that they had not received adequate support (4a)

> *"there is no help really out there where you can phone or speak to anybody"*

1.4 Reflections of the treatment received

1.4.1 Communication

Three women commented on the amount of information that was communicated to them. One said it was "*a lot of information to take in*". Surprisingly in two cases women said that they had not received enough information. One of these cases involved an uncertain chromosome result where no accurate information was available: the patient's feeling of inadequate information is therefore justified but inevitable. In the second case the woman felt she was told too little as they were "*afraid of upsetting her*". This may be due to HCPs withholding information as they do not understand it in order to convey it, and are concerned that they may convey something incorrectly. Information should not be retained so as not to upset someone as this information may be required in order to make important decisions, such as the termination of a pregnancy. Two women commented on the use of medical language in the consultation. One said she had to "look up new words", the second that HCPs should alter their language (9a)

> *"get past all the doctor jargon and get it into plain English"*

All HCPs should be aware of this important problem in their own practice, particularly when conveying complicated concepts.

1.4.2 Improvements

Women suggested that HCPs should be "*as open as possible as early as possible*", provide quicker follow up, provide access to support groups, counselling and direction to good websites, and that a letter should be sent after the appointment summarising what had taken place.

1.5 Emotions

1.5.1 Highs and lows and the effect of time on emotions

Women used many words to express their emotions during this entire experience. Some of the more common were "*devastated*", "*shocked*" and "*worried*". Three women described the effect that time had on their emotions; *"like a roller coaster*": some days things would be better but something like a hospital visit would put them back to "*square one*" (3b)

> *"It's peaks and troughs isn't it, it's not something that is in the forefront of your mind all the time, but coming to the hospital visits it becomes a lot more apparent"*

1.5.2 Uncertainty

Many women and their partners expressed uncertainty and lack of control over the situation.

Two women expressed distress at not knowing if their unborn child would live or die: One woman commented that uncertainty surrounding the prognosis meant she could not make decisions about continuation of the pregnancy, and one woman expressed her concern that the same thing could happen in a subsequent pregnancy. Five interviewees spoke of their uncertainty when the HCPs did not have the answers. (2a normal result)

> *"I assume nobody really knows and because they don't know they can't tell me"*

1.5.3 Blame

Women discussed the blame that they felt using phrases such as "*it's my fault*", "*you blame yourself "* and "*typical me*".

In two cases the baby had inherited a chromosomal problem from the woman. One woman seemed to find the information helpful. (5)

> *"Because I carry too much X chromosome I have got something to blame (for previous miscarriages)"*

The second woman however blamed herself (9b)

> *"then it came back that it was carried from me which is even harder to register really because I had given the baby whatever it is. I feel like it is my fault"*

Blame may be encountered when a structural anomaly is found on scan, as a mother attempts to find a cause, often blaming something she may have done or not done in pregnancy. Genetic diagnoses, particularly when inherited, are also associated with blame as they "passed it on". The combination of the two means that HCPs should be particularly alert to this and attempt to discuss and counsel where appropriate.

1.5.4 Hope

In two cases women discussed the hope that could be given to them by a HCP. For one woman it was the hope that research could provide answers in the future, and for another woman the hope that a subsequent pregnancy would not be affected. Two women discussed grasping or holding on to hope that everything would be alright.

Discussion

This qualitative work gives a first-hand insight into the experiences of women and their partners diagnosed with an abnormality on prenatal USS and accepting genetic testing including microarray testing (Figure 20). Previous qualitative work in this field is limited to women's experience of prenatal genetic testing *or* women's experience of having an abnormal ultrasound scan.

Women described being detached from the scan and being able to tell something was wrong as the scan was taking a long time. Women are alert to verbal and non-verbal expressions from health care professionals and other studies have similarly found silence creates anxiety (96). One study also described women feeling like passive objects "lying on the table" and having the scan "done" to them (97).

Couples found the wait between initial and tertiary appointment hard, especially if it was more than a few days. Other studies have shown that while the time between appointments is useful in terms of recovery from the shock of diagnosis, it is also a time of great stress when parents need support (98).

Women and their partners struggle to recall the details of genetic testing and the differences between genetic tests. Often women have only just had a diagnosis of an abnormal scan and under stressful circumstances it is unsurprising that little information is retained. Previously authors have commented that it is virtually

impossible to counsel in these circumstances (99). It is possible that when pregnant women find themselves in a stressful position, they cope by complying with what they believe is the health care professional's recommendations (100). They may also feel that complying with testing is their way of showing that they are, or will be, a "good parent" (101).

A recent publication by Bernhardt et al (66) also looked at prenatal microarray testing, but they interviewed women with abnormal or uncertain microarray results. The cohort also included women tested for reasons other than abnormal ultrasound scan findings (such as advanced maternal age or a positive screening result). One of the similarities when comparing this study with ours was the theme of support found in both papers. In the main both found HCPs, support groups, and friends and family to be supportive.

The internet as a source of information was often found to be unhelpful and even worried participants in the cohort. This is similar to other prenatal and postnatal cohorts that have found the internet confusing or that it did not yield much information (66).

Newer experiences specific to prenatal microarrays testing included examining the reasons that women accepted microarray testing (to gain extra information, to make informed decisions and prepare adequately). These women were sometimes

disappointed by a normal or a “negative result”. Some people requested the microarray for reassurance, which is a concern. Although the testing is at very high resolution a negative result does not mean there is *no* possibility of a chromosomal abnormality or single gene disorder.

My study showed that women were keen to have results as quickly as possible. This is a potential advantage of microarray as it can be performed on uncultured cells and therefore more quickly. When the result showed a chromosomal difference the women felt that the telephone call was sometimes complicated or uncertain. This is a difficult telephone call for the HCP and woman. Other studies have found poor understanding during such telephone calls due to strong emotions and grief (102).

Similar to the study by Bernhardt et al, women in this study felt that they chose to have microarray testing as it was an offer “too good to pass up” considering they had already decided to have an invasive procedure, and the test required no further risk of miscarriage. Bernhardt et al concluded that as there was no further risk of miscarriage, women and their partners may have opted for the microarray testing understanding less about the test than they might have done if it *had* conferred a risk. In their study this was the case, particularly when there was an abnormal ultrasound finding and the couples were under emotional stress (66). If this was the situation, it may account for why women and their partners could often recall little, or recalled misinformation, about the microarray test in the cohort. This could be a particular problem if they have not comprehended the possibility of a VOUS.

Communication of VOUS presents a particularly difficult challenge. People appeared to be particularly concerned when the HCP did not know the meaning of the result. Here patients require rapid follow up with a consultant clinical geneticist. Even when this has occurred people may make incorrect conclusions to fit in with their own schemata. In my experience having a VOUS did not lead to a termination of pregnancy, a fear previously expressed (103). The recent publication by Berhardt et al interviewed 14 women with VOUS. Many of them considered this knowledge to be information that they wished they did not have ("toxic knowledge") (66). Women were left feeling anxious, and these concerns lingered into worries about their child's development.

Interestingly, in a postnatal setting when a microarray was performed, parents expressed similar views regarding the understanding of the scientific uncertainties, regardless of whether the result was a pathogenic variant or a VOUS. This may be because similar uncertainties can surround both pathogenic results and VOUS, particularly if the pathogenic finding is rare, or newly identified, and limited prognostic information exists (104). In this study some parents reported feeling relieved when the result was a VOUS, and they also felt that the result (both pathogenic and VOUS) was valuable and empowered them particularly to access educational services (104). Perhaps then, VOUS should not be reported prenatally but only when testing is performed postnatally. Both in my study and the Bernhardt study women felt that after receiving initial normal results (in my study QFPCR) they were "blinded" by the normal results and expected the next set of results to be normal. They were therefore shocked if the results of subsequent studies were abnormal. The Bernhardt et al

cohort found this to be the case when testing was performed for a reason *other* than abnormal ultrasound scan findings (66). However in my cohort (with abnormal ultrasound scan findings) I found differently and women were still blinded by normal QFPCR results expecting subsequent results to be normal.

Limitations

This study was limited by the demographics encountered: all participants were Caucasian. Although 25 is a reasonable sample size for qualitative work, only nine had abnormal chromosomal results. Women often talked about the experience as a whole and it was therefore sometimes difficult to extract what was applicable to the scan, invasive test, or results of a test.

Conclusion

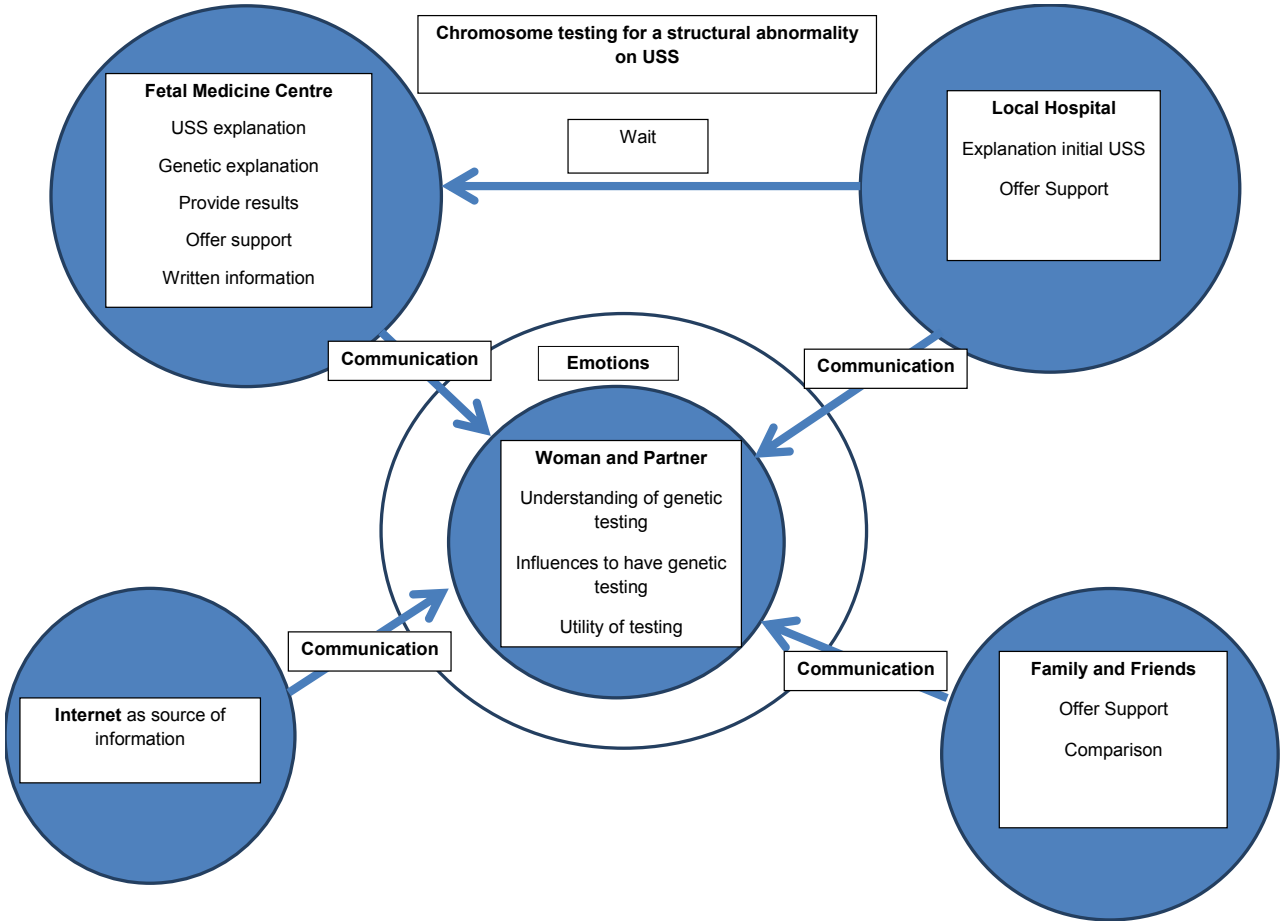
My study has given insight into the thoughts, feelings and perceptions of couples during this time. Interviewees often parcelled abnormal scan findings and chromosomal testing together, viewing the experience as a whole. There were frequent misunderstandings and misconceptions by couples regarding aspects of counselling/testing; proving that reinforcement with information to take home is paramount. Good clear communication by HCPs is essential.

Recommendations

Results from my qualitative analysis have led me to propose that in clinical practice:

- When counselling women and their partners for fetal chromosomal testing, HCPs should explain that although the most common, Trisomy 13,18 and 21 only account for some of the chromosomal changes resulting in abnormal scan findings
- HCPs should be aware that often people remember a small amount when counselled so it must be backed up with patient-friendly literature to take home
- If possible, women and their partners should be directed to good websites
- Partners should be involved in the counselling process where possible
- Information for support groups such as ARC (http://www.arc-uk.org/) and Unique (http://www.rarechromo.org/html/home.asp) should be given
- Medical language should be as minimal as possible
- When results of QFPCR testing are telephoned it should be reinforced that there are further results to come
- When a chromosomal abnormality is diagnosed, rapid referral to a clinical geneticist is paramount
- A letter should be sent to the woman following an appointment with a geneticist/genetic counsellor summarising what was discussed

Figure 20 Experiences of women undergoing CMA testing for a structural anomaly on ultrasound scan (USS)



Legend: Diagram of the influences and experiences undergone by women and their partners when they have CMA testing for a structural anomaly detected on fetal ultrasound. Women and their partners are at the centre of the diagram surrounded by their emotions. Other influences (family, medical and the internet) communicate with them.

Manuscript submitted from Chapter 5

The following manuscript from chapter 5 has been published:

Hillman SC, Skelton J, Quinlan-Jones E, Wilson A, Kilby MD. "If it helps..." the use of microarray technology in prenatal testing: patient and partners reflections. Am J Med Genet A. 2013 Jul; 161A(7):1619-27.

CHAPTER 6 HEALTH ECONOMIC MODEL BASED EVALUATION: CHROMOSOMAL MICROARRAY (CMA) USE FOR THE PRENATAL DETECTION OF CHROMOSOME ANOMALIES

Introduction

CMA technology is increasingly being used to discover and define chromosomal anomalies in the prenatal setting. It has increased detection rates of pathogenic CNVs over standard techniques (as described in Chapters 1, 2 and 3). However, when applied to clinical practice, the cost-effectiveness of CMA is yet to be established. I sought to assess the cost effectiveness of chromosomal microarray compared against standard cytogenetic tests (105).

In order to achieve this a decision tree was built (106). A decision tree is used in health economic modelling and graphically represents different pathways that patients can take through various decisions or chances that incur costs along their particular path. The path may diverge into branches at a “node” either because of a chance of a different outcome (chance node e.g. chance fetus had chromosomal abnormality vs. chance did not have a chromosomal abnormality), or because of different paths due to a decision (made by a HCP or patient e.g. decision to have karyotype testing vs. decision to have CMA testing). The branches of the tree have a probability attached, with the sum of the branches adding up to 1. Chance nodes are traditionally represented by a circle while decision nodes are typically represented by a square. The final outcome of a path is called a terminal node shown by a triangle.

A decision tree was built and populated using three different sets of data:

1) that of my cohort, comprising of 309 fetal samples, where testing was performed for structural abnormalities on fetal ultrasound scan (Chapter 2).

2) that of the literature, comprised of data from 22 journal articles and 13755 fetal samples, where there was a range of indication for testing (Chapter 4).

3) and finally, using the literature and data from 17 cohorts where the indication for testing had been abnormalities on fetal ultrasound scan (4276 fetal samples are included) (Chapter 4).

Developing the model structure

To assess the cost effectiveness of the various tests, or combination of tests, decision trees were created using TREEAGE software (Treeage software inc., Williamstown, MA, USA) (Figure 21). Three models were developed:

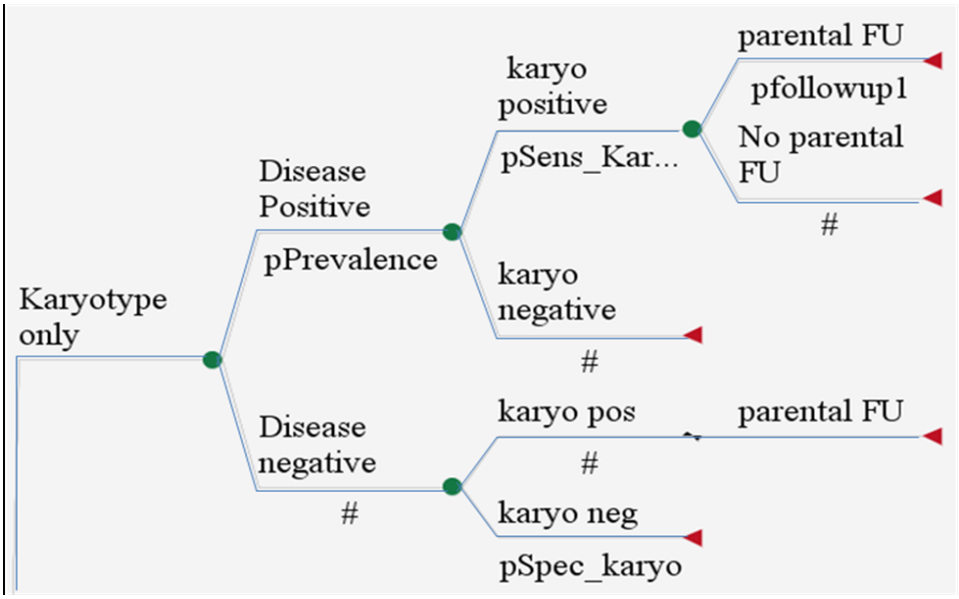
1) The first model is populated using data from my prospective cohort (described in Chapter 2). This was a study running from December 2009 until April 2012 at Birmingham Women's Foundation Trust. When a fetal anomaly scan showed a significant structural anomaly, women were counselled and offered fetal chromosome testing by invasive sampling. If accepted, women were also offered, counselled and consented to have chromosomal microarray testing in addition to standard cytogenetic testing. 309 women were recruited and had

cytogenetic testing performed. Nine options for testing within the model were considered using combinations of the tests; QFPCR (Quantitative Fluorescence Polymerase Chain Reaction), conventional G band karyotyping, CMA, FISH (Fluorescence In Situ Hybridization for 22q11.2 microdeletion syndrome on remaining fetal samples with cardiac abnormalities). The definition of these alternative options is presented in Table 12.

2) The second model was constructed following a systematic review of the literature. MEDLINE (1970–June 2012), EMBASE (1980–June 2012), Cinhal (1982-June 2012) were searched electronically. Selected studies had >5 cases and microarray testing was performed prenatally in addition to karyotyping. Search yielded 559 citations. Full manuscripts were retrieved for 85. Data from 21 primary studies (including the Birmingham BAC cohort described in Chapter 2) were used to populate the model (38;39;43-45;52;53;65;71-80;107). In total 13,755 results were included. In this model QFPCR and FISH were not included as diagnostic tests (as the majority of papers in the literature did not contain this information). The five options considered for testing were; karyotyping alone, CMA alone, karyotyping *then* CMA, CMA *then* full karyotyping, full karyotyping *and* CMA. Here the referral reasons for fetal chromosomal testing were a mixture of indications including a “high risk” on antenatal screening for Down’s Syndrome, a family history of fetal anomalies, parental anxiety, or advanced maternal age.

3) The third model was also constructed from data collated from the systematic review described above. Here data were extracted only when testing had been performed because a structural anomaly had been found on fetal ultrasound scan (USS). From the 22 primary studies 17 had this information (39;42-45;52;53;65;68;71;73;75-79). In total 4,276 results were included. The same five testing options were considered as in model 2; karyotyping alone, CMA alone, karyotyping *then* CMA, CMA *then* karyotyping, karyotyping *and* CMA.

Figure 21 An example of an "arm" of the decision tree for "karyotype only"



Legend: An arm of a decision tree. Circles represent chance nodes and triangles are terminal nodes, # is 1 minus the probability in the parallel arm (i.e. the first # is 1 minus pPrevalence).

Table 12 shows the different strategies of chromosomal testing that were analysed within model 1.

Option	Test 1	Followed by Test 2	Followed by Test 3
1	QFPCR	-	-
2	G-band karyotyping	-	-
3	CMA	-	-
4	QFPCR	*Then* G-band karyotyping (if QFPCR negative)	-
5	QFPCR	*Then* CMA (if QFPCR negative)	-
6	G-band karyotyping	*And* CMA (regardless of G-band karyotyping result)	-
7	QFPCR	*Then* G-band karyotyping (if QFPCR negative)	*And* CMA (regardless of G-band karyotyping result)
8	QFPCR	*Then* FISH (if QFPCR negative)	-
9	QFPCR	*Then* G-band karyotyping (if QFPCR negative)	*Then* FISH (if G-band karyotyping negative)

QFPCR= Quantitative Fluorescence Polymerase Chain Reaction
CMA= Chromosomal microarray
FISH= Fluorescence In Situ Hybridization for 22q11.2 microdeletion syndrome on remaining fetal samples with cardiac abnormalities

Assumptions for the model

Some assumptions were required in order to develop a workable model:

1) In cytogenetic testing a result may be positive or negative but (more so in CMA testing) it is possible to have a result of uncertain clinical significance (VOUS) (see Chapter 1). This result has the potential to be pathogenic but may, with future research, prove to be a benign variant, that is normal chromosomal variance within the population. In these models, due to the possible pathogenic nature of these results, I treated them as positive and allowed for the costs of parental follow up to be included and the effectiveness score attributed to the result was the same as a pathogenic one. Recent prospective studies have reported these results to patients (65). However not all VOUS will be true positive findings and over time some will be determined as being benign. By treating all VOUS as true positives I may well overestimate the effectiveness of CMA testing in detecting chromosomal variants. To allow for this all models were re-run treating the VOUS results as false positives and the effectiveness score attributed was zero (the same as an incorrect or false positive finding). The results of this “sub-analysis” are included.

2) That the starting point for the testing was that no prior testing had been performed on the sample acquired from the fetus. This was done to simplify an already complex model and so that all “samples” were entered at the same point in the tree.

3) That autosomal Trisomies, sex chromosome aneuploidies and Triploidies would require no parental follow up. In reality it may be that parental karyotyping is performed (for instance in a finding of Down's Syndrome) if array had shown trisomy 21 and the aetiology was unknown, or karyotyping had shown trisomy 21 from Robertsonian translocation. By testing parents HCPs can give important information as to the chance of recurrence in future pregnancies. However it will not affect this current pregnancy, only subsequent pregnancies, and was therefore not included.

4) That a true positive or true negative result gave an effectiveness score of one and a false positive or false negative gave an effectiveness score of zero. However for some patients if there was any uncertainty (a VOUS) associated with the results they may have ranked their efficacy at less than one. However this information is currently unavailable.

5) The CMA costed is the Birmingham BAC array (BlueGnome, Cambridge, UK) as this was the prenatal array being used in the WMGL. I acknowledged that this array is more expensive and more targeted then other prenatal arrays used and for this reason the analysis includes a threshold analysis, reducing the cost of the CMA test.

Test accuracy data

Tests accuracy data in the first model (Table 13)(Appendix J) was taken using the data from my own prospective cohort. When calculating the sensitivity of CMA *and* karyotyping it was assumed that the overall result would be positive if either test was positive on its own. The sensitivity therefore increased as all individuals that tested positive on either test were potentially positive cases. The specificity therefore falls as there are more false positives and it is lower than the specificity of each test alone. The CMA used in the first model is a targeted BAC focused constitutional array (Bluegnome Cambridge). This has a lower resolution than other prenatal array used but also limited the number of VOUS found (n=1).

Table 13 Test accuracy data taken from Birmingham BAC array prospective cohort

Screening test	Probability parameter (Sensitivity)	probability parameter (Specificity)
QFPCR	66/91 (73%)	218/218 (100%)
Karyotyping	82/91 (90%)	217/218 (99.5%)
CMA	87/91 (96%)	215/218 (99%)
Karyotyping and CMA	91/91 (100%)	214/218 (98%)
FISH	4/7 (57%)	34/34 (100%)

Test accuracy data for the second model (Table 14) was based on the values estimated by the systematic review (39;42-45;52;53;65;68;71;73;75-79). Here the indication for performing CMA is any clinical indication. As well as including those that were referred for abnormal scans it also includes; those that were referred for abnormal Down's Syndrome screening, those that had testing because of a family history of chromosomal problems, those that had testing for advanced maternal age and those that requested testing for parental anxiety. Here the resolution (and therefore detection rate) of the CMA varied depending on the platform used.

Table 14 test accuracy data when performing CMA for mixed referral reasons

Screening test	Sensitivity (95% CI)	Specificity (95% CI)
Karyotyping	54.5% (51.7-57.2)	99.9% (99-100)
CMA	86% (84.2-88)	99.9% (99-100)
Karyotyping and CMA	100% (100-100)	99.9% (99-100)

Test accuracy data for the third model (Table 15) were also based on values from the systematic review(39;42-45;52;53;65;68;71;73;75-79). Here all testing was performed because of at least one structural anomaly detected on fetal ultrasound scan and the sensitivity of CMA is somewhat increased. The resolution also varied depending on the platform used.

Table 15 Test accuracy data when testing performed for abnormal ultrasound findings

Screening test	Sensitivity (95% CI)	Specificity (95% CI)
Karyotyping	53.5% (50.1-56.9)	99.9% (99.8-100)
CMA	96%(94.9-97.5)	99.9% (99.8-100)
Karyotyping and CMA	100% (100-100)	99.8% (99.7-100)

Cost and resource data

In order to have cytogenetic testing a patient must be counselled by an appropriately trained health care professional (HCP) and have an invasive test performed (amniocentesis, chorionic villus sampling or fetal blood sampling). The sample is then sent to an approved laboratory for testing and results are disseminated to HCPs. If negative/normal they are telephoned to the patient, but if abnormal the patient re-attends for further counselling by HCPs and in some cases cytogenetic testing on parental samples is performed.

Here I have only included costing from when the sample arrives at the laboratory and then subsequent follow-up costs. The initial cost of a consultation and the invasive test procedure were not included as it was assumed that these would be the same for all cases. Base costs are calculated as if the invasive sample taken was amniotic fluid by amniocentesis. However, the models have been recalculated for CVS samples.

The costs of resources utilised were those that were directly incurred by the NHS. The laboratory costs were calculated by the West Midlands Genetics Laboratory (WMGL). Costs of the cytogenetic tests included; DNA extraction, cost of the base test and consumables, staffing costs, capital costs (such as the scanner service contract) and administration costs.

Staffing costs for the laboratory work were calculated by timing staff performing the procedures. The average salary of those routinely doing the work was then used and the cost per hour of their time calculated. For the staffing costs for those performing the analysis work, as this can be highly variable depending on the complexity of the case, the average time was estimated. Capital costs such as the scanner service contract were calculated by taking the cost of the contract and dividing it by the number of samples processed over the time of the contract. Administration costs have been previously calculated by the laboratory per test and are included. Consumable costs were then recorded as those directly incurred by the NHS. Cost of follow-up included costs of seeing a consultant clinical geneticist and specialist midwife. This was based on a thirty minute appointment. These costs were taken from Unit Costs of Health and Social Care 2011(108) (Table 16). Costs used were those calculated by the laboratory for the years 2011/2012.

It was assumed that follow-up with parental samples would be required for any abnormal cytogenetic test unless the results was an autosomal triploidy, trisomy or sex chromosome aneuploidy. If a trisomy 13, 18 or 21 was detected in the model by

CMA or QFPCR the cost of parental follow-up was not included (to determine if the trisomy was from a Robersonian translocation) as this cost is required for future counselling regarding recurrence risk but would not change the outcome for the current fetus. This is as opposed to parental follow up for VOUS which would alter the outcome for the current fetus when determining pathogenicity of the chromosomal anomaly.

Table 16 Cost resource data

Screening test	Unit cost	source
QFPCR trisomy 13,18 and 21 and sex chromosome aneuploidy	£129	WMGL
Karyotyping Amniocentesis	£223	WMGL
Karyotyping Chorionic villus sampling	£265	WMGL
CMA (1Mb BAC array)	£405	WMGL
FISH for 22q11.2 DS	£186	WMGL
Parental CMA	£350	WMGL
Parental karyotype	£382	WMGL
Follow up specialist midwife per 30 mins	£48.50	Unit Costs of Health and Social Care 2011(108)
Follow up clinical geneticist (consultant) per 30 mins	£81	Unit Costs of Health and Social Care 2011(108)

WMGL= West Midlands Genetics Laboratory

Outcome

In this analysis outcomes are reported in terms of the additional diagnoses provided by CMA compared to conventional G-band karyotyping. The results are reported in terms of the Incremental Cost Effectiveness Ratio (ICER) (Appendix J) based on the additional cost for every chromosomal abnormality detected.

Sensitivity analysis

In addition to the base case analysis I carried out deterministic sensitivity analysis. This is a form of sensitivity analysis where input parameters are assigned different values (109). Here the following changes were made:

1) In the CMA arm when VOUS were found they were removed from being true positive and treated as false positive results.

2) The cost for laboratory karyotyping were changed from £222 (karyotyping performed on cells obtained from amniotic fluid), to £265 (karyotyping performed on cells obtained from chorionic villus sampling (CVS)). This was to see the effect the type of invasive test was having on the cost effectiveness.

3) Threshold sensitivity analyses were carried out to establish the critical value of CMA that would change the deterministic results in terms of ICERs and may affect the decision of policy makers. This meant decreasing the cost of CMA testing and seeing its effect on the cost effectiveness of the technology. The

last few years have shown a decrease in the cost of this technology, whereas conventional karyotyping is a more stable price having been used for a much longer time period.

A probabilistic sensitivity analysis was also undertaken to determine the uncertainty of the model. A probabilistic sensitivity analysis is when probability distributions are applied to the ranges for a model's input parameters, samples from the distribution are then drawn at random to generate an empirical distribution of the relevant measure of cost effectiveness (109). A beta distribution was assigned to each true positive, true negative, false positive and false negative parameter. Cost effectiveness results were then calculated by simultaneously selecting random values from each distribution. A Monte Carlo simulation repeated the process 10,000 times. A Monte Carlo simulation evaluates the effect of uncertainty by running a large number of simulations for each of which values are drawn from distributions assigned to uncertain parameters, the aim of which is to construct an empirical probability for the overall results (109). This gave an indication of how variation in the test sensitivity and specificity lead to variation in the results of cost and effectiveness (109).

The decision uncertainty surrounding the use of CMA as a replacement for karyotyping was examined with the cost-effectiveness acceptability curve (CEAC). A CEAC curve is a graphical representation of the results from a cost effectiveness analysis allowing assessment of the probability of the assessed interventions being cost effective at various levels of a decision maker's willingness to pay for an

additional unit of health outcome (109) .This plots the probability that CMA will be cost effective as compared to conventional G-band karyotyping at a given threshold of willingness to pay (WTP) that decision makers may be willing to pay for a gain in effectiveness. In this case a gain in effectiveness is an extra case of fetal chromosomal anomaly detected.

Results

1) Incremental Cost Effectiveness Ratios (ICERS) treating VOUS as true positives and using amniocentesis as the invasive test performed

The base case deterministic results of the strategies based on the outcome of cost per case detected are presented in Tables 17-19 (respectively model 1-3). A strategy is said to be dominated if it is more costly but less effective than another strategy. Dominated strategies are omitted from the table. They are at the base cost of £405 for CMA and £222 for karyotyping. This is assuming all invasive samples were amniocentesis. In these analyses VOUS were treated as true positive results (and awarded an effectiveness score of 1). Here incremental cost and incremental effectiveness are relative to the previous strategy i.e. In Table 17 if the test of choice was QFPCR, then FISH for DiGeorge Syndrome, the ICER is £2500; so it would cost an extra £2500 per extra case detected over the previous strategy using QFPCR alone.

The effectiveness measure is the cost per case of chromosomal anomaly detected by a test (or a positive test result). The Incremental Cost Effectiveness Ratio is in pounds sterling and represents the cost per additional case of chromosomal anomaly detected.

Table 17 ICERs using model one populated using "Birmingham BAC" cohort data and amniocentesis. VOUS are true positives.

Strategy	Cost (£)	Incremental Cost (£)	Effectiveness	Incremental Effectiveness	ICER (£)
QFPCR only	173		0.9159		
QFPCR then FISH Di George syndrome	205	32	0.9288	0.0129	2500
Karyotype only	298	93	0.9644	0.0356	2600
QFPCR then karyotype then FISH DiGeorge	411	112	0.9774	0.0129	8700
CMA only	490	80	0.9806	0.0032	24600
Karyotype and CMA	722	232	0.9832	0.0026	90500

QFPCR=Quantitative Fluorescent Polymerase Chain Reaction
FISH= Fluorescence In Situ Hybridization
ICER = £ per additional chromosomal abnormality detected
Effectiveness = Proportion of chromosomal abnormalities correctly detected
Explanation of table: incremental cost and Incremental effectiveness are relative to the previous strategy i.e. if the test of choice was QFPCR, then FISH for DiGeorge Syndrome the ICER is £2500, so it would cost an extra £2500 per extra case detected over the previous strategy using QFPCR alone.

Table 18 ICERs using model two populated using systematic review data where prenatal chromosomal testing is performed for any clinical indication and the invasive test is amniocentesis. VOUS are true positives.

Strategy	Cost (£)	Incremental Cost (£)	Effectiveness	Incremental Effectiveness	ICER (£)
Karyotype only	238		0.9578		
CMA only	437	199	0.9871	0.0292	6800
Karyotype then CMA	646	209	0.9997	0.0127	16500

ICER = £ per additional chromosomal abnormality detected
Effectiveness = Proportion of chromosomal abnormalities correctly detected

Table 19 ICERs using model three populated using systematic review data where prenatal chromosome testing is performed for abnormal scan findings and the invasive test used is amniocentesis. VOUS are treated as true positives.

Strategy	Cost (£)	Incremental Cost (£)	Effectiveness	Incremental Effectiveness	ICER (£)
Karyotype only	255		0.9107		
CMA only	469	214	0.9925	0.0819	2600
CMA then karyo	655	185	0.9991	0.0066	28300

ICER = £ per additional chromosomal abnormality detected
Effectiveness = Proportion of chromosomal abnormalities correctly detected

2) Deterministic sensitivity analysis

2.1) Using Chorionic Villus sampling instead of amniocentesis.

As part of the deterministic sensitivity analysis the cost of the invasive test was changed from that of amniocentesis to chorionic villus sampling. The following three Tables (20-22) use the same populations as above but the cost of the karyotyping is increased to £265 as this is the cost of karyotyping chorionic villus samples. In these analyses VOUS were again treated as true positive results with an effectiveness score of 1:

Table 20 ICERs using Model one populated using "Birmingham BAC cohort" data using Chorionic Villus Sampling. VOUS are treated as true positives.

Strategy	Cost (£)	Incremental Cost (£)	Effectiveness	Incremental Effectiveness	ICER (£)
QFPCR only	173		0.9159		
QFPCR then FISH DiGeorge	205	32	0.9288	0.0129	2500
Karyotype only	341	136	0.9644	0.0356	3800
QFPCR then karyotype then FISH DiGeorge	444	103	0.9774	0.0129	8000
CMA only	490	46	0.9806	0.0032	14200
Karyotype and CMA	756	266	0.9832	0.0026	103800

ICER = £ per additional chromosomal abnormality detected
Effectiveness = Proportion of chromosomal abnormalities correctly detected

Table 21 ICERs using model two populated using systematic review data where prenatal chromosomal testing is performed for any clinical indication and using Chorionic Villus sampling. VOUS are treated as false positives.

Strategy	Cost (£)	Incremental Cost (£)	Effectiveness	Incremental Effectiveness	ICER (£)
Karyotype only	281		0.9578		
CMA only	437	156	0.9871	0.0292	5300
CMA then karyotype	688	252	0.9997	0.0126	19900

ICER = £ per additional chromosomal abnormality detected
Effectiveness = Proportion of chromosomal abnormalities correctly detected

Table 22 ICERs using model three populated using systematic review data where prenatal chromosome testing is performed for abnormal scan findings and using Chorionic Villus Sampling. VOUS are treated as true positives.

Strategy	Cost (£)	Incremental Cost (£)	Effectiveness	Incremental Effectiveness	ICER (£)
Karyotype only	298		0.9107		
CMA only	469	171	0.9925	0.0819	2100
CMA then karyotype	690	221	0.9991	0.0066	33700

ICER = £ per additional chromosomal abnormality detected
Effectiveness = Proportion of chromosomal abnormalities correctly detected

2.2) Treating VOUS as false positives

As part of the deterministic sensitivity analysis VOUS were treated as false positives with an effectiveness score of zero rather than as true positives with an effectiveness score of one. In reality women may view the “effectiveness” or “worth” of a VOUS as somewhere between zero and one, but as this information is not available the extremes were analysed.

Test accuracy data was determined as above; model 1 from the prospective BAC cohort (Table 23) and models 2 and 3 from systematic review data but here VOUS for CMA were treated as false positive results (Table 24 and 25).

Table 23 Test accuracy data "Birmingham BAC cohort” when VOUS are treated as false positive

Screening test	Sensitivity	Specificity
QFPCR	72.5%	100%
Karyotyping	90%	99.5%
CMA	95.6%	98.5%
Karyotyping and CMA	100%	98.2%
FISH	57%	100%

Table 24 Test accuracy data using systematic review data (any indication for chromosomal testing) and VOUS treated as false positives

Screening test	Sensitivity	Specificity
Karyotyping	64.6%	99.9%
CMA	83.6%	98.4%
Karyotyping and CMA	100%	98.4%

Table 25 Test accuracy data using systematic review data and testing for abnormal fetal ultrasound findings, VOUS treated as false positives

Screening test	Sensitivity	Specificity
Karyotyping	66%	99.9%
CMA	95.5%	95.6%
Karyotyping and CMA	100%	95.5%

The base case deterministic results of the strategies, based on the outcome of cost per case detected, are presented in Tables 26-28 (respectively models 1, 2 and 3). The strategies presented are again undominated (so not dominated by a more cost-effective strategy). They are at the base cost of £405 for CMA and £222 for karyotyping. This is assuming all invasive samples were amniocentesis. In these analyses VOUS were treated as **false positive** results.

Table 26 ICERs using model one populated using "Birmingham BAC cohort" data using Amniocentesis as the invasive test and treating VOUS as false positives.

Strategy	Cost (£)	Incremental Cost (£)	Effectiveness	Incremental Effectiveness	ICER (£)
QFPCR only	173		0.9191		
QFPCR then FISH DiGeorge	205	32	0.9320	0.0129	2489
Karyotype only	298	93	0.9676	0.0356	2616
QFPCR then karyotype then FISH DiGeorge	411	112	0.9806	0.0129	8670
QFPCR then karyotype and CMA	719	309	0.9871	0.0065	47697

ICER = £ per additional chromosomal abnormality detected
Effectiveness = Proportion of chromosomal abnormalities correctly detected

Here CMA alone is dominated by the strategies above due to the reduced effectiveness of CMA if the VOUS results were treated as false positives.

Table 27 ICERs using model two populated using systematic review data where prenatal chromosomal testing is performed for any clinical indication and using amniocentesis as the invasive test. VOUS are treated as false positives.

Strategy	Cost (£)	Incremental Cost (£)	Effectiveness	Incremental Effectiveness	ICER (£)
Karyotype only	238		0.9723		
karyo then CMA	646	408	0.9852	0.0129	31500

ICER = £ per additional chromosomal abnormality detected
Effectiveness = Proportion of chromosomal abnormalities correctly detected

Here CMA alone is dominated by the strategies above due to the reduced effectiveness of CMA if all VOUS results were treated as false positives.

Table 28 ICERs using model three populated using systematic review data where prenatal chromosomal testing is performed for abnormal ultrasound scan and using amniocentesis. VOUS are treated as false positive.

Strategy	Cost (£)	Incremental Cost (£)	Effectiveness	Incremental Effectiveness	ICER (£)
Karyotype only	255		0.9474		
CMA only	469	214	0.9558	0.0084	25400
CMA then karyo	655	185	0.9623	0.0065	28300

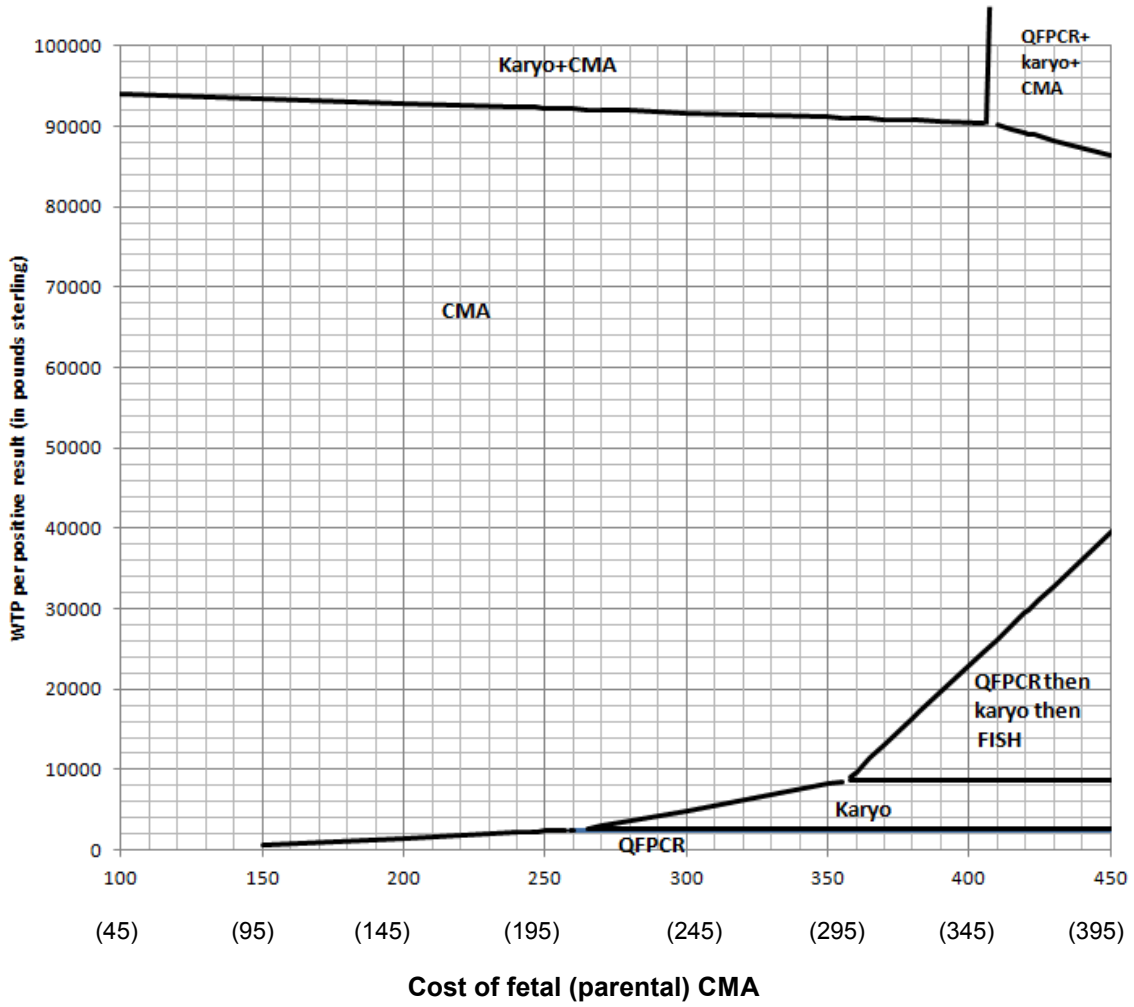
ICER = £ per additional chromosomal abnormality detected
Effectiveness = Proportion of chromosomal abnormalities correctly detected

2.3) Threshold analysis reducing cost of CMA testing

As the cost of CMA is decreasing it was appropriate to model this decrease in cost and the effect it would have on the willingness to pay (WTP) for a chromosomal anomaly to be detected.

This “threshold analysis” was done twice for the Birmingham BAC cohort, once when VOUS treated as true positives (Figure 22) and once with the VOUS treated as a false positive (Figure 23). It was also performed for models 2 and 3 using systematic review data, here the VOUS are treated as true positives (Figure 24 and 25). The cost of CMA was reduced from its base case value of £405 to £100.

Figure 22 Threshold analysis reducing the cost of fetal and parental CMA. Using "Birmingham BAC cohort". VOUS treated as true positive findings.

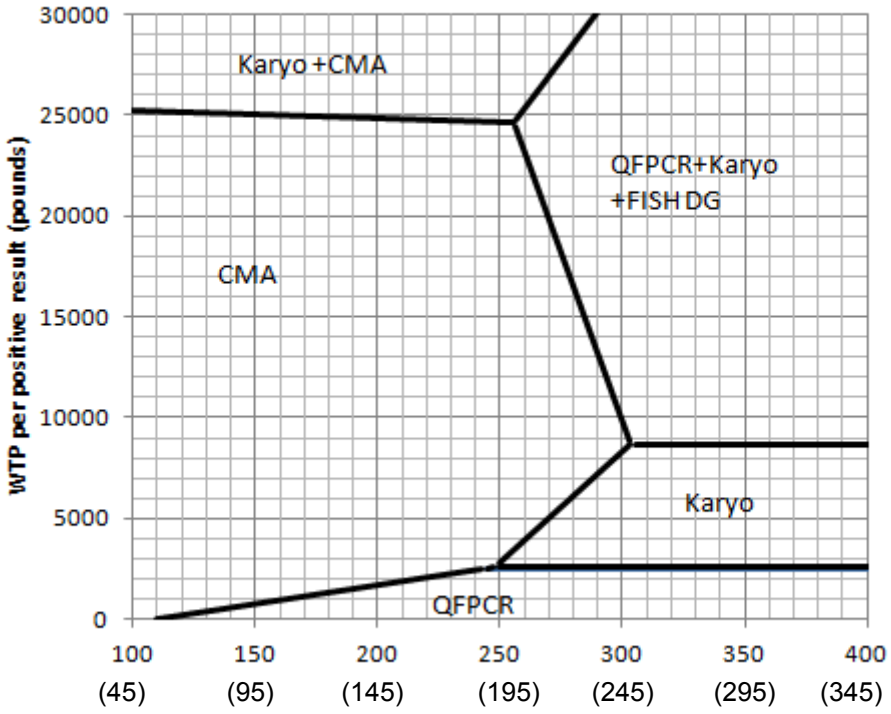


CMA= Chromosomal Microarray
FISH= Fluorescent In Situ Hybridisation
Karyo= Conventional G Band Karyotype
QFPCR= Quantitative Fluorescence Polymerase Chain Reaction
VOUS= Variant Of Unknown Significance

Legend: The threshold analysis above shows the effect of decreasing the cost of fetal CMA and parental CMA (presented in brackets) and the effect of this on the willingness to pay for fetal chromosomal anomaly to be detected. In this analysis VOUS are treated as true positives.

Graph Interpretation: at a given cost of fetal CMA of £400when the willingness to pay for a chromosomal abnormality to be detected is <£2000 the test of choice is QFPCR. When the WTP is between £2000 and £8000 then the test of choice is karyotyping. When it is above £8000 but below £22,000 the tests of choice are QFPCR followed by karyotype (if QFPCR negative) followed by FISH for Di George (if other tests negative and a cardiac anomaly). If the WTP is above £22,000 but below £90,000 the CMA is the test of choice.

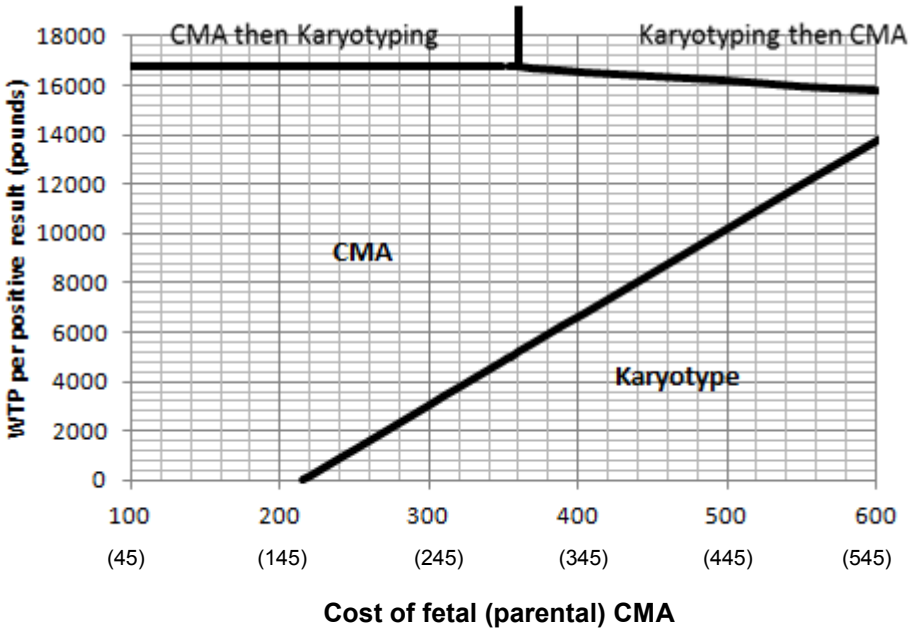
Figure 23 Threshold analysis reducing the cost of fetal and parental CMA. Model one "Birmingham BAC cohort", but VOUS treated as *false* positives.



CMA= Chromosomal Microarray
FISH= Fluorescent In Situ Hybridisation
Karyo= Conventional G Band Karyotype
QFPCR= Quantitative Fluorescence Polymerase Chain Reaction
VOUS= Variant Of Unknown Significance
WTP= Willingness To Pay

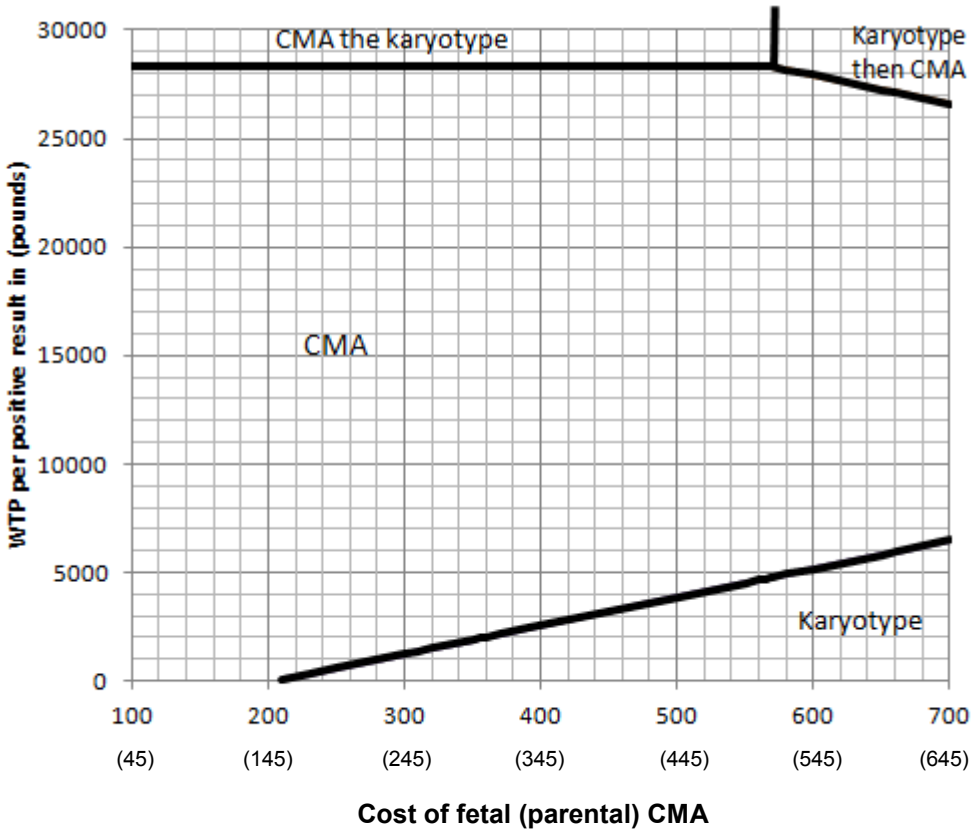
Legend: The threshold analysis above shows the effect of decreasing the cost of fetal CMA and parental CMA (presented in brackets) and the effect on the willingness to pay for detection of a chromosomal anomaly. In this analysis VOUS are treated as false positives.

Figure 24 Threshold analysis reducing the cost of fetal and parental CMA. Model two using systematic review data and any indication for testing, VOUS treated as true positives



Legend: The threshold analysis above shows the effect of decreasing the cost of fetal CMA and parental CMA (presented in brackets) and the effect on the willingness to pay for detection of a chromosomal anomaly. In this analysis VOUS are treated as true positives and the analysis was populated with systematic review data where the indication for testing was “any clinical indication”.

Figure 25 Threshold analysis reducing the cost of fetal and parental CMA. Model Three using systematic review data and abnormal ultrasound scan as an indication for testing, VOUS treated as true positives



Legend: The threshold analysis above shows the effect of decreasing the cost of fetal CMA and parental CMA (presented in brackets) and the effect on the willingness to pay for detection of a chromosomal anomaly. In this analysis VOUS are treated as true positives and the analysis was populated with systematic review data where the indication for testing was abnormal fetal ultrasound findings

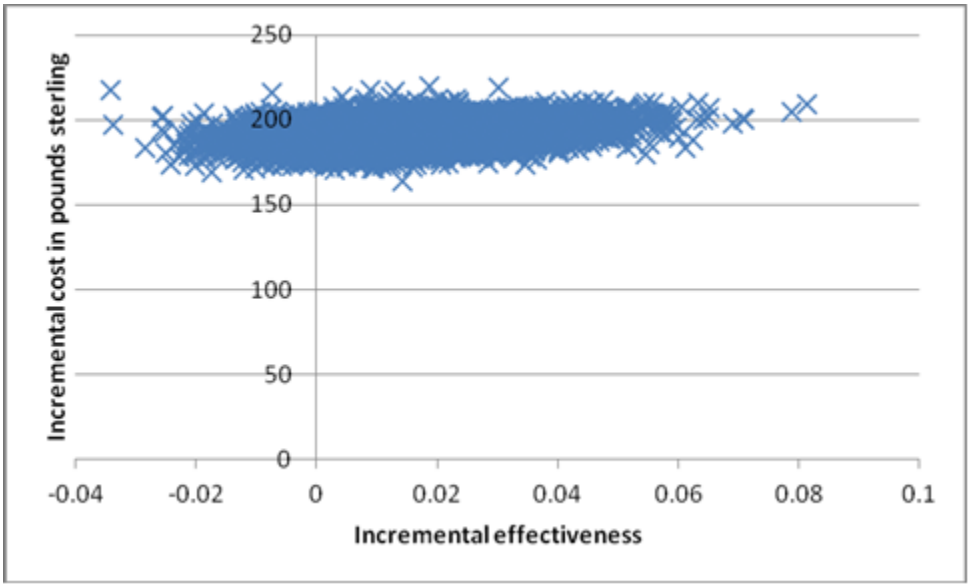
3) Results of probabilistic sensitivity analysis

A sensitivity analysis was performed as previously described using a Monte Carlo simulation. This allows the extent of the uncertainty surrounding the result to be graphically represented. The probabilistic sensitivity analyses are presented for the Birmingham BAC cohort Model 1 (both when VOUS are treated as true positive and false positive findings) (Appendix H). These sensitivity analyses were performed 4 times looking at the probability that CMA is cost effective over 4 different strategies; 1) Karyotyping, 2) QFPCR, 3) QFPCR then FISH for DiGeorge, 4) QFPCR then Karyo then FISH for DiGeorge.

The probability that CMA is positive over karyotyping for Model 1 both when VOUS are treated as true positives and false positives is described and represented as Figures 26-29.

Probabilistic sensitivity analyses are also performed for Models 2 (Figures 30-31) and 3 (Figures 32-33) using systematic review data. Here the probability that CMA is cost-effective over karyotype is also considered.

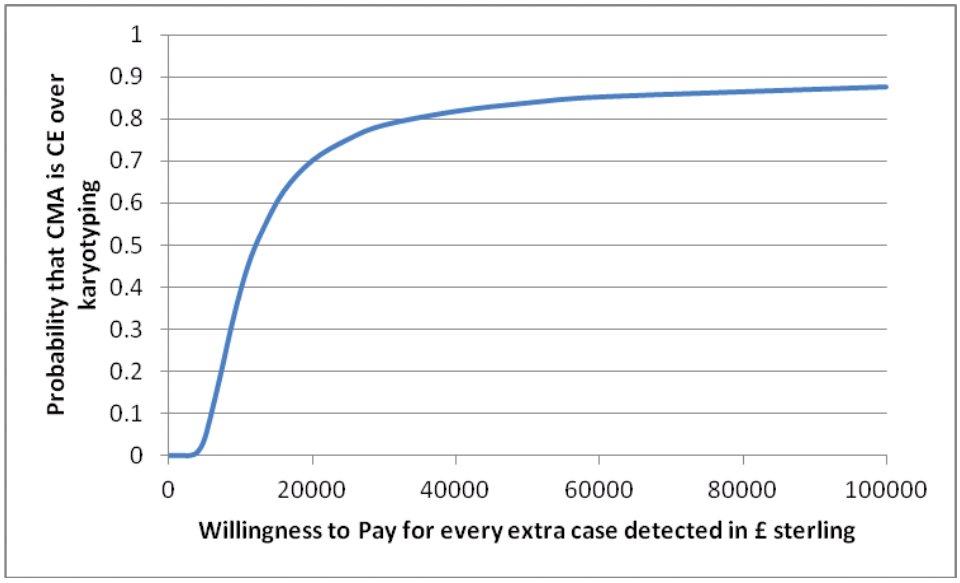
Figure 26 Cost effectiveness scatter plot. Model 1 "Birmingham BAC cohort". VOUS treated as true positives, CMA over karyotyping



Legend: Each point plotted is one sample. The area covered by the plot represents uncertainty.

Interpretation: Here the effectiveness of CMA over karyotyping is mostly positive around an incremental cost of £180.

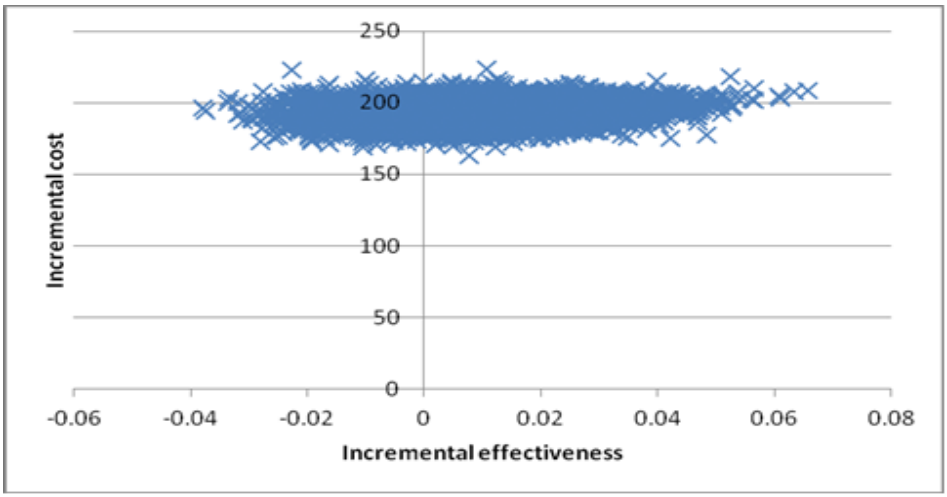
Figure 27 CEAC of CMA over karyotyping "Birmingham BAC cohort" model 1, treating VOUS as true positives



Legend: Cost effectiveness analysis curve showing the probability that CMA is cost effective over karyotyping as the willingness to pay (WTP) for every extra case detected by CMA increases. Here the case rate cost of CMA is £405. There is an 80% probability that CMA is cost effective over karyotyping when the WTP for a positive diagnosis is £40,000 and a 50% probability that it is cost effective when the WTP is £13,000.

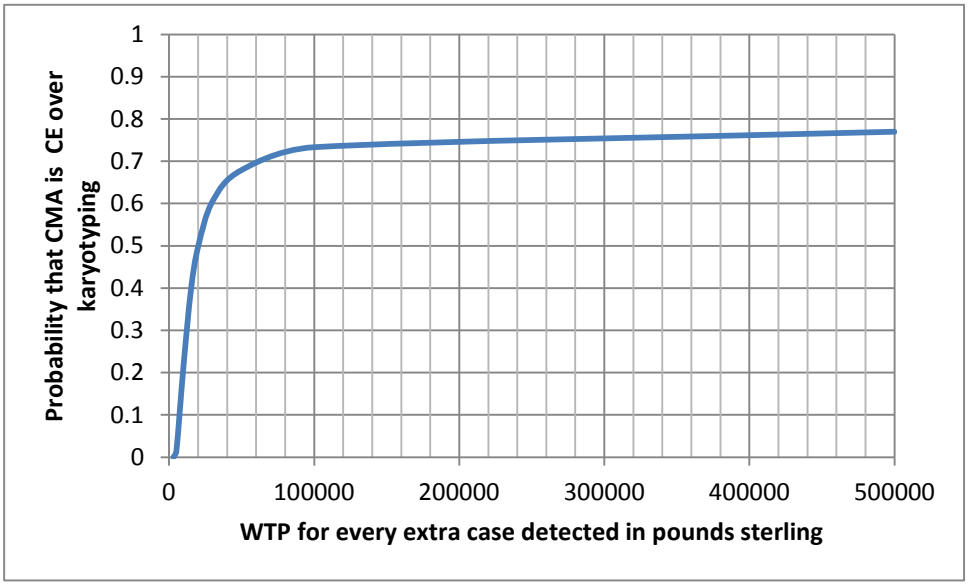
Birmingham BAC cohort Model 1 VOUS treated as false positive:

Figure 28 Cost effectiveness scatter plot. Model 1 "Birmingham BAC cohort" VOUS as false positives CMA over karyotyping



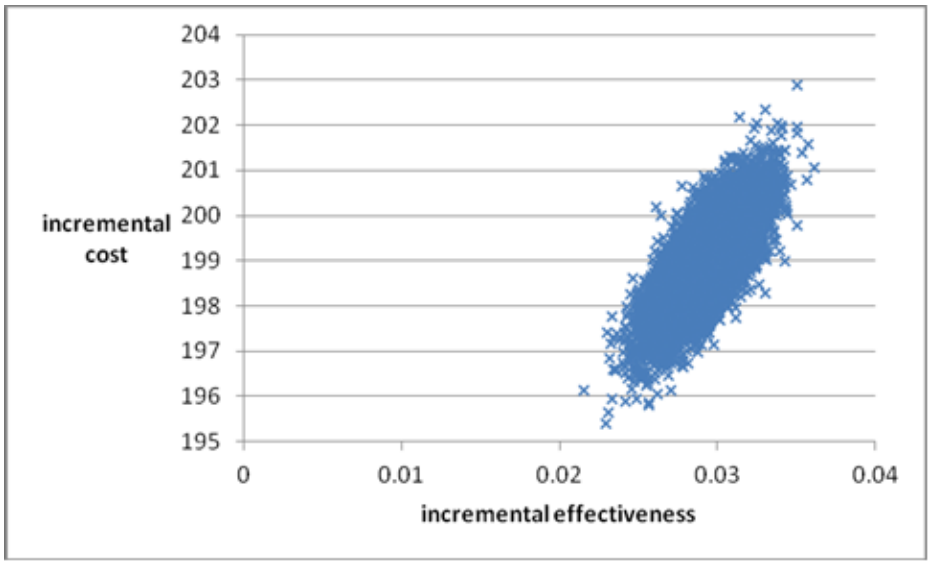
Legend: Here the effectiveness of CMA over karyotyping is mostly positive around an incremental cost of £180

Figure 29 CEAC Model 1 "Birmingham BAC cohort", VOUS as false positives, CMA over karyotyping



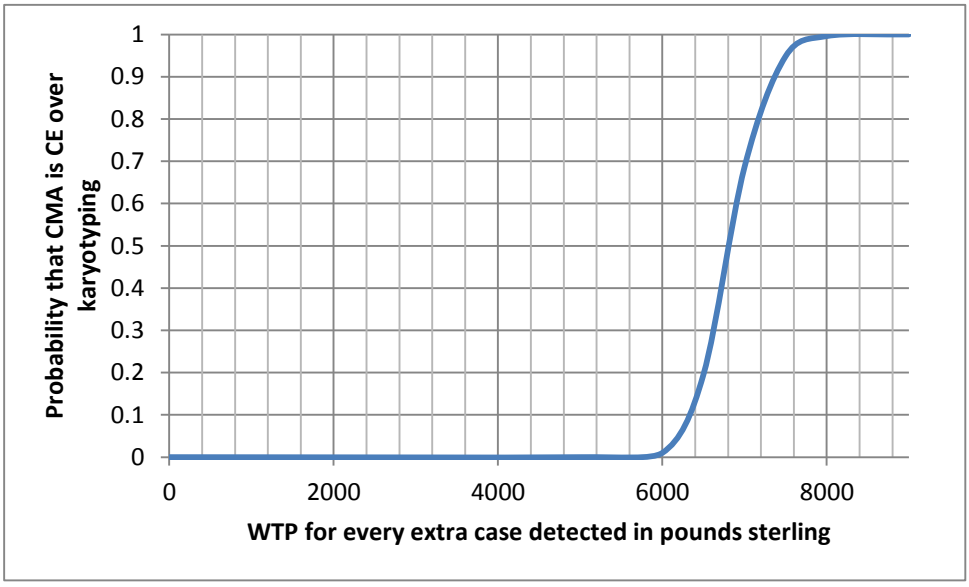
Legend: Cost effectiveness analysis curve showing the probability that CMA is cost effective over karyotyping as the willingness to pay (WTP) for every extra case detected by CMA increases. Here the case rate cost of CMA is £405 but VOUS are treated as false positives. There is a 77% probability that CMA is cost effective when the WTP for a case to be detected is £50,000 and 50% probability that it is cost effective at a WTP of £20,000.

Figure 30 Cost effectiveness scatter plot. Model 2 using systematic review data (all indications for testing) VOUS treated as true positives, CMA over karyotyping



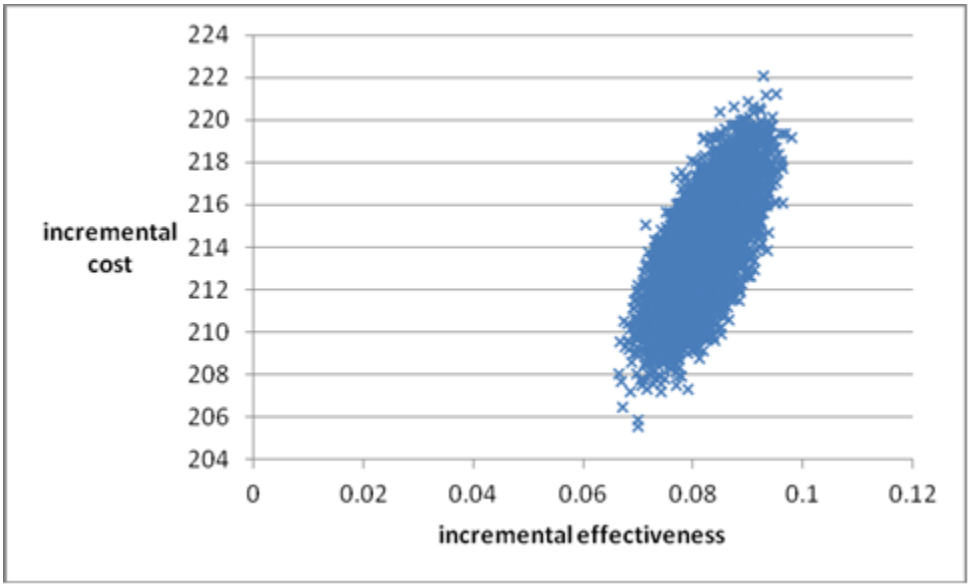
Legend: Here the effectiveness of CMA over karyotyping is always positive around an incremental cost of £199. Note there is no zero on incremental cost scale purposefully to aid interpretation of the plot.

Figure 31 CEAC Model 2 Systematic review data, all indications for testing, treating VOUS as true positives



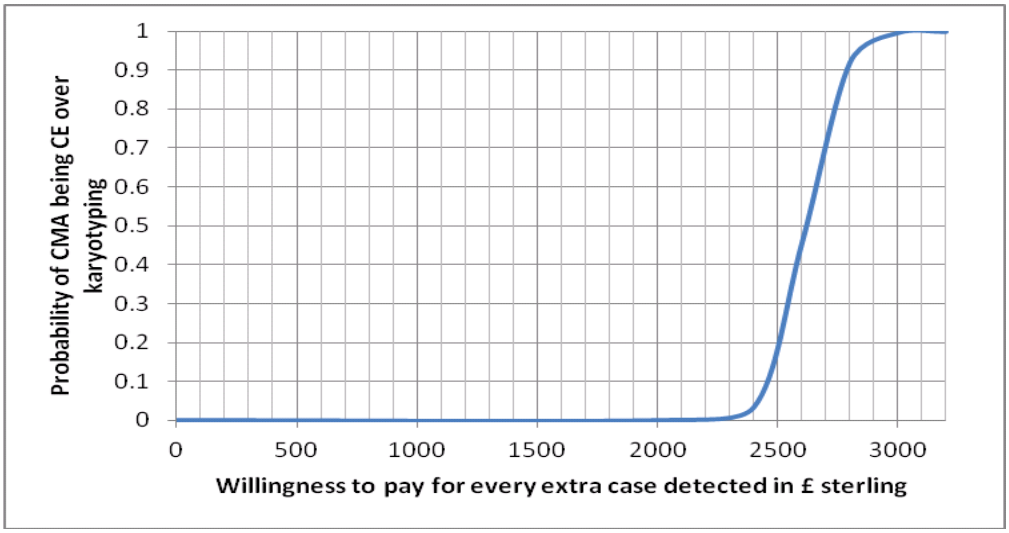
Legend: Cost effectiveness analysis curve showing the probability that CMA is cost effective over karyotyping as the willingness to pay (WTP) for every extra case detected by CMA increases. Here the base rate cost of CMA is £405. This curve is populated by data taken from systematic review analysis when the indication for chromosomal testing is any indication. CMA has a 95% probability of being cost effective over karyotyping when the willingness to pay is £7500.

Figure 32 Cost effectiveness scatter plot. Model 3 systematic review data when testing performed for abnormal ultrasound anomalies, CMA over karyotyping



Legend: Here the effectiveness of CMA over karyotyping is always positive around an incremental cost of £214. Again there is no zero on the incremental cost scale.

Figure 33 CEAC Model 3 systematic review data, testing performed for abnormal ultrasound scan findings, treating VOUS as true positives CMA over karyotyping



Legend: Cost effectiveness analysis curve showing the probability that CMA is cost effective over karyotyping as the willingness to pay (WTP) for every extra case detected by CMA increases. Here the base rate cost of CMA is £405. This curve is populated by data taken from systematic review analysis when the indication for chromosomal testing if an abnormal fetal ultrasound scan finding. CMA has a 95% probability of being cost effective over karyotyping when the willingness to pay is £2830.

Discussion

To summarise, decision modelling was used to examine whether prenatal CMA was a cost effective option, either when referral occurred for any indication (including advanced maternal age, parental anxiety, family history of increased prenatal screening result), or referral was for abnormal findings on ultrasound when compared to karyotyping or other conventional cytogenetic testing (QFPCR for common trisomy, FISH for Di George). This was performed using data from the Birmingham BAC cohort and also using data available from systematically reviewing the literature. These methods produced differing results and the benefits and limitations of these will be discussed.

Treating VOUS as true positives

Data accuracy

When looking at the sensitivity of karyotype this was lower for the systematic review data (models 2= 54.5% and 3= 53.5%) than for the BAC cohort (Model 1 = 89%). This appears to be because the higher detection rate by CMA in the systematic review data (both as a combination of higher resolution arrays and more pathogenic results but also higher VOUS rate) increased the prevalence of chromosomal anomalies and decreased the sensitivity of karyotyping.

CMA had a lower sensitivity when calculated from systematic review data for any indication (model 2 = 86%) compared with both the BAC cohort of abnormal scan

findings (model 1 = 96%) and the systematic review data array performed for abnormal scan findings (model 3 = 96%). This may be because the data used to calculate CMA sensitivity when performed for any indication included samples where there was a known abnormal karyotype that would not be detected by CMA (such as a balanced rearrangement) and this may have artificially lowered the sensitivity of the array. Although balanced rearrangements will exist within the population, these had been selected to test on CMA and the numbers may not be representative of the incidence within the population. Balanced rearrangements are also unlikely to be pathogenic and lead to a phenotypic effect.

ICER treating VOUS as true positives

The ICER was significantly higher for CMA testing using model 1 rather than models 2 or 3. Using model 1 the limited resolution of the Birmingham BAC array (and therefore limited detection rate) and the finding of 4 submicroscopic deletions in the DiGeorge region (detectable by FISH analysis as well as CMA), the ICER of CMA *over* a combination of QFPCR followed by karyotype followed by FISH (for DiGeorge in known fetal cardiac anomalies) was £24,600. This means that it would cost an extra £24,600 for every extra case detected by CMA over the above combination of tests (QFPCR then Karyotype then FISH Di George).

This result is very different to Models 2 and 3 using SR data where the ICER for CMA *over* karyotype is £6,800 for every extra case detected by CMA where there was any

indication for testing and £2,600 for every extra case detected by CMA *over* karyotyping where the indication was abnormal scan finding.

This could be due to the fact that:

1) Models 2 and 3 could not include QFPCR and FISH (for DiGeorge region) data as there was insufficient information available from the published cohorts.

2) VOUS will have increased overall prevalence rates in models 2 and 3 (as higher resolution array were used which will have increased the VOUS rate and artificially lowered the sensitivity of karyotype and over-inflated the sensitivity of CMA).

3) VOUS were given an effectiveness score of 1, the same as pathogenic results. This is because we lack the information on how women view these results and the value that they would afford them. Current qualitative data seems to suggest that parents have mixed feeling about their worth, depending upon whether they are given pre- or postnatally and that prenatally they may be given a limited value, or even a negative value (66;90) (Chapter 5).

Deterministic sensitivity analysis

Treating VOUS as false positive

Data Accuracy

When VOUS are treated as false positive findings karyotype still has a lower sensitivity when compared with CMA. This is more profound in the systematic review models 2 and 3, then the Birmingham BAC model 1. This is because the higher resolution CMA platforms in the systematic review data will increase the prevalence of chromosomal anomalies in the model and decrease the karyotype's sensitivity.

CMA sensitivity was higher for the Birmingham BAC model 1 and the systematic review for abnormal scans model 3 (96%) than the systematic review model for all indications model 2 (84%). This was because model 2 included samples with a known balanced rearrangement on karyotype and therefore none detectable by CMA which would lower the CMA sensitivity.

ICERs treating VOUS as false negatives

CMA alone is dominated by other strategies (more costly but less effective) when VOUS are treated as false positives in models 1 and 2. The only model in which CMA alone featured as an undominated strategy was model 3 (systematic review data testing performed for an abnormal scan finding). Here the ICER for CMA alone over karyotype alone would cost £25,400 for an extra positive diagnosis by CMA. However treating all VOUS as false positives will underestimate the specificity of

CMA as some VOUS will in time be determined as benign but others will be pathogenic.

Threshold analysis

The current base rate cost of CMA in these 3 models was the cost of £405 for a Birmingham BAC array. However the cost of CMA is rapidly decreasing and higher resolution CMA platforms are now being costed at £350 or lower. A threshold analysis was therefore performed to see what would happen to the cost effectiveness of the tests as CMA decreased in price. Using data from the Birmingham BAC cohort model 1 when CMA costs £360 or lower and the willingness to Pay (WTP) for a positive diagnosis is £9768 then CMA is cost effective over karyotyping. Using the same cohort when VOUS are treated as false positive results, then CMA has to cost £302 or less and the WTP for a positive diagnosis has to be £9185 or less for CMA to be cost effective over karyotyping. If the WTP stays below £9768 and the CMA cost stayed above £360 then karyotyping is the test of choice. If the WTP increases but the cost of CMA stays at £360 or lower then QFPCR, followed by Karyotyping (if QFPCR negative), followed by FISH for DiGeorge syndrome (if karyotype negative and cardiac anomaly) becomes the testing strategy of choice.

In comparison when the threshold analysis was performed for systematic review testing for all indications (model 2) when CMA was under £210 it was always the test of choice and at its current cost of £405 or lower if the WTP for a positive diagnosis was at least £6807 then CMA was the test of choice.

Using model 3 (systematic review abnormal scan data) CMA becomes the most cost effective test at or below £210. At its current cost of £405 if the WTP was at least £2613 for a positive test CMA would be the test of choice.

Models 2 and 3 found CMA to be more cost effective at a lower WTP is because:

1) Models 2 and 3 included higher resolution CMA platforms and therefore had a higher detection rate by CMA making it more cost effective.

2) Models 2 and 3 included VOUS as true positives and therefore overestimated the effectiveness of CMA.

3) Models 2 and 3 were not as complex and could not include further testing (QFPCR FISH for DiGeorge syndrome) as the information was not extractable from the published articles.

Probabilistic sensitivity analysis.

Cost Effectiveness Analysis Curves (CEAC)

CEACs were calculated for the base rate cost of £405 for CMA. Using data from the Birmingham BAC Cohort (model 1) there is an 80% probability that CMA is CE over karyotyping when the WTP for a positive diagnosis is £40,000 and 50% probability that it is CE when the WTP is £13,000.

In contrast using systematic review data, when chromosomal testing is performed for any indication there is a 95% probability that CMA is cost effective over karyotyping when the WTP is >£7500 (model 2). When chromosomal testing is performed for abnormal fetal ultrasound (also using systematic review data) there is a 95% probability that CMA is cost effective over karyotyping when the WTP is >£2830 (Model 3).

The reason there is such a difference in CEAC curves for models 1 vs. 2 and 3 is because of the higher resolutions of CMA included in models 2 and 3 and therefore their high detection rates of both pathogenic variants and VOUS. It must be stressed that these curves were produced for the base case cost of £405. When the Birmingham BAC model was reduced to £350 there was an 80% probability that CMA was cost effective when the WTP for a positive diagnosis was £25,000.

Strengths and Limitations

The first model's strength was that only one array platform was used and therefore there was no heterogeneity in the data that would have existed had various arrays been used at differing resolutions. It was primary data taken from a complete data set so I was able to perform complex modelling using various testing strategies. This was in contrast to models 2 and 3 using systematically reviewed data that used heterogeneous CMA Platforms and lacked the information to provide more detailed modelling of tests other than CMA and karyotype.

There were limitations and assumptions made. Firstly, that the outcome was measured as effectiveness in terms of extra cases detected. In economic evaluation this is seen as an intermediate outcome because the final pathway that is followed based on the detection of a positive case is not explored in terms of the additional costs and effects that are likely to be incurred. Yet chromosome testing is done and the purpose of the current evaluation is to explore which is the most cost effective option for detecting an anomaly, so for that end the intermediate outcome is justified. Furthermore, no numerical figure could be placed on the benefit that women and their partners would state for a positive result, particularly if that positive result had some uncertainty attached (such as a VOUS). Also this analysis treated all chromosomal anomalies as equivalent and in reality some may be more severe than others and so the WTP might vary with severity of the chromosomal anomaly detected. Quality Adjusted Life Years (QALYs) are not appropriate as the resulting conditions are heterogeneous. For instance the lifetime social and medical cost for

caring for someone with Down's Syndrome will be higher than a chromosomal anomaly leading to a perinatal lethal condition. Also there is no "treatment" for a positive result at present, the only action that can be taken is to terminate the pregnancy. This is a complex medical problem with many ethical dimensions and it cannot be reduced to an economic argument based on an outcome measure.

Secondly, it is acknowledged that the 1 Mb BAC array is more expensive than other prenatal arrays used. For this reason the analysis includes reduction in the cost of the CMA test which is also included within the sensitivity analysis. I also acknowledge the targeted nature and conservative resolution of the array (and hence its decreased detection rate) but this platform was chosen in the knowledge that the rate of variants of unknown significance (VOUS) would also be significantly decreased. This would therefore not create potentially unnecessary uncertainty/worry for women and their partners.

Conclusion

Prenatal targeted CMA platforms (such as the targeted BAC array) may not presently offer good value for money in terms of cost per case detected. However increasing resolution and decreasing costs of CMA mean that it is likely to become a cost effective option in the future. Before this can be ascertained VOUS must be awarded an effectiveness score. Current qualitative data seems to suggest that parents have mixed feeling about their worth, depending upon whether they are given pre or postnatally and that prenatally they may be given a limited value, or even a negative value (66). If awarded a negative value it may be better to not report them to parents at all.

If VOUS continue to be reported to patients they should continue to be modelled as I have done (as true positive results) but with an adjusted effectiveness score. If an international decision is made to not report VOUS they could be re-modelled as true negative results but with associated parental follow-up costs. Modelling VOUS as false positives (as I have done in my sensitivity analysis) may be inaccurate as many will turn out to be pathogenic findings.

Submitted manuscript from Chapter 6

The following manuscript has been submitted to the Fetal Diagnosis and Therapy and is pending amendments:

Chromosomal microarray for prenatal detection of chromosome anomalies in fetal ultrasound anomalies: an economic evaluation Hillman SC, Barton PM, Roberts TE, Maher ER, McMullan DJ and Kilby MD

CHAPTER 7 THE ETHICS OF PRENATAL MICROARRAY TESTING

Introduction

In this Chapter I will discuss some emerging ethical concerns related to chromosomal microarray testing (CMA) and draw from this discussion some tentative recommendations for guidelines for future practice.

As I have previously discussed, in pregnancy couples seek information regarding their fetus' chromosomes due to; a "high risk" screening result, a family history of genetic conditions, structural abnormalities seen on ultrasound scan, advanced maternal age or even for parental anxiety/reassurance. Prenatal chromosomal testing in some form has been available since the 1960s.

The main ethical argument for providing this testing is that of reproductive autonomy by providing people with an option of termination of pregnancy should a fetal chromosomal anomaly be found. For some they would view this as prevention of any suffering that a child with a chromosomal anomaly may experience, as well as prevention of financial and emotional stress on a family. For those that oppose termination of pregnancy it would still be of benefit if this knowledge could be used to plan for the future, prepare for the birth and tailor medical treatment of the child effectively (110).

Counter ethical arguments lie in the concern that women may be coerced into prenatal testing either by society as a whole, by the medical profession, or by a

patriarchal partner (110-112). There is also an argument that prenatal testing and termination of pregnancy of fetuses with chromosomal disorders may be seen as a way of “dealing” with disability, and that chromosomal disabilities could be viewed as increasingly rare with limited services being distributed to people with these diagnoses. Society may view people with chromosomal anomalies as the ones that were “missed” by prenatal testing. The attempt to eliminate chromosomal anomalies may even be viewed as a form of eugenics (110;111).

Debate has always existed around prenatal testing and how the reproductive autonomy of women can be balanced against any rights that may be afforded to the fetus (111;113). I will, however, leave these mentioned issues aside in favour of highlighting the newer more specific challenges posed by microarray technology.

CMA allows high resolution of analysis, detecting chromosomal differences that would be missed by conventional testing. It also allows thousands, even millions of loci throughout the genome to be analysed simultaneously and is amenable to high throughput. Results may show a pathogenic chromosomal variant known to cause a definite syndrome in the baby. They may, however, also show a Variant of Unknown Clinical Significance (VOUS), where current databases/literature suggests a possible effect in the baby but the findings are still uncertain. Microarrays can also reveal incidental findings such as a susceptibility to adult onset disease. The detail that can be generated by this testing creates ethical concerns that, although not new, will be more commonly encountered with microarray technology. How do we gain informed

consent for a prenatal test that is so detailed it would take an unfeasible amount of time to discuss every possible outcome? Should VOUS be communicated to parents? Do we report incidental findings that may not manifest until adulthood? Are we (further) removing the child's right to an open future?

The technology to detect fetal chromosome abnormalities has left a "therapeutic gap" (110); the information generated can only be used as the basis for a decision about whether or not to terminate a pregnancy rather than to inform options for treating the abnormality detected. Those offering the test therefore have a responsibility to ensure that couples/women are counselled accurately about the possible outcomes of the test prior to consent for testing and that results are conveyed to women clearly and honestly.

Genetic testing in both the prenatal and postnatal setting remains controversial and chromosomal microarray testing shares all of the controversial features of more routine testing. Since the invention of prenatal chromosome analysis in the 1960s women have had the opportunity to have prior knowledge of their fetus' chromosomes.

As described in Chapter 2, at Birmingham Women's Hospital Foundation Trust in the United Kingdom we have been providing microarray testing alongside standard full, conventional karyotype testing under local research ethics approval between 2009-

2012. I interviewed some of the research participants and where appropriate the words of these women or their partners have been used to illustrate my key points (Qualitative work in full is presented in Chapter 5 and Appendix G).

Gaining consent to microarray testing

To give consent, the patient must have an adequate understanding of what the test involves, what the possible benefits, drawbacks and outcomes are and any realistic alternatives (114). Information needs to be tailored to the individuals so that s/he can make a well-informed decision. The patient must have the capacity to understand the information and form a reasoned judgment. This must be done voluntarily without any coercion. Informed consent protects self-determination and rational decision-making (115).

Prenatal microarray testing poses particular challenges for the Health Care Professional (HCP) aiming to gain valid consent for the test. Pregnancy is a highly emotional time for women and their partners in “normal” circumstances. When something in the pregnancy has already caused concern, such as following a “high risk” screening result or an abnormal finding on ultrasound, then counselling for genetic testing becomes even more difficult. Stress may limit the amount of information retained (99).

“I think because in the last few weeks we have been to that many appointments and tried to absorb so much and at the same time trying to come up with a decision, it has been hard to decipher” (Interview 9b)

It is for this reason that women and their partners need to be given opportunities to revisit the idea of testing at subsequent visits if initially unsure, and the opportunity to withdraw from the testing should they change their mind. Of course this indecision regarding the testing cannot be endless given the time frame of pregnancy or even possible in some pregnancies. Likewise once the microarray test has been processed and analysed it should be reported. Women should also be given written information to take home. This written material is a source of reference not only so patients can reflect on what the microarray test is but what their results may show and how these results will be disseminated back to them. It should also contain contact details should the patients have any further questions.

This already stressful situation is further compounded by the biological timetable of pregnancy, the scheduling of testing and the greater clinical and emotional difficulties of late abortion all of which create a sense of time pressure (86).

"Because of the Christmas holidays that was my 24 weeks (twenty four completed weeks is the legal limit for the majority of clauses for the Abortion Act, 1967), if it was really bad (the result) you just have the time factors, there wasn't going to be anytime to have a result and make a decision so you had to think it through probably further than you would have wanted to." (Interview 11a)

The current lack of guidance for prenatal microarray testing may encourage the standard of information given during counselling to fall as its use becomes more widespread. Current guidance from the American College of Obstetrics and Gynaecology (published 2009) and the Italian society of Human genetics (2011) state that in the prenatal context CMA should not be first line and not without conventional karyotyping. Neither give any advice on the reporting of results to patients when the result is of an uncertain nature (48;83). Many obstetricians receive no training in medical genetics beyond that given in medical school, and some qualified before chromosomal microarray technology was available. A lack of understanding on behalf of HCPs will lead to misunderstanding or miscommunication when performing pre-test counselling, leading to a failure to gain truly informed consent.

It can be a challenge to gauge how much information to give, treading a fine line between "information-overload" and too little for the patient to have a realistic notion of what is being consented to. The National Institutes for Health – Department of Energy (NIH-DOE) Task Force on genetic testing by the Ethical, Legal and Social

Implications (ELSI) branch of the Human Genome Project developed guidelines for ensuing quality and appropriate use of genetic tests (116). It contains a large number of recommendations for validation of tests, quality assurance, genetic education, confidentiality and consent. These, however, refer to testing for a single disease and may not readily translate to microarray testing, which tests many regions of the chromosome in parallel (117). It would be impossible to explain in detail all the possible syndromes and diseases that could be detected by the array before the test is performed; predictive testing for the BRCA 1 and BRCA 2 gene mutations (utilised to discuss risk of developing breast cancer) commonly require 1-2 hours of counselling alone for instance (117).

Instead a model of generic consent before the testing could be used with detailed information provided when a positive result is found. Such generic consent would need to include examples of potential findings so that the woman and her partner can put the counselling into context. This approach would be similar to that prior to a physical examination (115) where the patient is unaware of every positive finding that could be discovered but gives consent knowing that the purpose of the examination is to determine potential problems. Larger cohort studies have shown that microarray testing will give a normal or a “negative” result between 88%-98% of the time (52;65;68;74); so hours of explanation prior to testing will ultimately be irrelevant for the majority of patients and may arguably also waste valuable clinical resources. On the other hand, the minority of patients for whom a positive result is found may feel that this approach was paternalistic, or even deceptive, in the hindsight of their positive result.

Pre-test counselling for chromosomal microarray testing however does need to be more detailed than counselling that normally occurs for prenatal cytogenetic testing, and with the average reading age in the UK being that of an educated nine year old a conscious effort needs to be made to keep counselling and literature as simple as possible. Darilek et al suggest that pretest counselling should include a review of chromosomes and genetic structure, a description of the CMA to be used, and the extent of its coverage as well as the spectrum of disorders that can be detected (118). In addition I suggest that pretest counselling should include why parental blood samples are needed (to assess inherited anomalies), the chances of finding an abnormality and examples of abnormalities that can be detected by genome-wide array including:

• Pathogenic chromosomal changes explaining or likely to result in fetal (ultrasound) anomalies

• De novo chromosomal changes of unknown clinical significance (VOUS)

• Inherited chromosomal changes of unknown significance

• Incidental findings-chromosomal variants that are health debilitating and may cause adult onset conditions (99).

Accepting testing

Prenatal screening and testing has become so embedded into the routine of prenatal care that there is an expectation that women will inevitably accept an offer to learn more about the health of their unborn child (66).

HCPs and patients may have different reasons for offering or accepting microarray testing. Hunt et al found that clinicians valued prenatal genetic testing as a means of enabling patients to prepare for an affected birth, or to use information to decide whether to continue the pregnancy. As microarray testing provides more information than standard karyotyping (31) its "detection rate" is higher and HCPs are therefore more likely to offer it. Conversely patients put emphasis on protecting and nurturing their baby: they sought reassurance rather than to isolate a potential problem (85).

"Once I had those tests and if they came back clear I could mark out and put those sorts of things to the back of my mind" (Interview 8a)

"You just want as many tests done as you can to see like if the baby's gonnna be OK" (Interview 9a)

Patients may not be as interested as HCPs in this higher "detection rate" and may prefer fewer results that can be fully interpreted. This mismatch between the HCPs' quest for positive results to provide information versus the patients' quest for a

negative result to provide reassurance presents a potential tension that must be explored during counselling before testing. Accepting microarray testing solely for reassurance, particularly when the test is being performed as a screening tool, should be discouraged as the rationale of testing is to find chromosomal abnormalities. Women and their partners must be left in no doubt that the test may detect a pathogenic finding that will present them with difficult decisions. Furthermore a negative microarray result will not guarantee a baby without a chromosomal/genetic abnormality and there is a possibility that if a child with the negative microarray result subsequently exhibits developmental problems these will be dismissed as behavioural on the erroneous assumption that any genetic element would have been detected by the microarray test.

"Now I definitely know that everything to do with the chromosomes is ok" (Interview 14a)

Microarrays have a limit to their resolution and will still miss some genetic problems - a further piece of information that women need to understand before consenting to testing.

The participants in my study, in all of whose fetuses structural abnormalities had already been found on ultrasound scanning, gave four broadly interpreted reasons for undergoing microarray testing the third of which was they felt they had "no other

choice" but to agree to testing [2]. My cohort was using microarray testing as a diagnostic tool (post-abnormal scan) rather than a screening tool of the kind that might be introduced for patients with advanced maternal age, and their views may not, therefore, represent those of a screening population.

Other studies have similarly found that women and their partners feel they have "no choice" (111) but to accept testing. Women may not be describing coercion from HCPs or family (although this is a possibility) but rather "the possibility that my child could be born with a chromosomal problem that I could have prevented is so unacceptable to me that I have no choice but to accept testing" (111). This was my impression during the qualitative work performed locally.

"Taken into consideration the reason and the risks there wasn't really any alternative choice"

(Interview 4a).

Coercion to accept testing is a possibility and it is not unlikely that as microarray testing becomes widely available parents will feel under obligation to accept testing (119). Refusing prenatal testing may leave parents feeling responsible for knowingly risking bringing a disabled child into the world, where suffering could have been avoided (by terminating the pregnancy) (120). One coping strategy of pregnant

[2] Their other reasons were: i) they were willing accept every test in order to find an explanation for the problems on ultrasound scan; ii) they wanted reassurance that the structural abnormality was not caused by a chromosomal difference (that would worsen the prognosis) ; and, iii) they benevolently wanted to help those in their position the future by participating in the research

women who find themselves in a stressful position is to comply with what they believe is the health professional's recommendations (100).

"We just agreed to everything" (Interview 6a)

It is therefore important that counselling for microarray testing is non-directive so that women and their partners do not feel that accepting or declining testing is conforming to a professional recommendation. We aim to be non-directive when counselling and consenting women for chromosomal testing, but in reality this is extremely difficult to achieve. Prior knowledge of the circumstances surrounding the referral will alter the way that HCPs counsel women. Lippman et al looked at the counselling of women undergoing genetic testing for Down's Syndrome (Trisomy 21) prenatally compared with postnatally. In both situations the information was factually correct but the before birth information was largely negative, focusing on medical complications and physical limitations, where the after birth information tended to be more positive highlighting medical resources (121). Jameton and colleagues found that HCPs often weave in professional and personal biases with what they present to patients (122).

Reporting of variants of unknown significance

Up to 12% of the human genome contains variance (27). Much of this is benign variation in the population, with specific ethnicities having variation throughout the genome. Pathogenic results can be conveyed with a degree of certainty. These occur when the variation causes disruption of genes with known pathogenic significance

causing structural problems for the fetus visible on ultrasound or causing a well-recognized syndrome. There are, though, many areas of the genome where variation is not thought to be benign but the effects of the variation are unknown. These so called “Variants Of Unknown Significance” (VOUS) present probably the biggest dilemma associated with microarrays both for HCPs and patients. They are a particular problem in the prenatal setting because of limited evidence available to us from ultrasound scan. For instance, we are not able to judge from the scan if the child has clinical signs of autism.

There is no national or international guidance on whether VOUS should be communicated to women in the prenatal setting. The recent US National Institute of Child Health and Human development NICHD study involved 4406 participants: only 25% of these had received an abnormal ultrasound scan, microarray testing was conducted in the remainder for advanced maternal age (47%), positive Down syndrome screening (19%) and other reasons including elective request (9%) (65). In this study VOUS were reported back to women and their partners.

In my cohort women were referred for microarray testing due to structural abnormalities seen on fetal ultrasound scan (such as a congenital cardiac anomaly). The pregnancy had therefore already become “abnormal” and microarray testing was performed to find out if a chromosomal anomaly had caused the abnormalities found on the scan so as to further inform the prognosis. In my cohort only one VOUS was defined and reported to the patient. In this case the couple seemed to base their

decisions on the “certain” information - the scan findings - rather than the uncertain array findings and they chose to continue the pregnancy. This one case is insufficient to allow generalisation but some of the women who were given information about VOUS in the much larger NICD study are reported to have considered it “toxic knowledge” (66). There is, of course, a danger that if the parents continue the pregnancy after having been informed of a VOUS, they will experience additional anxiety. The NICHD study found that even after delivery of a healthy baby 50% of these women (n=16) admitted to lingering worries about their child’s development (66).

Although potentially paternalistic, “protecting” patients from uncertain results may not only prevent some of the additional anxiety associated with the uncertainty of those who decide to go to term, it may also prevent them from making a decision to terminate that they may later regret. I make this point not to suggest that those who decide to terminate *should* or *necessarily* will feel regret. Rather, to echo the view of Shuster, suggesting that the quest for a healthy baby may ironically result in never having a baby at all, as CMA testing could lead to increasing numbers of terminations of pregnancies where the fetus actually has uncommon benign chromosomal variation (103). Informing patients, who have an otherwise normal fetus of a VOUS, may change their perception of the pregnancy from being a normal to abnormal one, which is by no means the case. Yet what needs to be stressed here is that what is being withheld is not *information* as such, but is rather *uncertainty*. Accordingly, I suggest that *not* telling potential parents about VOUS is justified. Recent work emerging (66) suggests that this approach may also reflect what women feel. Others

disagree such as McGillvray et al who suggest that if a woman chooses to avoid the birth of a child when confronted by uncertainty, than this is a legitimate exercise of her reproductive autonomy, just as she may choose to terminate a pregnancy because of uncertainty surrounding her social circumstances (123).

It could be argued that there are advantages to parents of having VOUS reported ahead of the child's birth: any potential problems such as learning difficulties may be identified earlier due to the high index of suspicion and access to specialist services and schooling will, therefore, be easier. This argument seems flawed. If the child has a clinical condition it should be detected regardless of previous microarray results, and if the condition warrants it the array could be re-run using updated and more accurate databases. We would therefore try to elicit causes for a condition *when* they exist rather than *before* they exist (as of course they may never exist and a child is inappropriately labelled as having some unknown condition). This issue relates to concerns that children should have an open future, which is discussed further below.

If the decision is made not to tell parents about VOUS this must be very explicit in the pre-test microarray counselling, potentially even going as far as directing women to an online database describing which conditions will be reported and examples of those that will not.

Late onset diseases

The aim of prenatal CMA testing is to detect pathogenic copy number variants that are associated with conditions that have either caused congenital malformations, will lead to a genetic syndrome, or are a large deletion/duplication that will affect many genes causing a phenotype that will affect the child from birth. It is not to find susceptibility to adult onset diseases. As the array is genome-wide it is able to detect variants that predispose the child to conditions in later life. Examples include the BRCA1/2 mutation giving susceptibility to breast and/or ovarian cancer, or a deletion of the gene PMP22 leading to adult onset hereditary neuropathy with liability to pressure palsies. Although relatively rare (1-2 per 1000 (99)), they present an significant ethical concern about disclosure: should we inform woman and their partners about these incidental findings? Are they entitled to this information about their unborn children? These questions maybe even more difficult to answer in the pre vs. postnatal setting.

The answers to these questions depend on how we balance the parental autonomy and the child's potential autonomy or "right" to an open future (124;125). These latter are "rights in trust" for the child until they reach adulthood and can exercise their rights for themselves (124). Knowledge without consent intrudes on the privacy of the adult that this child will become, but more than this is the impact it may have on their future life affecting their ability form relationships, seek employment, and gain health insurance. It may also affect subjects own reproductive decisions. This limitation of the child's open future may be unnecessary if the concern is regarding a condition

that the child, as an adult, never develops (as the chromosomal change may show susceptibility rather than an absolute risk of getting the disease). Most HCPs would not recommend genetic testing for BRCA1/2 gene mutations in a child as it predicts the risk of a condition for which there are no interventions in childhood. There are inconsistencies between restrictions regarding genetic testing in children and testing for the same conditions prenatally (126). Finding this information in a prenatal setting may well therefore impact on their right to an open future. By informing the woman of the fetal chromosomal status we may be inadvertently informing women and their partners' of their own chromosomal status, information which may not be wanted. Where practitioners feel, in contradiction to my argument, that they must disclose this information it should be discussed in pre-test counselling.

On the other hand it has been argued that subjects *are* entitled to information about their children that might limit the child's right to an open future, since as children are always guided by their parents' beliefs and wishes their future can never truly be open (127).

One of the potential solutions to this problem may be a selective filter on the arrays to avoid diagnosing known serious and non-treatable late onset disorders such as Huntington's disease. The filters could then be removed or reset (or tests repeated) in later life at the request of the now adult nee fetus, if new information came to light that would put them at increased risk (such as a parent developing breast cancer).

This would ensure that results are not ‘lost’ but merely withheld until the relevant adult wishes to have them.

Recording of array findings

The child’s open future is also potentially compromised by the way in which the findings of prenatal microarrays are recorded. Results of known pathogenicity will be recorded in the mother’s notes and automatically transferred to the child’s records at birth, thereby recording that the nature and origin of their condition to enable future medical and educational interventions to be appropriately targeted. The much more difficult decision is whether VOUS or susceptibility to adult onset conditions should be transferred. It may be that leaving these findings in the mother’s medical records, and not retesting the baby, and transferring them to the live born child’s records could limit the impact these results have. However this would be of no merit if the parents choose to tell the child. Of course if these findings were not reported to the parents after testing they would not be recorded in either record.

The need for guidance

International guidance is required regarding the reporting of VOUS, adult onset disorders and record keeping as well as the minimum information required in order to consent for prenatal microarrays. Until guidance regarding prenatal arrays is published these issues will continue to be contentious and practice will continue to vary dramatically. If medical and ethical leaders within their professional institutions within this field fail to provide guidance it is likely that standards will be set by courts

of law in reaction to currently prevailing standards and practices applied to difficult cases, which is a suboptimal solution for HCPs and patients.

Conclusion

CMA is quickly becoming part of the prenatal diagnostic process. They ought to be used to gain knowledge of chromosomal differences that are known to have caused problems seen on fetal ultrasound scan, or by finding known chromosomal differences that will cause well recognised problems for the baby. It is not appropriate to use them to provide reassurance and patients should be counselled as such. I feel that results disclosed to patients should only include any pathogenic chromosomal changes and not VOUS. As far as possible prenatal CMA design should avoid areas of adult onset conditions. Appropriate pre-test counselling remains of paramount importance but needs to be tailored to the complexity of the results achievable and be feasible to deliver on a larger scale than is currently the case. This may mean providing detailed generic information rather than information on every possible outcome as would usually precede genetic testing. A clinical geneticist must be involved with any abnormal CMA results in addition to an obstetrician. Guidance on the disclosure of VOUS and incidental findings would be welcome by both HCPs and patients alike.

Recommendations

In relation to consent

- Pre test information must include an explanation about VOUS and make clear that they will not be reported
- Pre test information must make clear that predisposition to adult onset diseases will not be disclosed OR must make clear what the implications for the parents as well as the child will be if they are disclosed.

For reporting

- Abnormal findings should trigger an urgent appointment with a clinical geneticist as well as an obstetrician
- VOUS revealed by prenatal microarray testing should not be reported to women and their partners
- Filters should be set as far as possible to minimise the findings of adult onset conditions
- Reports of known pathogenicity (i.e. recognised syndromes) should be transferred from the mother's notes to the child's notes; all other reports should stay in the mother's records.

CHAPTER 8 DISCUSSION OF PRENATAL MICROARRAY TESTING AND THE FUTURE OF PRENATAL DIAGNOSTICS

Evidence for prenatal CMA testing presented within the thesis

There is increasing evidence in postnatal and prenatal cohorts that CMA will increase the detection rates of pathogenic microscopic chromosomal anomalies that will adversely affect children's outcome, including long-term morbidity.

This research proposal set out to define the detection rate of pathogenic CNVs using a 'relatively conservative', targeted CMA platform over conventional, full G-band karyotyping when there was a structural anomaly present on fetal ultrasound scan. These data defined this in a prospective cohort as 4.1% using the 1Mb BAC array (Bluegnome Cambridge, UK) but I indicated the limitations of this platform in that it was prospectively chosen to target disease regions known to be associated with congenital structural anomalies. The1Mb targeted BAC array was purposefully not analysing regions/genes known to cause adult onset diseases and areas of the genome where little is known about the pathogenic nature of a variance. This strategy was outlined to limit the detection of VOUS and indeed in the prospective cohort of 243 samples, I only encountered one difficult counselling situation because of such an association. In 2009, when the project was initiated, there was no national or international guidance as to how (or if) VOUS should be reported to women and their partners. However, there is emerging evidence from qualitative work in the prenatal period (66) that giving uncertain information is viewed negatively by parents, leading to increased anxiety in pregnancy that extends after the birth of the child.

I have acknowledged in Chapter 2 that other published prenatal studies using the same 1Mb BAC array noted higher pathogenic detection rates over G-band karyotyping (51;52), but that these studies had lower chromosomal detection rates using conventional G-band karyotyping. At our Regional cytogenetic laboratories we have high quality assurance and good quality prenatal G-band preparations that would have would have lowered the comparative detection rate of CMA. My prospective dataset confirmed an increased detection rate of fetal chromosomal anomalies by CMA within the UK when a structural anomaly is identified on ultrasound scan.

Critical appraisal of the published literature for cohorts between 2011-2012 are examined and meta-analysed (Chapter 4) (using a variety of CMA platforms). The excess CMA detection rate over full karyotyping is 7.2% (95%C 5.4 - 9.8). This figure does include the reporting of VOUS (which may account for up to 2.1% of the overall figure). It is therefore likely from the literature that prenatal diagnosis using CMA would note 5% more information of certain pathogenicity that would be reportable to parents and potentially adversely affect fetal outcome. It is possible therefore that the 1 Mb BAC targeted array used may not have such a decreased detection rate compared with other platforms and in addition would eliminate much uncertainty at the analysis/counselling stage of testing due to its avoidance of VOUS.

The second aim of this thesis was to perform a relatively high resolution CMA (60K oligonucleotide array, the International Standard for Cytogenomic Array (ISCA)

design v2.0) in a small sub-set of the prenatal cohort (performed after birth) to compare this to the BAC array and to compare the detection rates and also VOUS rates. Using this 60K array my data noted an extra 4.8% (3 out of 62) pathogenic CNVs but, in addition, a 13% (8 out of 62) VOUS rate. Thus, utilising a prenatal CMA platform of increasing resolution is likely to increase pathogenic CNV detection in babies with congenital malformations but also increase the VOUS rate significantly.

Within Chapter 4, I demonstrated evidence that an increase in CMA resolution results in an increase in VOUS rate. I conclude that when prenatal array testing is undertaken and in the absence of national/international guidance, particularly as CMA resolutions increase, a decision should be made prospectively as to the reporting of VOUS to patients locally (in collaboration with clinical genetic support). Alternatively international consensus may indicate that not to report VOUS may be the preferred option. I do not believe that giving parents a choice about reporting of VOUS prior to testing is wise, as many will not comprehend or have full insight into the added anxiety that uncertain knowledge (in the presence or absence of a fetal structural anomaly) will give them and that their feelings in retrospect may change (66).

The application of new technologies has a financial impact upon all health economies. However, in the United Kingdom, the National Health Service (NHS) usually only implements new technologies after independent review by agency such as the National Institute of Clinical Excellence (NICE). One of my chapters (Chapter

6) presented the results of a formal, objective health economic analysis to examine the added costs per additional case detected by CMA over the present prenatal karyotype regimen. When the health economic model was populated using data from the Birmingham BAC cohort study (model 1), this analysis indicated that when CMA is costed at £360 or lower (per test) and the willingness to Pay (WTP) for a "positive diagnosis" is £9768; the new technology of CMA is cost effective over full, conventional karyotyping. In this analysis, I used the targeted BAC array, the present cost being £405 (per test). However CMA technology is becoming more widely introduced leading to decreasing cost of the platforms. This effect is compounded by an increase in the number of samples it is possible to analyse on a single slide and increased automation. Current, contemporary oligonucleotide arrays are available at less than £340 per sample. CMA analysis is currently still a skilled process, as intelligent decisions relating to the use of 'software' to identify and classify CNVs are required, and consideration of the detected CNVs in relation to the associated phenotype is important. However in the future analysis will be a much speedier process involving less reliance on the analyst and more on complete databases and intuitive software.

One cost that was not included was the cost of retraining clinical staff (obstetricians, paediatricians and clinical geneticists) in genetic technologies that have developed since the onset of their careers. This will need to be undertaken to ensure thorough and correct counselling of women and their families both when consenting for testing and reporting of results. Studies such as those described are helping to inform a generation of clinicians through peer review publications and presentations at clinical

conferences. It is vitally important that clinicians are the advocates of patients as new technologies become available and it is their responsibility to ensure they understand and counsel accurately about a test they offer. Clinicians are in a privileged position to ensure that as prenatal diagnostic testing becomes available it is offered in the interest of patients and not merely because it can be done.

The ‘impact’ upon new prenatal diagnostic technologies on the individual patient is potentially high and qualitative research will go some way to answering the question “How do women and indeed couples, feel about the use of CMA testing?” In this qualitative analysis, most couples sampled were positive about the new CMA testing and keen for as much information as possible from the fetal DNA sample that was taken by prenatal testing, so as to inform ‘choices’ relating to their unborn baby with a congenital malformation. Many expressed that a “quick result” was important in pregnancy and this is an advantage the CMA has over conventional G-band karyotyping. Analysis raised the unanticipated finding that some women choose to have CMA testing for “reassurance”. The data though presented in this thesis (and indeed in the literature) indicates that such feeling should probably be discouraged as the reason for testing is to try and find a chromosomal cause for the abnormalities seen on scan and there is the potential to identify ‘anomalies of unknown significance’ (VOUS). Women and their partners also highlighted that they struggled to recall and retain information prospectively delivered by healthcare professionals at the time of the prenatal test. This is not a unique point to prenatal CMA testing (as indeed it applies to all prenatal diagnosis) but reinforces that as testing options get more complicated it is becoming increasingly important to counsel women and their

partners on more than one occasion and make sure they have written information to take home. Signposting to relevant websites rather than the patient “becoming scared” with inaccurate or irrelevant web material was also a highlighted point from the qualitative work. These qualitative studies will aid our professions to understand how women and their partners felt about VOUS. In one case vignette, one couple struggled with the uncertainty of such information and fortunately, after further detailed analysis, the identified CNV was rare but no gene involvement was identified. The conservative nature of the BAC array used in my prospective case cohort study meant that only a single true VOUS was identified. However, other studies that revealed VOUS, such as the NIH-funded prospective study (65), performed qualitative interviews with patients who underwent prenatal diagnosis using a higher resolution 44K array where more VOUS were identified. The qualitative theme was identified that the information became “toxic knowledge” and this ‘uncertain information’ had a negative value causing them anxiety(66).

There are potential ethical dilemmas related to the introduction of prenatal array testing and this was examined in one of my chapters (Chapter 7). Working with Dr Heather Draper, in the University Department of Medical Ethics, this work has made potential recommendations for practice. These include the recommendation that “Pre-test information” must include counselling about potentially unexplainable information (i.e. VOUS) and make the case for the consideration of not reporting such information. Pre-test information should be clear and document if a predisposition to adult onset diseases (by the application of predefined filters) will be

disclosed and must make clear the potential implications this information has for the parents as well as the child.

Recommendations also include that an “abnormal findings” should trigger an urgent appointment with a multidisciplinary team of individuals, including a clinical geneticist.

The future

I have examined in my PhD thesis the potential for the introduction of CMA into prenatal practice when a congenital, fetal anomaly is identified using ultrasound scanning in pregnancy. However prenatal diagnosis is a rapidly evolving speciality and already ‘newer technologies’ such as whole genome and exome sequencing are available for use. So where does the future lie for such technologies in prenatal diagnosis?

Next generation sequencing

Until relatively recently the overwhelming majority of DNA sequencing was performed on a version of Sanger sequencing (128;129). However in the past 3 years exome and genome sequencing have rapidly matured. Several countries are starting to use exome sequencing in paediatric and adult clinical practice when there is a high chance of a monogenic disorder. An example would be for the investigation of a child with unexplained developmental morbidity (130). In the UK, the Health

Innovation Challenge Fund has supported the “Deciphering Developmental Disorders” (DDD) project which is recruiting in a multicentre case-cohort to attempt to identify diagnoses for children with development disorders (www.ddduk.org/). The first 288 trios of which have had a diagnostic rate of 15-30%, depending on whether CMA had been previously performed. However in the last few months, a single case cohort has been published indicating the use of whole genome sequencing in prenatal diagnosis(131). By comparison our group at Birmingham with the Sanger Institute Cambridge have performed a relatively large and comprehensive preliminary analysis of thirty prenatal cases with congenital anomalies that were included in the Birmingham BAC cohort. Parents gave informed consent (and their DNA) to have exome sequencing performed in fetal ‘trios’. The analysis and interpretation of results took many weeks and they were not available for discussion within the time frame of the pregnancy. However, any results of potential pathogenicity that were considered to be causative to the findings of the ultrasound scan or post mortem (if applicable) will be relayed to families.

Thirty fetal “trios” (fetal samples, maternal and paternal samples) were sent to the Wellcome Trust Sanger Institute (WTSI) in Cambridge in a collaboration with Dr M. Hurles. Of these, 27 ‘trios’ were processed and 3 failed initial quality control criteria for DNA. The staff at the WTSI analysed the results and observed a spectrum of pathogenicity. The analysis concentrated on *de novo* mutations, autosomal recessive and X linked inheritance models. In two cases the exome sequencing found genes were disrupted causal to the phenotype. The first was in a male fetus with scan findings of skeletal dysplasia where a *de novo* mutation of the gene *FGFR3* was

found (132). The second was in a fetus with increased nuchal translucency and severely abnormal hands and feet in which a *de novo* mutation of the gene *COL2A1* was found (133). In 8 cases the genes that were found had potential pathogenicity but without further studies (such as modelling in zebrafish or mice) information surrounding causality is uncertain. In one cases a potential pathogenic mutation in *SMARCC1* was flagged up but on follow up with the mother the child (who *in utero* had a hydrothorax) was asymptomatic and had normal development aged 2 ½ years. This may represent a false positive. Finally, in 17 cases nothing was found by the exome sequencing.

This small pilot study is proof of principle for the use of exome sequencing in prenatal diagnosis and indicates that some causative candidate genes may be identified but also highlights the identification of 'candidate genes of unknown significance'. A larger prospective study will be needed in order to answer questions such as 1) whether the average amount of DNA acquired from an invasive sample would be enough for sequencing or if culturing would be required, 2) whether exome sequencing has the potential to be performed within a realistic timeframe of a pregnancy (i.e. 4 weeks), 3) how much certain causative information it could provide, and how much uncertain information (akin to the VOUS elicited by CMA) it would detect 4) how often adult onset disorders would be uncovered and, of course, the ethical implications of sequencing in the prenatal period. To address these issues a collaborative grant between research groups at the University of Birmingham, Birmingham Women's Foundation Trust, the WTSI and Great Ormond Street Hospital has been submitted (and shortlisted) to the Health Innovation Challenge Fund for

funding to exome sequence 1000 fetal samples (with maternal and paternal samples).

Non invasive prenatal diagnosis (NIPD)

The goal in prenatal diagnosis is to provide the maximum amount of accurate information causing the least amount of risk to the mother and her unborn baby. To this effect NIPD is an ultimate goal. In 1997, cell-free fetal DNA (cffDNA) was discovered in maternal plasma (134). This opened up the possibility of non-invasive testing of fetal DNA, eliminating the risk of miscarriage (~1%) associated with invasive testing. Since then clinical applications have included fetal sex determination (135), fetal rhesus status in mothers with a history of haemolytic disease of the newborn (136) and, in a very few, single gene disorders (137). Fetal DNA accounts for just 3-10% of the total free DNA the vast majority being maternal. The proportion of cffDNA increases with gestation and disappears after delivery.

This approach has rapidly been introduced into clinical practice in the US and Asia for the non-invasive prenatal testing (NIPT) of aneuploidies and is also used in the UK for some single gene testing, e.g. for achondroplasia(138). However robust genome-wide detection of all types of causal variant has proven challenging, especially for *de novo* single nucleotide variants and indels (insertions and deletions) (139). Nevertheless it is possible that, within the next 5-10 years, exome sequencing of the fetal genome will be achievable through none invasive means.

Conclusion

I have shown that CMA is a robust, accurate tool within prenatal diagnostics, with a quick turnaround time. The technology can be implemented within the NHS and will lead to a higher rate of diagnoses due to chromosome abnormalities within the prenatal setting. I have shown that prenatally its main advantage lies in detection of chromosome anomalies when a structural difference is found on prenatal ultrasound scan. I have highlighted the potential problems caused by the discovery of variants of unknown significance (VOUS) and that I believe these variants should not be reported to women and their partners. As the cost of CMA is currently decreasing it is likely to present a cost effective option for prenatal chromosome testing and provide the necessary “bridging” technology between traditional G-band karyotyping and sequencing.

APPENDICES

Appendix A Original Proposed protocol

Structural chromosomal anomalies leading to chromosomal copy number imbalance are often associated with perinatal mortality and morbidity. It is well described that congenital structural malformations are associated with chromosomal anomalies. The more structural abnormalities that exist in a fetus, the greater the risk of chromosome abnormality. Congenital structural malformations are associated with a significant risk of aneuploidy but our group has also described an association with subtle chromosomal rearrangements, more likely to be detected when fetal lymphocytes are obtained by cordocentesis for cytogenetic evaluation.

Clinical cytogenetics is being transformed by widespread implementation of array comparative genomic hybridisation (array CGH). This technique represents arguably the most powerful direct application of the human genome mapping project in medicine today, both in terms of design of microarrays to interrogate the human genome in one assay (at previously unthinkable resolution) and in terms of establishing very accurate phenotype-genotype correlations from the results obtained.

Over the last 10 years, our multidisciplinary group has published numerous large cohort studies investigating congenital heart disease and prognosis appears worse in those babies who are prenatally diagnosed. This appears to be because of associated anomalies and in particular, chromosomal anomalies; a ‘marker’ significantly worsening prognosis and increasing associated morbidity.

We wish to investigate prospectively a cohort of fetuses with a prenatally detected structural abnormality to examine the association with subtle chromosomal anomalies identified using microarrays. We wish to see if this is so using microarray studies. The primary research question is:

"Will the addition of microarray testing in prenatal chromosomal assessment of fetuses with structural malformations give clinically relevant information (in terms of diagnostic and prognostic information) in a timeframe and format acceptable to patients and health care providers?"

The aims of the three year research proposal are:

To determine the prenatal detection of significant chromosomal abnormalities associated with prenatally diagnosed structural malformations using Affymetrix 60K oligonucleotide array

2) To evaluate the health economics of prenatal diagnosis using microarray analysis in the above cohort of fetuses.

3) To assess the acceptability of the test to the patients both pre- and postnatally.

4) To develop a care pathway for the application of microarray.

5) If novel genes are suspected from results of array testing to perform further analysis in the form of sequencing to discover unique genes.

The project has evolved from the longstanding collaboration between Mrs Davison and Professor Kilby on studies of cytogenetic anomaly associated with congenital malformations in the fetus. That collaboration is in turn based upon the extensive

clinical and research expertise of Professor Kilby in the area of fetal growth and malformations syndromes, together with ready availability of clinical cases through the tertiary referral activity of the Fetal Medicine and Genetic service at the Birmingham Women's NHS Foundation Trust.

The proposed research will be a prospective cohort study. The study will consist of ten phases in order to achieve the above aims:

1) Women who have been found to have a fetus with an anomaly at their routine 12 or 20 week scan will be referred to Birmingham women's hospital/ City Hospital /University hospital Coventry and Warwickshire. The anomalies include:

a. Central nervous system (brain) anomalies i.e. hydrocephalus, holoprosencephaly, cerebellum anomalies and agenesis of the corpus callosus).

b. Diaphragmatic hernia.

c. All cardiac disease.

d. All anterior abdominal wall defects.

e. Urinary tract anomalies (lower urinary tract obstruction, severe upper urinary tract obstruction).

f. Nuchal fold/translucency over 3.5mm/ cystic hygromata

g. fetal hydrops

h. severe skeletal dysplasia

i. multiple soft markers (i.e. at least 3)

After confirmation of the anomaly (through re-scan) patients will be counselled as they would be routinely regarding the significance of the anomaly and the effect this may have on the baby once born. It will be explained that some structural anomalies are associated with chromosomal problems and that these babies have a poorer prognosis. The patients will then be counselled regarding invasive testing for chromosomal testing and the risks associated with this. Patients will be offered karyotyping (as routine) and also microarray analysis. They will also be offered and consented to genetic sequencing on umbilical cord blood at delivery. This will be included in the consent form but patients will be able to opt for microarray testing prenatally and decline genetic sequencing after delivery.

Full genetic counselling from a fetal medicine specialist (trained in counselling for genetic tests) will take place prior to taking informed consent. This will include information about basic genetics, the sort of problems that may be detected, the accuracy/limits of the test, the possibility of detecting a genetic “difference “ of unknown significance, and the possibility of detecting susceptibility to a disease that we are not routinely looking for. The implications not just for the baby but also the parents and wider family will also be discussed. Genetic counselling will be given not just to women who are having the test performed but also for their partners. Sample acquisition and failure rates due to inability to culture cells (although not such a problem with microarray technology) will also be routinely discussed.

Patients will then be given a patient information sheet. Patients will be consented prior to the testing by the members of the fetal medicine team as stated above. The patients will be informed that in order to analyse the significance of some chromosomal aberrations it may be necessary to also run the microarray testing on

parental blood samples. Parental samples will be taken (EDTA and Lithium heparin from both). These are sent along with the fetal sample with a form requesting karyotyping and array analysis (“blue” sticker).

Patients that opt for genetic sequencing on cord blood at delivery will be given a “pack” to bring with them at time of delivery, this will include; a letter for the midwife looking after them in labour explaining the request for cord blood, two blood bottles (lithium heparin and EDTA) and a form with a “green” sticker on it for the West Midlands genetics laboratory. Also a “pink” sticker is included in the patients hand held record to inform health care professionals that the patient is to cord blood taken at delivery.

Results from the karyotyping will be fed back by telephone. Results from the microarray testing will be fed back to the patient within 14 days again by telephone and if abnormalities are found a consultation will be organised. A standard 60K oligonucleotide array (currently becoming standard use for prenatal samples) will be used.

2) The results will be fed back to patients within 14 days.

3) When the parents have received the results of all the tests (including genetic tests) in combination with full counselling from a fetal medicine specialist they may opt to stop the current pregnancy. If patients decide to terminate the pregnancy they will be asked to consider genetic sequencing. Full genetic counselling as described earlier will take place. Results for this will not routinely be fed back to patients and are for research purposes.

There will be a time period of at least 24 hours to one week before they have an appointment for the termination procedure and consent for sequencing will be taken at this time by the above members of the fetal medicine team. A fetal blood sample will be acquired at the time of termination of pregnancy (acquiring a blood sample at this time is currently standard practice) if a patient has fetocide. If patients have a medical termination of pregnancy (not requiring fetocide) the sequencing will be performed on a placental sample. A letter will be given to the patient to give to the nursing staff on the ward as well as a universal container and a cytogenetics form with a “green “ sticker on it so that a placental sample can be sent to the west midlands genetics laboratory.

4) At a follow up appointment patients will be invited to take part in a semi-structured interview to establish parental views on the array testing.

5) For those pregnancies that continue women will have fetal cord blood taken at the time of delivery (either at normal delivery or at caesarean). This blood will be used for either high resolution CMA and/or exome sequencing. This consent will be taken as part of the initial consent form. If cord blood is required for a standard clinical test then the standard test will take priority. Results of this testing will not routinely be fed back to patients and are for research purposes.

6) If the baby is born alive, consent will be sort to examine the medical records of the baby. If the patients opt for a termination or the baby is stillborn, post-mortem examination will be discussed.

7) Semi structured interviews will also take place postnatally if the infants is born alive to determine whether their views on the testing are altered by the birth of their baby.

8) If found to be cost effective and acceptable to patients a care pathway will be developed for the introduction of microarray analysis in the above cohort of patients.

9) Dissemination of results nationally and internationally through the use of peer review scientific publications, guidelines and conferences.

Appendix D Laboratory Methods for the 1Mb targeted BAC array (Blue Gnome Cambridge)

Typically >10mg of CVS or >12mls of amniotic fluid were required to perform CMA.

DNA was extracted using QIAGEN EZ1 DNA extraction as per manufacture's protocol.

Quality Control

DNA was the quality controlled (QC). If the sample was from tissue QC on a 1% agarose gel was performed in addition to the method described. DNA was incubated on a hot block at 37^{o}c for 30 minutes to equilibrate. DNA was then vortex and spun and 1.5 µl was transferred onto the Nanodrop spectrophotometer to give 260/280 (<2.2 and >1.8 ideally) and 260/230 (<2.2 and >1.5 ideally) ratios. Concentration of DNA is also estimated and ideally should be ≥38ng/µl.

DNA clean up procedure

If DNA concentrations or ratios are particularly low/high then DNA clean up procedures can be applied. Using the information generated by the nanodrop spectrophotometer the volume of DNA required to give 10,000ng of DNA is calculated. Samples are topped up to 100µl with sterile water. 10µl of cold 3M sodium acetate and 200 µl of 100% ethanol are added. Tubes are inverted and stored at -80^{o}c for 30 minutes. Samples are then centrifuged at 4^{o}C for 30 minutes. Samples are examined for a pellet and the supernatant removed. 500µl of cold 70% ethanol are added and tubes inverted. Samples are centrifuged for 15 minutes again at 4^{o}C. The samples are then once again examined to identify the pellet and the

supernatant removed. Tubes are left open for a maximum of 5 minutes to let the remaining ethanol dissolve. DNA pellet are dissolved in TE buffer, the volume depending on the volume of DNA precipitated. Tubes are sealed and placed on a 37^{o}C hot block overnight. The following day samples are vortexed and spun and then re-tested on the nanodrop spectrophotometer according to the method described above.

BlueGnome constitutional BAC array procedure

Random prime labelling

Test DNA is thawed and briefly centrifuged as is the reference DNA. 400ng of DNA is transferred and topped up to 23µl with water. Labelling "master mixes" (Cyanine; Cy3 and Cy5) are prepared. The volume required for 1 reaction is 10µl of reaction primer, 10µl or random primer, 5µl of deoxycytidine triphosphate **(**dCTP) mix and 1 µl of Cy3 or Cy5 dCTP dye (all reagents are provided by the company Bluegnome). 26µl of labelling master mix is then transferred to the corresponding DNA sample, Cy3 to fetal test DNA and Cy5 to reference DNA. Each tube is mixed and denatured on a hot block at 94^{o}C for 5 minutes and then transferred immediately to ice for 5 minutes. Tubes and pulse spun and 1µl of klenow enzyme (formed by the treatment of *Escherichia coli* DNA polymerase) is added to each tube before being spun again and added to a hot block overnight at 37^{o}C. The reaction is stopped the next morning by adding 5µl of ethylenediamine tetraacetic acid **(**EDTA) stop buffer. Samples are mixed and spun. An Autoseq G50 gel column is then prepared for each sample as per manufacturer's guidance. Each labelling reaction is then added carefully to the

gel and are centrifuged at room temperature for 1 minute. Gel columns are then discarded and the tube sealed vortexed and spun. The sample is then tested on the Nanodrop spectrophotometer as described above but successful labelling reactions should meet the criteria: Concentration >150ng/µl, pmol dye/µl >3. Each labelled fetal test DNA is then combined with its corresponding labelled reference DNA. 25µl of COT1 DNA (used to block repetitive elements to prevent non-specific hybridisation) are added to each sample. For precipitation 12.5µl of 3M sodium acetate are added followed by 312µl of 100% ethanol and inverted. They are then stored at -80^{o}C for 2 hours. Samples are then centrifuged at full speed (~14,000rpm (equivalent to ~18,000 rcf/xg) for 30 minutes at 4^{o}C.

Array hybridisation

Samples are centrifuged at full speed and 4^{o}C for 10 minutes. Supernatant is disguarded and 312 µl of 70% ethanol are added inverted and centrifuged again at 4^{o}C for 5-10 minutes. The supernatant is decanted and disguarded and excess ethanol is removed with the tube left open for no more than 5 minutes for the remaining ethanol to evaporate. The sample is then re-suspended in 21µl of 15% denaturation Solution hybridisation solution (the solution is pre warmed in a 75^{o}C water bath). Tubes are sealed and placed in the 75^{o}C water bath for 5-10 minutes until the pellet is completely dissolved and allowed to denature for a further 10 minutes in the water bath. Samples are then spun for 20 seconds and allowed to cool to room temperature. 18µl of labelled DNA is then transferred to the cover slip of the microarray slide and the array slide is then lowered onto the cover slip. The slide is

then placed into a chamber and the chamber incubated in the hybridisation oven at 47^{o}C for 16-21 hours. Four separate wash steps are then prepared and the slide is washed in sequence as follows; wash 1 2x saline-sodium citrate (SSC)(/0.05% at room temperature for 15 minutes, Wash 2 1xSSC at room temperature for 15 minutes, Wash 3 0.1SSC at 60^{o}C (hybex) for 5 minutes. Wash 4 0.1%SSC at room temperature for 1 minute. The slides are then transferred to the mini slide centrifuge and spun for 20 seconds to dry. The slide is then removed a plastic slide holder is applied. Slides are then transferred to the scanner (Agilent scanner, model G2939A). After scanning raw TIFF images are transferred to Bluefuse multi software.

Quality Control of array Image

To get an impression of array quality and exclude mosaicism standard devistion of the autosomes should be between 0.03 and 0.07, percentage BAC inclusion should be >95%.

Appendix E Laboratory Methods for the higher resolution 60K cytochip oligonucleotide array (Bluegnome Cambridge)

The CMA used was the 8x60K cytochip oligonucleotide (Bluegnome Cambridge).

Diluting

DNA samples are incubated on a hot block for 30minutes set at 37^{o}C to equilibrate DNA before dilution. 0.5ml microtubes are labelled for each sample and for each reference. Each genomic DNA and corresponding reference DNA is then diluted using sterile water for injection (equivalent to 0.4-0.5µg of DNA in a total volume of 13µl).

Labelling

DNA and references samples are vortexed and pulse spun. All labelling reagents (provided by Bluegnome) are thawed on ice, with exception of the klenow enzyme kept in a -20 ^{o}C freezer until required. 2.5µl of random primers are then added to each reaction tube. The contents of each reaction tube are denatured at 95^{o}C for 10minutes and then transferred immediately to ice for 5 minutes.

On ice “master mixes” of Cy3 and Cy5 are prepared (42 µl of 5x buffer, 21 µl of 10xdUTP and 12.6 µl of Cy3/Cy5).

On Ice 9 µl of Cy3 master mix is added to each patient sample and 9 µl of Cy5 master mix are added to each reference. 0.5 µl of exo-Klenow fragment are added to

each reaction tube. Reaction tubes are then incubated at 37^{o}C for 4 hours. To stop the reaction samples are then incubated at 65 ^{o}C for 10 minutes.

A new set of 1.5ml microtubes are labelled and an AutoseqG50 gel column prepared for each labelling reaction by inverting the column, snapping off the bottom closure, placing each column in a collection tube and spinning at room temperature at 2000g for 1 minute and then placing each column into a prelabelled 1.5ml microtube.

Labelled DNA (patient and reference) are loaded onto the centre of the gel in the column.

Columns are then spun at room temperature at 2000g for 1 minute. Gel columns are discarded and the remaining microtubes mixed and pulse spun. The nanodrop spectrophotometer is then used to measure dye incorporation. The concentration should be>100ng/ µl pmoldye/ µl>2.5.

Test and reference combination and precipitation.

Cy5 reactions (reference sample) are then transferred to Cy3 reactions (patient sample). 5.5 µl of cold 3M sodium acetate pH 5.2 followed by 138 µl of 100% ethanol to each combined sample and the samples inverted.

The combined sample is stored at -80^{o}C for 30 minutes to allow the precipitate to form. Combined samples are spun at 4^{o}C for 30 minutes. The supernatant is

discarded leaving a dark purple pellet. 312 µl of 70% ethanol is added to the sample and inverted.

Oligonucleotide Array Hybridisation

The samples are centrifuged for 5-10 minutes. The supernatant is discarded and the sample pulse spun to collect any residual ethanol, which is removed using a p10 pipette. Samples are left uncapped for <5 minutes to allow the pellets to dry.

Pellets are re-suspended in 16 µl of DNA hydration solution and incubated at 73-75^{o}C for 2 minutes. 2 µl of COT human DNA, 4.5 µl of 10xblocking agent and 22.5 µl of 2x Hi-RPM buffer. Samples and mixed and pulse span and placed on a 95^{o}C hot block for 3 minutes. Tubes are transferred to a 37^{o}C hot block for 30 minutes. Tubes are allowed to cool to room temperature.

Each sample is transferred to the correct gasket. The array slide is then placed down onto the gasket. The hybridization chamber is placed to cover the gasket and array. The chamber is then rotated to allow the sample to cover the slide. Chambers are then placed in the hybridization oven at 65^{o}C for 24 hours.

Oligonucleotide Array Washing

The chambers are disassembled and the slide and gasket are submerged into the disassembly wash. Whilst submerged the gasket and microarray slide are separated. The slide is then loaded into a slide rack submerged in fresh wash buffer. When all slides are loaded a stirring bar is added to the dish and the wash stirred for 5 minutes. The slides (in the rack) are then transferred to the second wash buffer at a temperature of 37^{o}C for 1 minute. Slides are then transferred to the aglient scanner. After scanning raw TIFF images are transferred to Bluefuse multi software.

Analysis

(performed by myself and overseen by two members of staff at the WMRGL Mr Lee Silcock and Mr Dominic Mcmullan)

Quality control of array image

Derivative Log Ratio fused; good<0.15, OK 0.15-0.22, fail>0.22.

Standard deviation robust; good 0.07-0.1, OK 0.1-0.17, fail <0.07 or>0.17

As described in chapter 1 and chapter 2 CNVs were classified as benign, VOUS or pathological in accordance with the American College of Medical Genetics (ACMG) guidelines (50). Pathological CNV are documented as clinically significant in multiple peer-reviewed publications. Penetrance and expressivity of the CNV is well defined, even if known to be variable. They may include large CNVs not described in the

medical literature at the size observed in the fetus, but overlap a smaller interval with clearly established clinical significance.

A benign CNV will have been reported in multiple peer-reviewed publications or curated databases as being a benign variant, particularly if the nature of the copy number variation has been well characterised and will typically represent a common polymorphism (documented in greater than 1% of the population).

VOUS will have included findings that are later demonstrated to be either clearly pathogenic or clearly benign, but insufficient evidence was available for unequivocal determination of clinical significance at the time of reporting (50) (see table below).

Table taken from microarray 4.4 CNV classification guidelines version 1.1 WMGL

Class	**DGV**	**Local databases**	**Clinical Databases**	**Gene function**	**HI index score**
Uncertain: likely Benign	<1% of cases, <3 studies or no similar cases	>1% cases; same dosage in non syndromic regions	Multiple similar cases classified as benign in ISCA/DECPIHER (a consenus classification)	No Genes/genes of irrelevant function Intronic imbalance in unrelated genes	>30%
Uncertain	No similar entries	No similar cases Cases equally uncertain representing <1% of cases	No similar cases and /or cases classified as uncertain in ISCA	Uncertain funtion No clear link to phenotype but possible i.e. in Mouse Genome informatic curated models	10-30%
Uncertain Likely pathogenic	No similar entries	Limited number of similarly affected cases in local database No simlar cases but complelling biology	Limited number of similar cases In DECIPHER/ISCA/literature	Clear link to phenotype but not a recurrent syndrome Clear link to susceptibility/late-onset disease	<10%

In addition to using DECIPHER, ISCA and DGV databases described above further analyses can be performed using further online tools. phenExplorer (http://compbio.charite.de/phenexplorer/) allows generation of a list of genes relevant to the phenotype see on ultrasound scan. Toppgene then allows prioritisation or ranking of candidate genes based on functional similarity between those in the training gene list (obtain by phenExplorer) and those in the test gene set (those within the CNV region).

STRING can be also used to search for interactions between known genes associated with the phenotype derived from phenExplorer and gene/s of interest from copy number imbalance.STRING v9.0 (http://string-db.org/)(140), is a database that contains known and predicted protein interactions. Interactions are classified as either direct (Physical) or indirect (functional); they are derived from genomic context, high-throughput experiments, co-expression analysis and previous knowledge and assigns a confidence score based on these criteria.

The above are quick solutions to obtaining further evidence for causality but will often still result in a classification of VOUS. Functional work may be a longer term solution but is not possible within the time frame of a pregnancy and is therefore more amenable to research then diagnostic work.

Appendix G Thematic analysis of interview transcripts

Provided on CD in excel spread sheet format

Appendix H Further CEACS and sensitivity analyses

Figure 34 Sensitivity analysis Model 1 Birmingham BAC cohort VOUS true positives, CMA over karyotyping

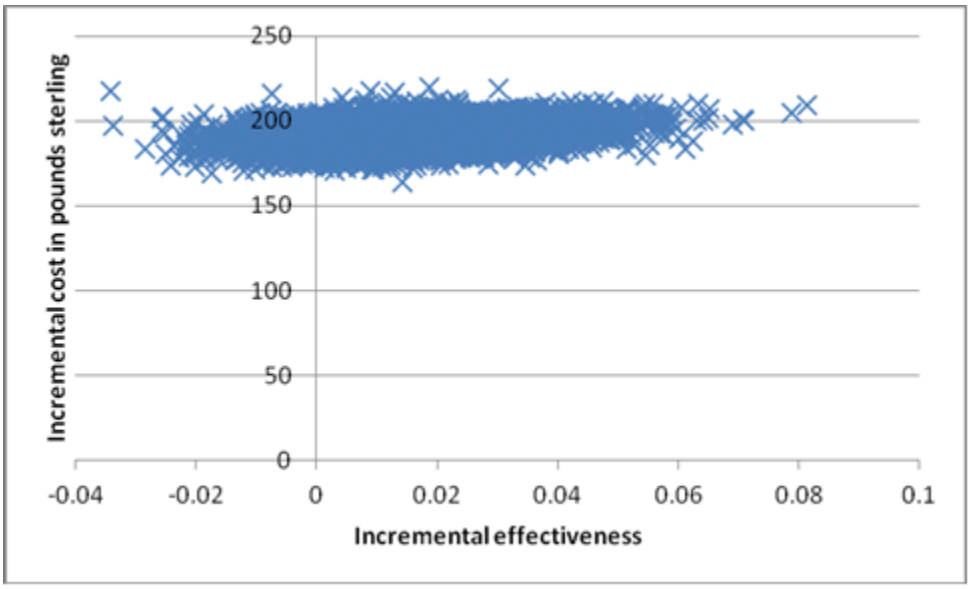


Figure 35 CEAC of CMA over karyotyping Birmingham BAC cohort model 1, treating VOUS as true positives

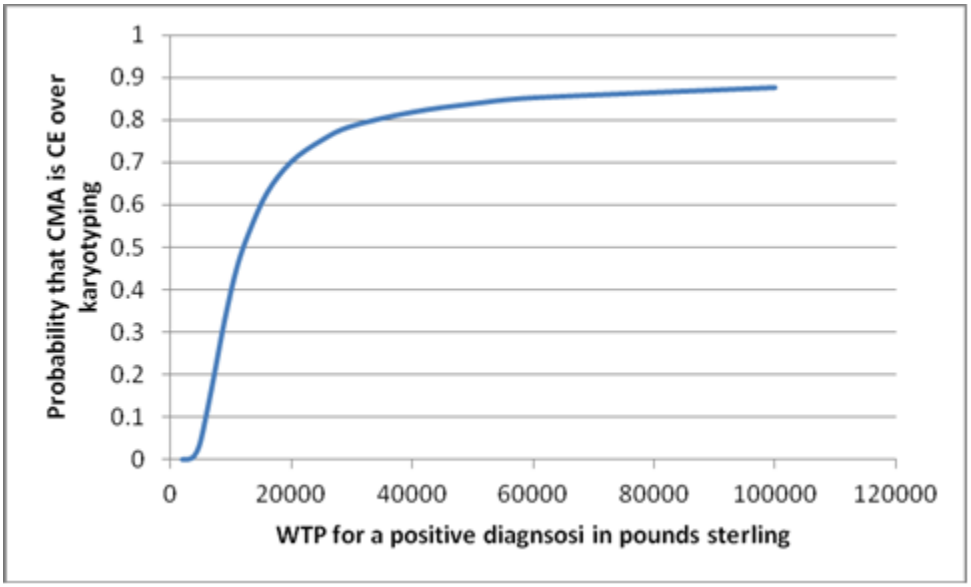


Figure 36 Sensitivity analysis, Birmingham BAC cohort model 1, treating VOUS as true positives, CMA over QFPCR

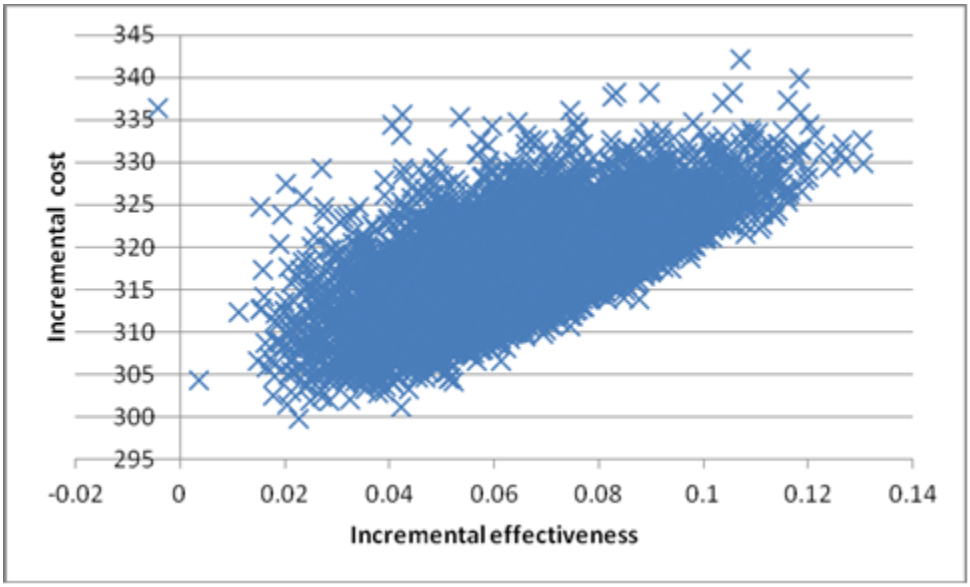


Figure 37 CEAC of CMA over QFPCR, Birmingham BAC cohort Model 1 treating VOUS as true positive

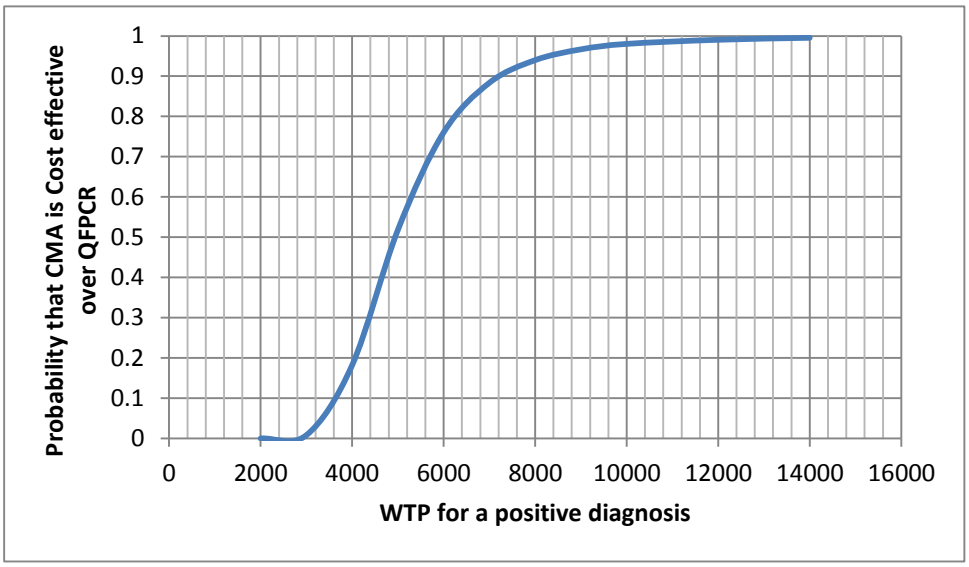


Figure 38 Sensitivity analysis Birmingham BAC cohort model 1, VOUS as true positives. CMA over QFPCR then karyotype then FISH Di George syndrome

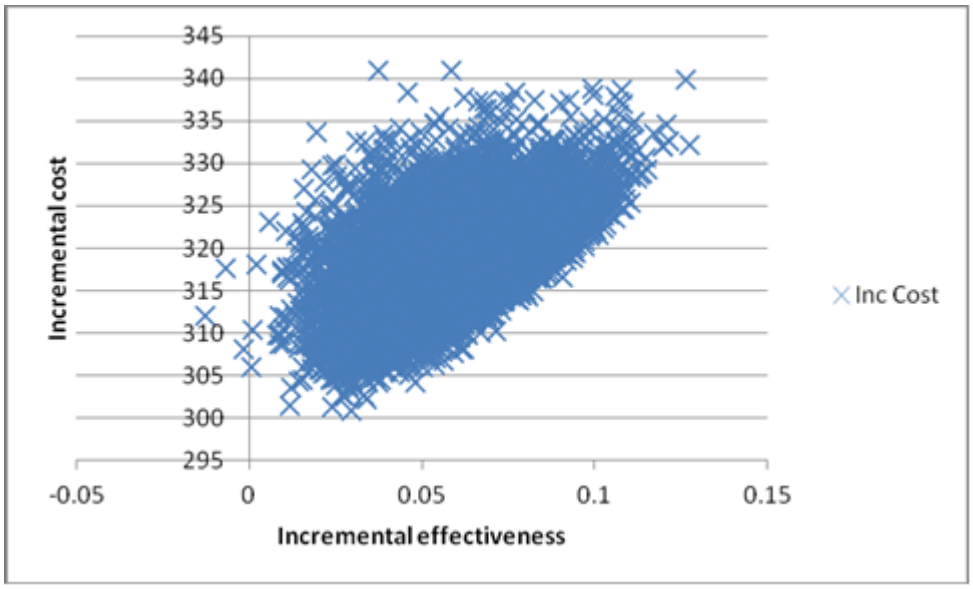


Figure 39 Birmingham BAC cohort model 1, VOUS as true positives. CEAC of CMA over QFPCR then karyotyping then FISH Di George Syndrome

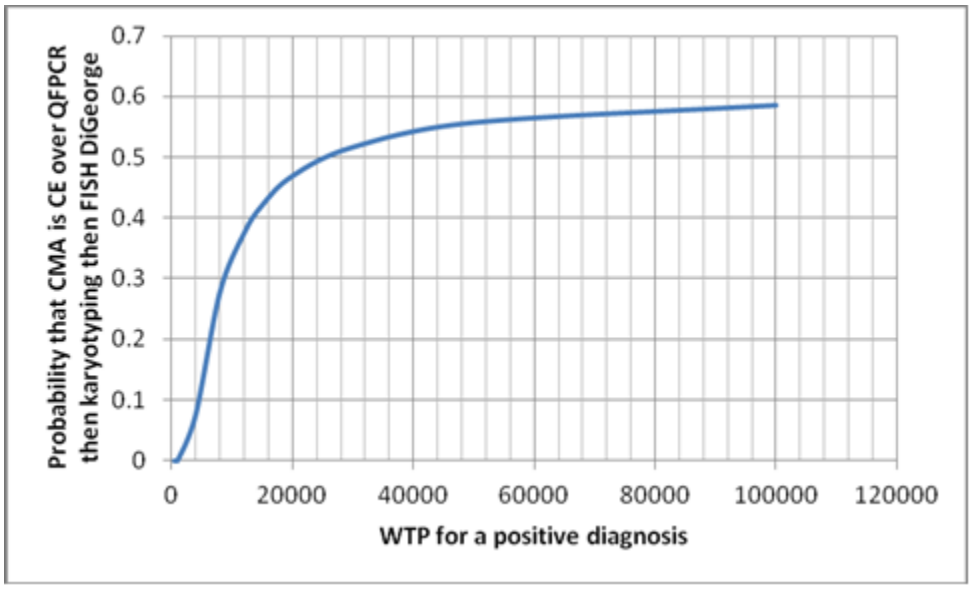


Figure 40 Sensitivity analysis Model 1 Birmingham BAC cohort treating VOUS as true positives, CMA over QFPCR then FISH for Di George Syndrome

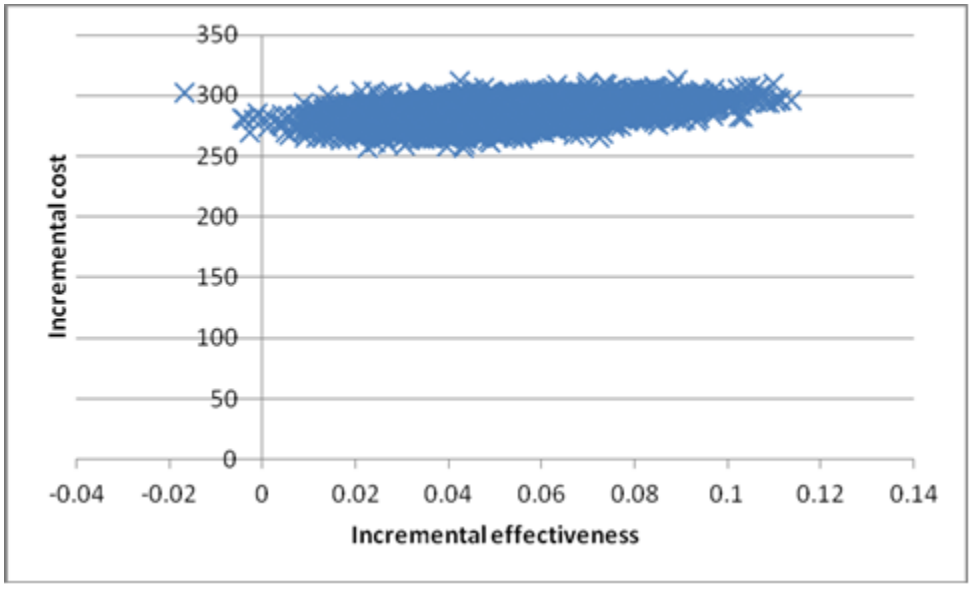
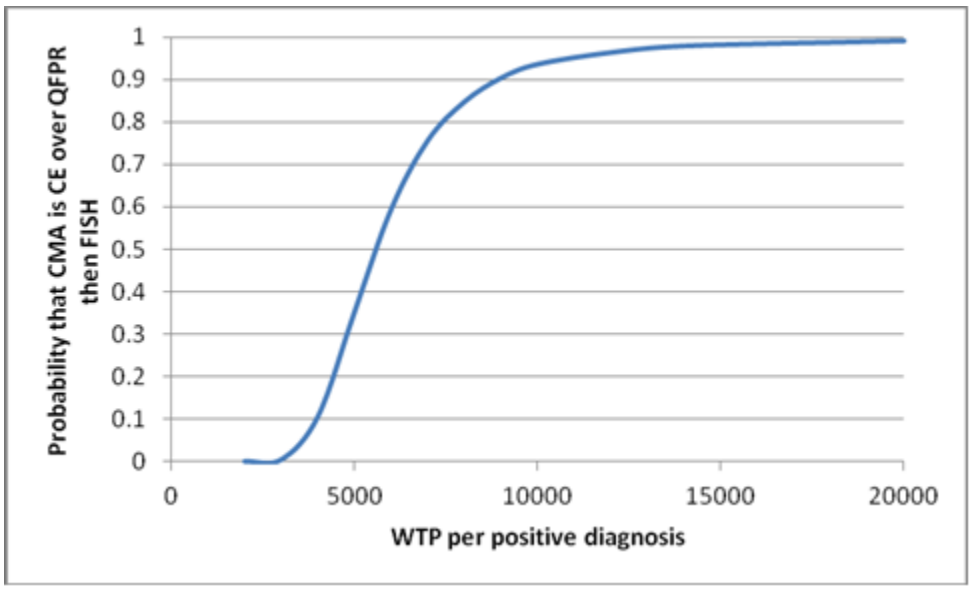


Figure 41 CEAC Model 1 Birmingham BAC Cohort, VOUS as true positives, CMA over QFPCR then FISH for Di George syndrome



Birmingham BAC cohort Model 1 VOUS treated as false positive:

Figure 42 Sensitivity analysis Model 1 Birmingham BAC cohort VOUS as false positives CMA over karyotyping

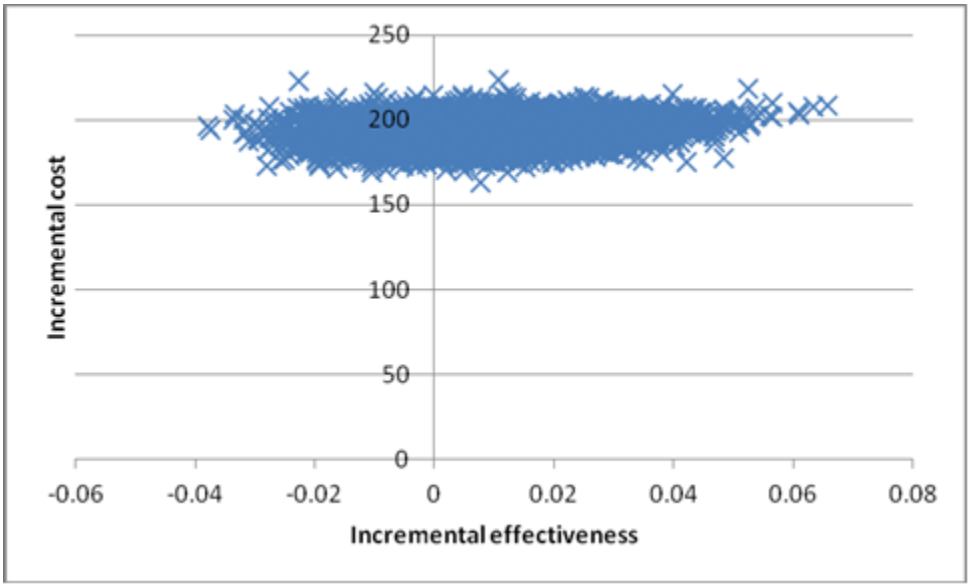


Figure 43 CEAC Model 1 Birmingham BAC cohort, VOUS as false positives, CMA over karyotyping

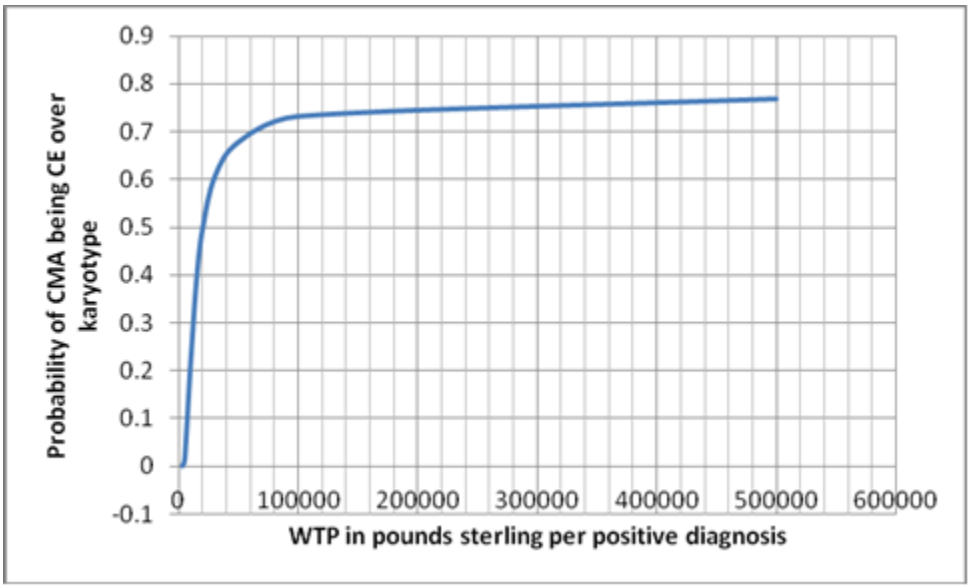


Figure 44 Sensitivity analysis Model 1 Birmingham BAC Cohort VOUS as false positives CMA over QFPCR

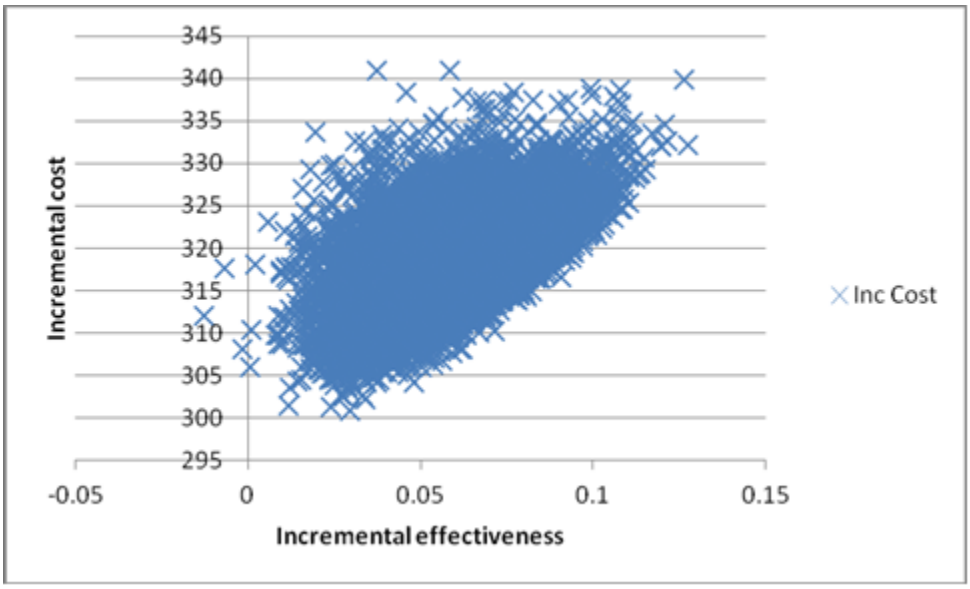


Figure 45 CEAC Model 1 Birmingham BAC cohort VOUS as false positive CMA over QFPCR

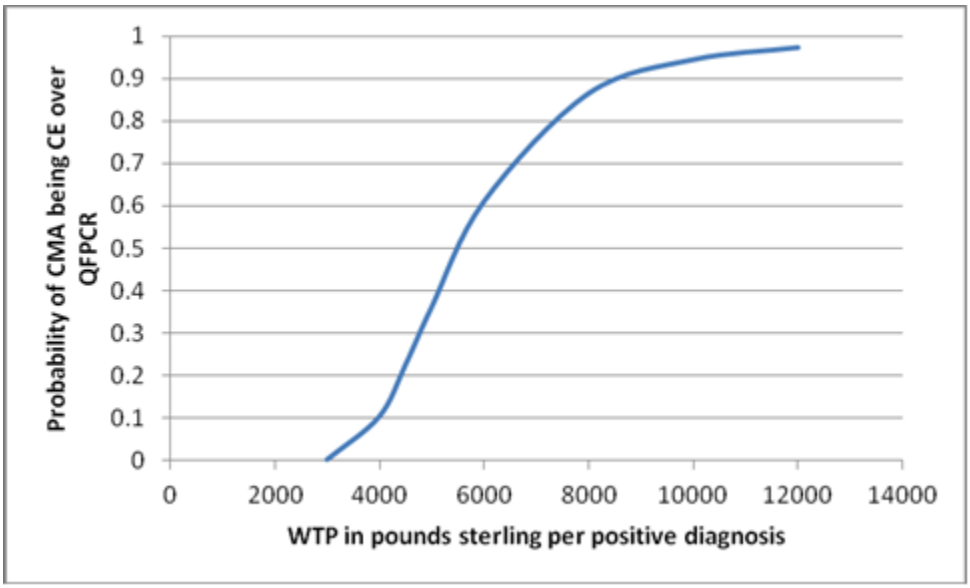


Figure 46 Sensitivity analysis Model 1 Birmingham BAC cohort VOUS as false positives, CMA over QFPCR then karotyping then FISH for Di George

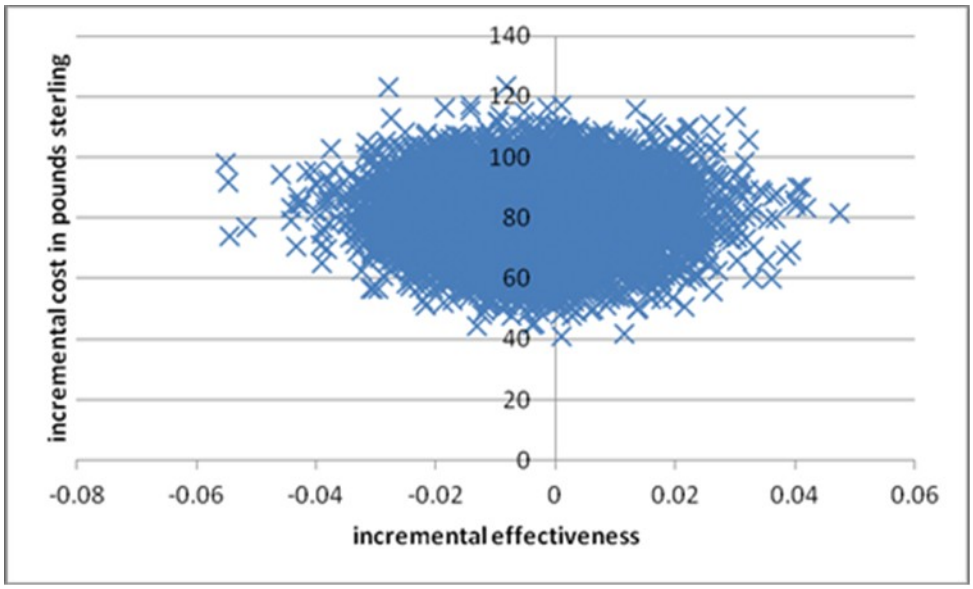


Figure 47 CEAC Model 1 Birmingham BAC cohort VOUS as false positives, CMA over QFPCR then karyotype then FISH for Di George

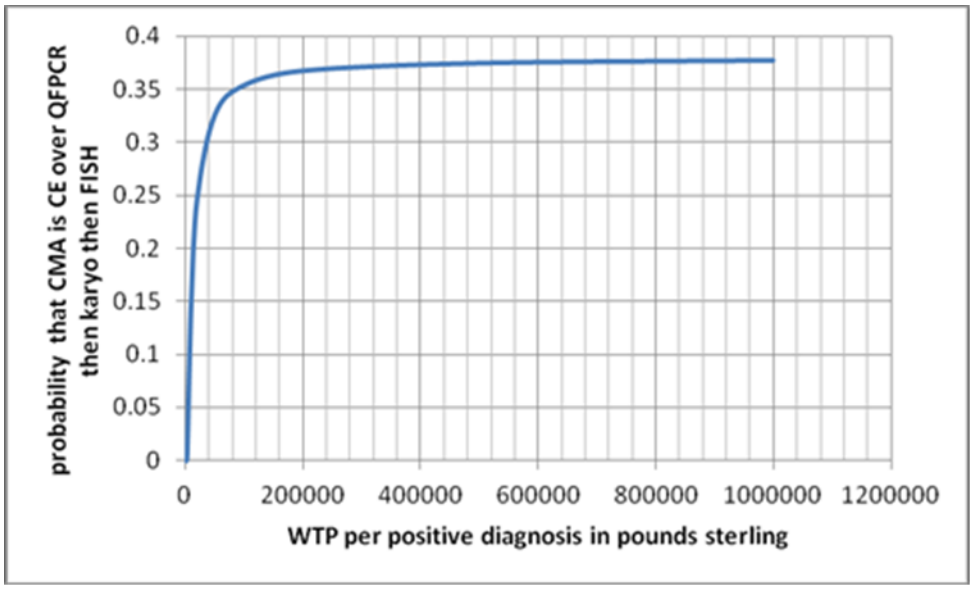


Figure 48 Sensitivity Analysis Model 1 Birmingham BAC cohort treating VOUS as false positives CMA over QFPCR then FISH for Di George syndrome

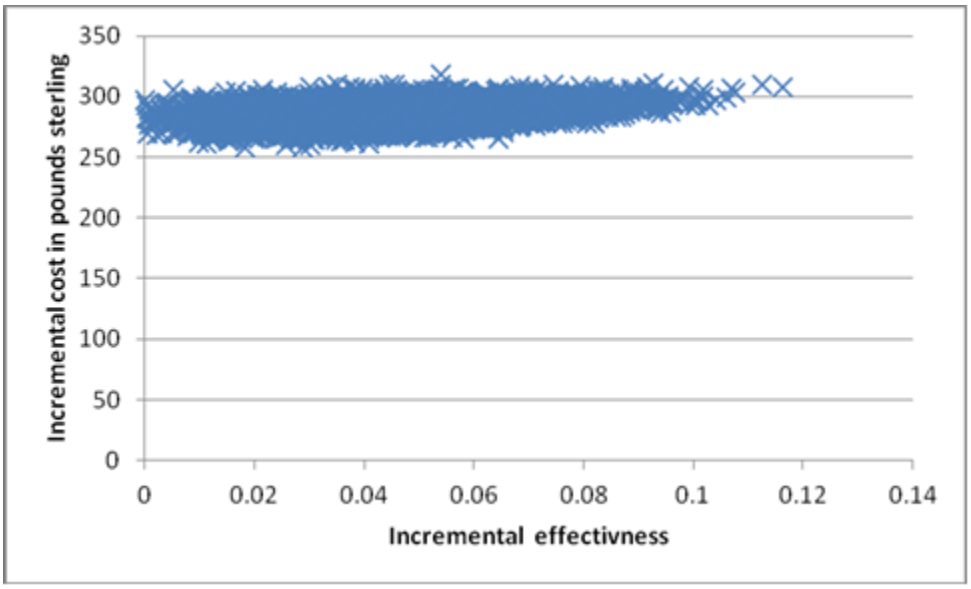


Figure 49 CEAC Model 1 Birmingham BAC cohort treating VOUS as false positives CMA over QFPCR then FISH for Di George syndrome

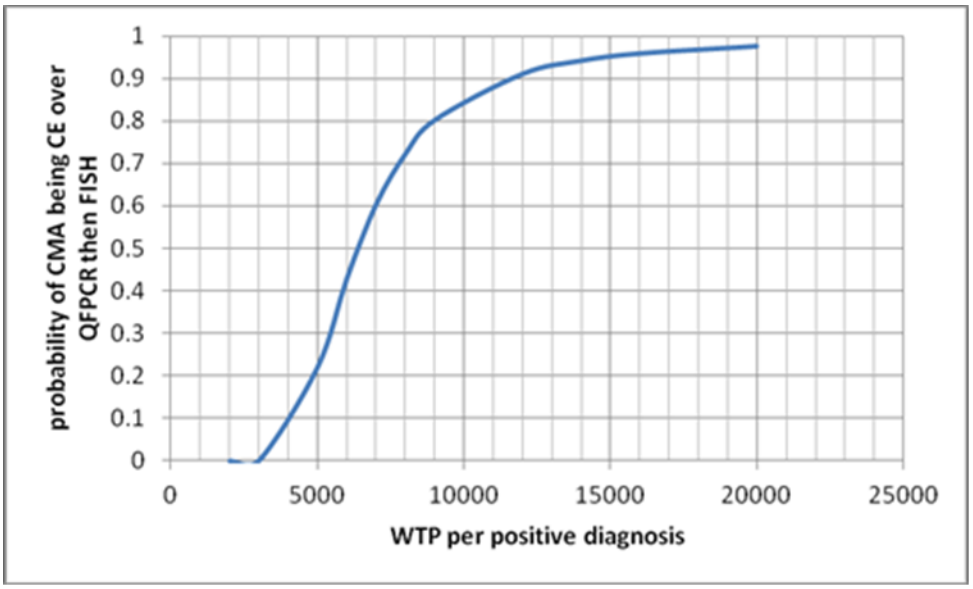


Figure 50 Sensitivity analysis Model 2 using systematic review data, all indications for testing, VOUS treated as true positives, CMA over karyotyping

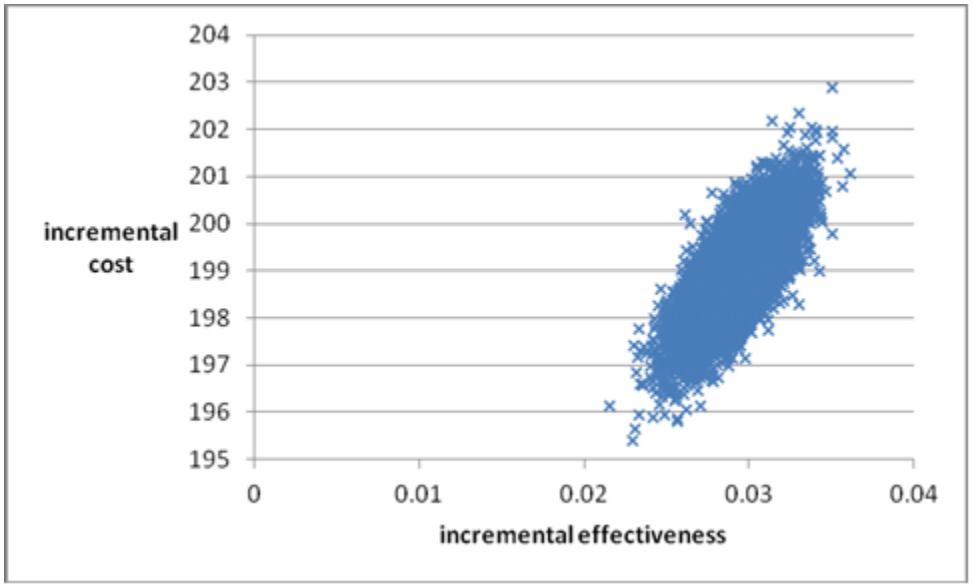


Figure 51 CEAC Model 2 Systematic review data, all indications for testing, treating VOUS as true positives

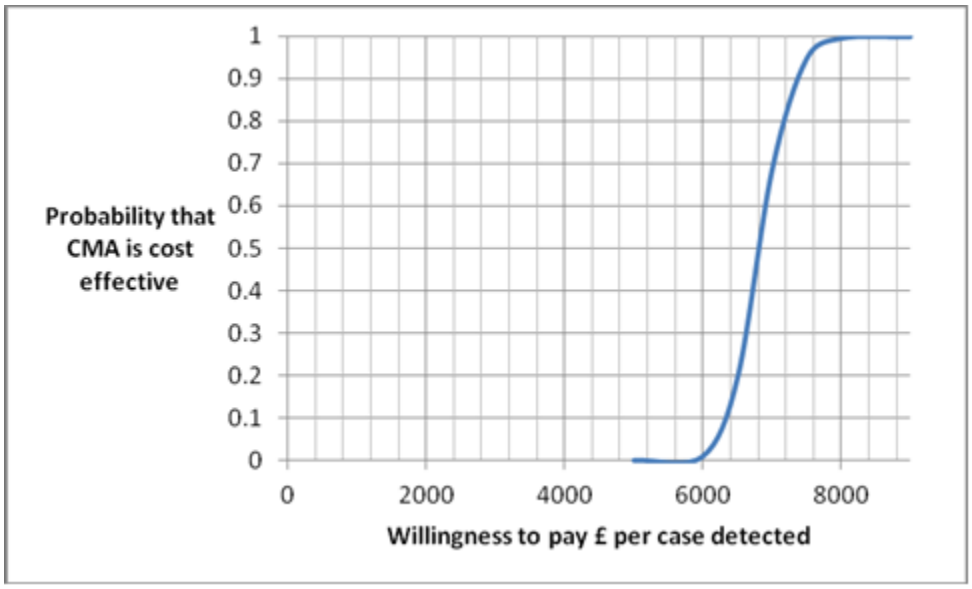


Figure 52 Sensitivity analysis Model 3 systematic review data when testing performed for abnormal ultrasound anomalies, CMA over karyotyping

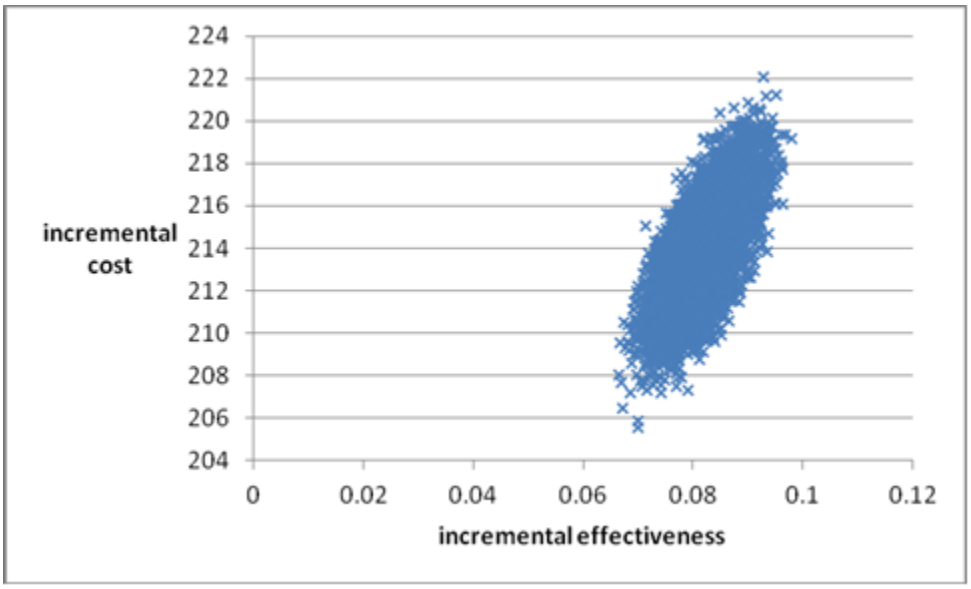
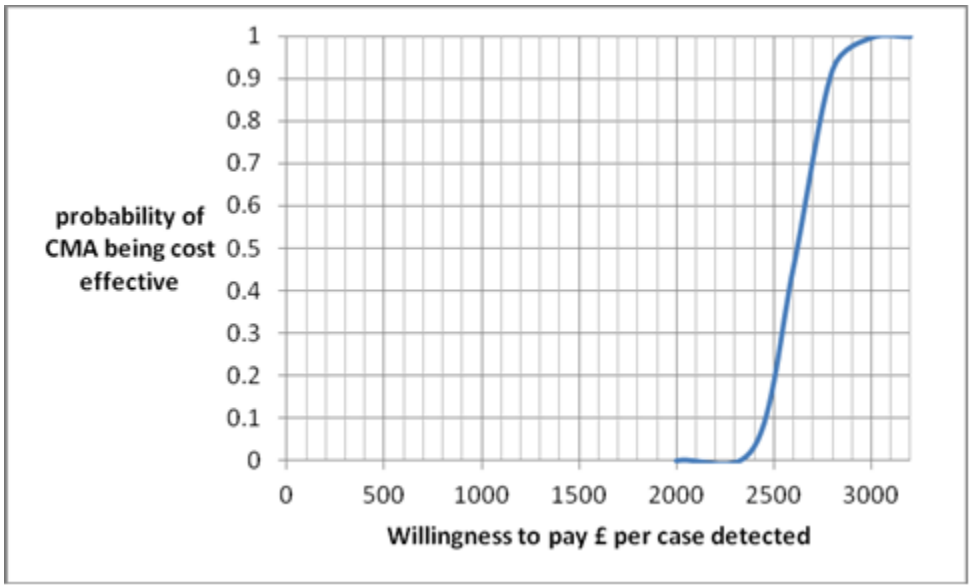


Figure 53 CEAC Model 3 systematic review data, testing performed for abnormal ultrasound scan findings, treating VOUS as true positives CMA over karyotyping



Appendix I Manuscripts Submitted from PhD work

From Chapter 1

Published

1) Additional information from array comparative genomic hybridization technology over conventional karyotyping in prenatal diagnosis: a systematic review and meta-analysis. Hillman SC, Pretlove S, Coomarasamy A, McMullan DJ, Davison EV, Maher ER, Kilby MD. Ultrasound Obstet Gynecol. 2011 Jan;37(1):6-14

Published

2) Microarray comparative genomic hybridization in prenatal diagnosis: a review. Hillman SC, McMullan DJ, Williams D, Maher ER, Kilby MD. Ultrasound Obstet Gynecol. 2012 Oct;40(4):385-91

Published

3) The use of Chromosomal Microarray (CMA) in prenatal diagnosis Hillman SC, McMullan DJ, Maher ER, and Kilby MD The Obstetrician & Gynaecologist. 2013 April; 15(2):80-84

Published

4) Prenatal microarray technology for the identification of chromosomal anomalies. Hillman SC, McMullan DJ, Maher ER, and Kilby MD. Current progress in Obstetrics and gynaecology 1. Chapter 10: 167-181

From Chapter 2 and chapter 4

Published:

1) S.C.Hillman, D.J.McMullan, G. Hall, F.S. Togneri, N. James, E.J Maher, C.H. Meller, D. Williams, R.J. Wapner, E.R. Maher and M.D.Kilby. Use of Prenatal chromosomal microarray use: a prospective cohort of fetuses and a systematic review and meta-analysis. USOG 2013 Jun;41(6):610-20

From Chapter 3

Published:

1) Hillman SC, McMullan DJ, Silcock L, Maher ER, Kilby MD.
How does altering the resolution of chromosomal microarray analysis in the prenatal setting affect the rates of pathological and uncertain findings?
J Matern Fetal Neonatal Med 2013; Aug 19 (e-pub ahead of print).

From Chapter 5

Published:

1) Hillman SC, Skelton J, Quinlan-Jones E, Wilson A, Kilby MD. "If it helps..." the use of microarray technology in prenatal testing: patient and partners reflections. Am J Med Genet A. 2013 Jul; 161A(7):1619-27.

From Chapter 6

Awaiting amendments fetal diagnosis and therapy

1) Chromosomal microarray for prenatal detection of chromosome anomalies in fetal ultrasound anomalies: an economic evaluation Hillman SC, Barton PM, Roberts TE, Maher ER, McMullan DJ and Kilby MD

Appendix J Statistical analysis

1)Systematic review and meta-analysis

a)2 x 2 tables were created for each study

Karyo result

Array result		+ve	-ve
	+ve	a	b
	-ve	c	d

b) STATA Version 11.0 Copyright 1984-2009 Statistics/Data Analysis StataCorp (http://www.stata.com) was used to perform the metanalysis. The following commands were used:

To calculate percentage agreement between tests:

gen logr = log ((a+d)/(a+b+c+d)

gen selogr = 1/sqrt(a+b+c+d)

metan logr selogr, fixedi second (random) eform

To calculate percentage detected by karyotype over CMA and meta analyse results

gen logr = log ((c)/(c+d)

gen selogr = 1/sqrt(c+d)

metan logr selogr, fixedi second (random) eform

To calculate percentage detected by CMA over karyotype and meta analyse results

gen logr = log ((b)/(b+d)

gen selogr = 1/sqrt(b+d)

metan logr selogr, fixedi second (random) eform

To calculate percentage of VOUS and meta analyse results

gen logr = log(n/t)

gen selogr =1/sqrt(t)

metan logr selogr, fixedi second (random) eform

log r is the log ratio of the output i.e. b/b+d (or extra information detected by CMA over karyotype. Se logr id the standard error of the log ratio. Metan is the main STATA meta-analysis command. This command produces both a fixed effect result (fixedi = inverse variance fixed effect method) as well as random effect method (random = Der Simonian and Laird random-effects method). The command eform then produces the output on a ratio scale (141).

2)Heterogeneity

A Chi-squared test was used to examine heterogeneity. Data under analysis in meet the assumptions of the test.

Heterogeneity was expected due to the known a priori variability in study characteristics, conduct and measurement methods. Such heterogeneity is expected in prognostic factor research (142). Hence our protocol pre-defined that a random effects approach was to be used. In relation to testing for heterogeneity, the Cochrane Handbook (143) states that: ‘'the choice between a fixed-effect and a random-effects meta-analysis should never be made on the basis of a statistical test of heterogeneity' A random effects analysis does estimate and therefore account for all unexplained statistical heterogeneity.

3)Publication Bias

Publication bias was assessed using Eggers test and the data meet the assumptions of the test.

Egger’s test, is based on the Galbraith plot. This is a plot of difference over standard error against one over standard error. It is a regression analysis by calculating the regression of study difference over standard error on 1/standard error (144).

4)Fishers exact Test

The test is useful for categorical data that result from classifying objects in two different ways; it is used to examine the significance of the association (contingency) between the two kinds of classification. It is used in preference to the Chi-squared

test when sample sizes are small (under ten). The data meet the assumptions of the test.

5)Sensitivity and specificity

Sensitivity relates to the test's ability to identify positive results.

The sensitivity of a test is the proportion of people that are known to have the disease (or chromosomal abnormality) who test positive for it:

$$= \frac{\text{number of true positives}}{\text{Number of true positives+ number of false negatives}}$$

Specificity relates to the test's ability to identify negative results.

The specificity of a test is defined as the proportion of patients that are known not to have the disease who will test negative for it:

$$= \frac{\text{Number of true negatives}}{\text{Number of true negatives+ number of false positives}}$$

6) Incremental Cost effectiveness ratio (ICER)

The ICER is universally accepted as the standard summary ratio that should be used for reporting the results of economic evaluations. The additional cost per additional

unit of benefit can be applied to any evaluation as long as there is a comparison of costs and outcomes of at least two alternative pathways or strategies.

$$= \frac{\text{Cost 1 minus Cost 2}}{\text{Effectiveness 1 minus Effectiveness 2}}$$

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