

Advanced Genomic Testing For Fetal Ultrasound Anomalies

**Geavanceerde genetische testtechnieken
bij foetale echoscopische
afwijkingen**

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Advanced Genomic Testing For Fetal Ultrasound Anomalies

Geavanceerde genetische testtechnieken
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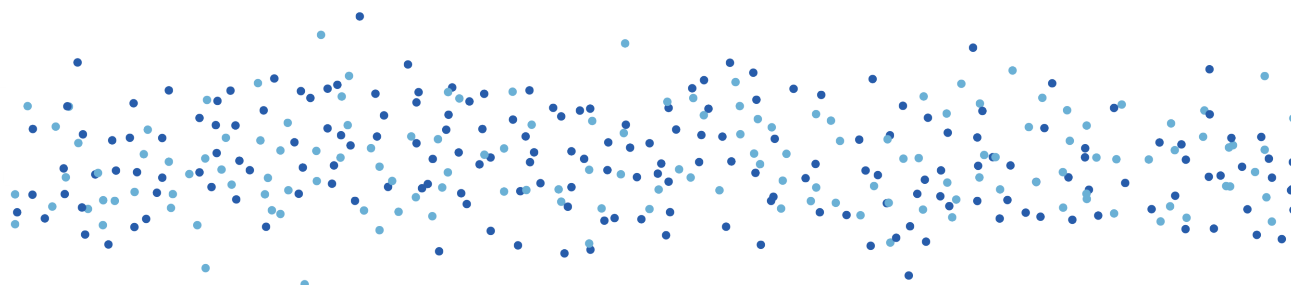
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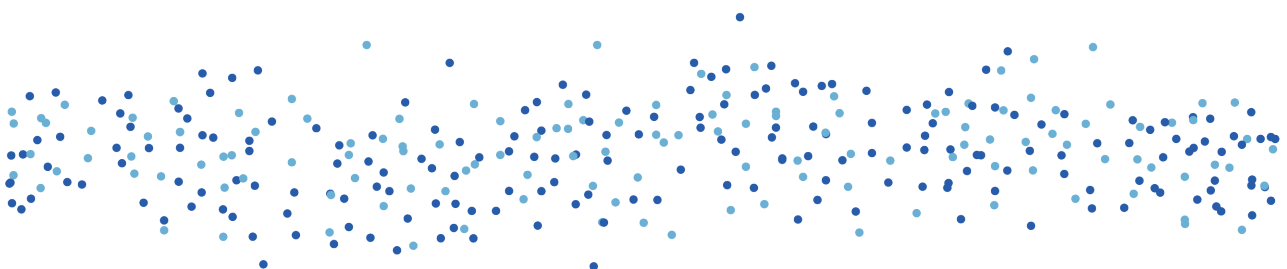
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Chapter 1

General introduction



General introduction

Aims of the thesis

Major congenital malformations, defined as having medical, surgical or cosmetic significance occur in two to four percent of livebirths and their prevalence in fetal life is even higher¹. Although the etiology of these malformations often remains elusive due to a multifactorial nature, a significant proportion is caused by distinctive genetic abnormalities such as chromosomal anomalies and single gene disorders. Common examples are an atrioventricular septal defect in the case of Down syndrome^{2, 3} or a pulmonary stenosis associated with Noonan syndrome⁴. The presence of such a genetic anomaly can strongly influence the fetal prognosis as it can cause other entities to arise such as intellectual disability and comorbidities. In the Netherlands, as in most Western countries, prenatal ultrasound is routinely offered to detect congenital malformations. Prenatal genetic testing is offered upon the diagnosis of most of these malformations.

Prenatal genetic testing employs different ways of determining the genetic makeup of a fetus. It serves two objectives; improving the quality of (medical) care for the mother and fetus and providing information for reproductive decision-making. The offer of prenatal genetic diagnosis is preceded by pre-test counseling in which a patient is informed about matters including the medical facts of the disorder(s) tested for, possible benefits and harms of testing, test performance (reliability and limitations), implications of testing, and alternative choices⁵. Furthermore, pretest counseling aims to explore the patients' attitude towards testing and to provide guidance in their deliberation process. Based on pretest counseling, a patient can either opt in or opt out of testing.

For prenatal genetic testing karyotyping has long been the gold standard. Karyotyping is a technique to visualize and examine metaphase chromosomes under a light microscope in order to detect large quantitative imbalances and rearrangements. Fetal karyotyping requires an invasive procedure to harvest fetal DNA such as chorion villus biopsy or amniocentesis, which is associated with a miscarriage risk of 1-2:1000⁶. In recent years, technical advances have led to 1) new genomic techniques with an increase in testing resolution over karyotyping and 2) new techniques for non-invasive, risk-free testing of cell-free fetal DNA.

This thesis involves studies on the integral value of new advanced genomic testing techniques in fetuses presenting congenital abnormalities on prenatal ultrasound. The aims of this thesis can be summarized as follows:

1. To evaluate the additional value of invasive single nucleotide polymorphism array over conventional invasive karyotyping or current non-invasive next generation sequencing based testing in specific fetal anomalies detected by ultrasound.
2. To evaluate the potential additional value of invasive fetal whole exome sequencing in specific fetal anomalies detected by ultrasound.
3. To provide an ethical deliberation on the offer of invasive testing for fetal whole exome sequencing in case of fetal anomalies detected by ultrasound.

The results of these studies can be used to improve pretest counseling thereby helping parents to make an informed choice in prenatal genetic testing. Furthermore, professionals can use the results to decide which testing modalities should be offered in case of specific fetal anomalies on ultrasound.

This thesis starts with a short introduction to the techniques used in its studies, in the context of a historic timeline of prenatal genetic diagnosis.

Prenatal genetic diagnosis: a historic timeline

The concept of fetal genetic testing was first clinically introduced 50 years ago by Steele and Breg with their 1966 Lancet publication "Chromosome analysis of human amniotic-fluid cells"⁷. They obtained amniotic fluid via a puncture of the amniotic sac between 20 and 37 weeks of gestation and cells of fetal origin were isolated, cultured and used for karyotyping. As the genetic origins of prevalent conditions such as Down's syndrome (trisomy 21)⁸ and Edwards syndrome (trisomy 18)⁹ were already known by that time, clinical diagnostic amniocentesis brought the opportunity to test for these syndromes before birth. Amniocentesis was offered based on the presence of an elevated risk for aneuploidies such as a positive familial history or advanced maternal age. However, most children with genetic diseases were and still are born to mothers without these risk factors. Excluding these low-risk mothers from access to prenatal diagnosis seemed unfair, yet amniocentesis held a risk for pregnancy loss and was too costly. Therefore, it was deemed unsuitable for population-wide screening. The introduction of ultrasound in prenatal diagnosis¹⁰⁻¹² in the early 1970s brought the opportunity to safely screen for fetal congenital malformations in an unselected population. Due to poor image quality, initially it was not considered clinically useful but as the technique developed it became an excellent tool to examine the fetus. This led to a breakthrough in identifying patients eligible for amniocentesis as the presence of congenital malformations is one of the important risk factors for concurrent genetic disease. From the 1990s onwards, the mid-pregnancy anomaly scan increasingly became a part of antenatal care in Europe, with an overall detection rate for fetal anomalies of 61%¹³. The first trimester nuchal translucency

measurement, first introduced in 1985¹⁴ enabled early, non-invasive screening for aneuploidies. Both the mid-trimester anomaly scan and the nuchal translucency measurement (as part of the combined test) were added to the Dutch population-screening program in 2007.

From the identification of the first chromosomal syndrome in 1959, proceeding genetic research continued to discover chromosomal loci associated with clinically distinct syndromes, such as Wolf-Hirschhorn syndrome, Cri-du-Chat syndrome and DiGeorge syndrome¹⁵⁻¹⁷. By 1992, karyotype analysis showed a clinically relevant chromosomal anomaly in $\geq 18\%$ of pregnancies presenting multiple fetal anomalies on ultrasound¹⁸. Although karyotyping remained the standard for fetal genetic testing for decades, it had important disadvantages in the purpose of prenatal testing. Firstly, its turnaround time was long due to the need for cell culturing and manual interpretation. Furthermore, prenatal karyotyping does not allow for the detection of aberrations below 5-10 megabases and rearrangements in certain areas, such as telomeres, are difficult to detect due to staining properties. Because of these limitations newly invented techniques have been reviewed and proposed as alternatives. The in the 1980s presented technique of Fluorescent in Situ Hybridization (FISH) can examine preselected target areas at a higher resolution than karyotyping with the potential to identify microdeletions^{19,20} and microduplications. FISH allowed a major advantage in the turnaround time as it did not require cell culturing, however due to its targeted approach, high costs and labor-intensiveness, it was difficult to scale to a genome wide approach. The principle of comparative genomic hybridization (CGH) was presented in 1992²¹ and posed another possible alternative for karyotyping. With CGH, the genome of interest and a reference genome are competitively hybridized to detect quantitative

differences. Although CGH was a genome wide approach that could potentially reach a slightly higher resolution, it did not reach a resolution below 5 Mb because the hybridization targets were metaphase chromosomes. Moreover, it was still labor-intensive. The combination CGH with microarray from the late 1990s onwards, however, meant a revolution. Array CGH (aCGH) used cloned DNA segments, the spacing and size of which determined resolution. These arrays were highly sensitive and, because of automation, fast. The first proof of principal and validation studies for aCGH in prenatal diagnosis were published between 2005 and 2007²²⁻²⁵. A variety of array platforms and targets (bacterial clones, oligonucleotides, etc.) were presented. The early designs were usually targeted on known disease causing loci, but unbiased genome wide approaches were also published. Subsequently, another type of genomic microarray, based on single nucleotide polymorphism (SNP) genotyping (Box 1), was introduced to prenatal testing in 2010^{26, 27}. This type of array has diagnostic advantages as it is able to detect stretches of homozygosity (e.g. uniparental disomy, consanguinity) and triploidy (Box 1). Collectively, the use of genomic microarrays meant a 5.2-10%²⁸⁻³⁰ overall increase in diagnostic yield in fetuses with abnormalities on ultrasound.

BOX 1:

Single Nucleotide Polymorphism Array

Single nucleotide polymorphisms (SNPs) are variations in single nucleotides that occur at a specific position in the genome. Two possible nucleotides types, alleles, that each appear in a significant portion of the human population. For every SNP, an individual inherits one allele from each parent. If these alleles are simplified to the values "A" and "B", an individual's

genotype at a certain SNP site is either "AA", "AB", or "BB", depending on which alleles the individual inherits. A kit for a SNP array contains probes designed to hybridize with the sequence before the targeted SNP and it contains single-base extensions complementary with both possible alleles (A or B) of the targeted SNP. Each base has a fluorescent tail which, upon excitation by light absorption, results in the emission of light. Each type of single base extension leads to a certain color signal (for simplicity called green and red) when hybridized with the SNP. These signals are measured and compared to the expected intensity based on a pool of references.

Because the alleles at each SNP are determined, not only the raw copy number can be established, but also the genotype at each SNP. This information forms the B allele frequency (BAF) plot. A BAF plot shows the individual's alleles for each SNP: 100% A or 100% B (homozygous) or 50% A and 50% B (heterozygous). This BAF plot helps validating a finding from the raw copy number plot, but can also be used to identify large stretches of homozygosity due to uniparental disomy or consanguinity, it can help identifying maternal cell contamination and it is the only microarray method to detect triploidy.

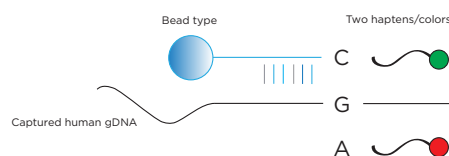


Illustration 1. Single nucleotide polymorphism array

BOX 2:**Whole exome sequencing**

Next generation sequencing (NGS), high throughput sequencing or massive parallel sequencing (MPS) requires a library of partially overlapping fragments of the human genome which are all simultaneously amplified. There are many different sequencing techniques available, such as pyrosequencing, sequencing by ligation or ion semiconductor sequencing. In the most used approach, sequencing by synthesis, analysis relies on the measurement of fluorescent signals secondary to nucleotide incorporation.

NGS allows much higher and faster throughput than chain termination or Sanger sequencing. It is used for large panels of genes of interest, for the whole exome or whole genome. NGS can potentially detect all types of sequence mutations (with a few exceptions, i.e. repeat expansions) as well as copy number variants. Technical disadvantages relate mostly to target coverage and sequence depth.

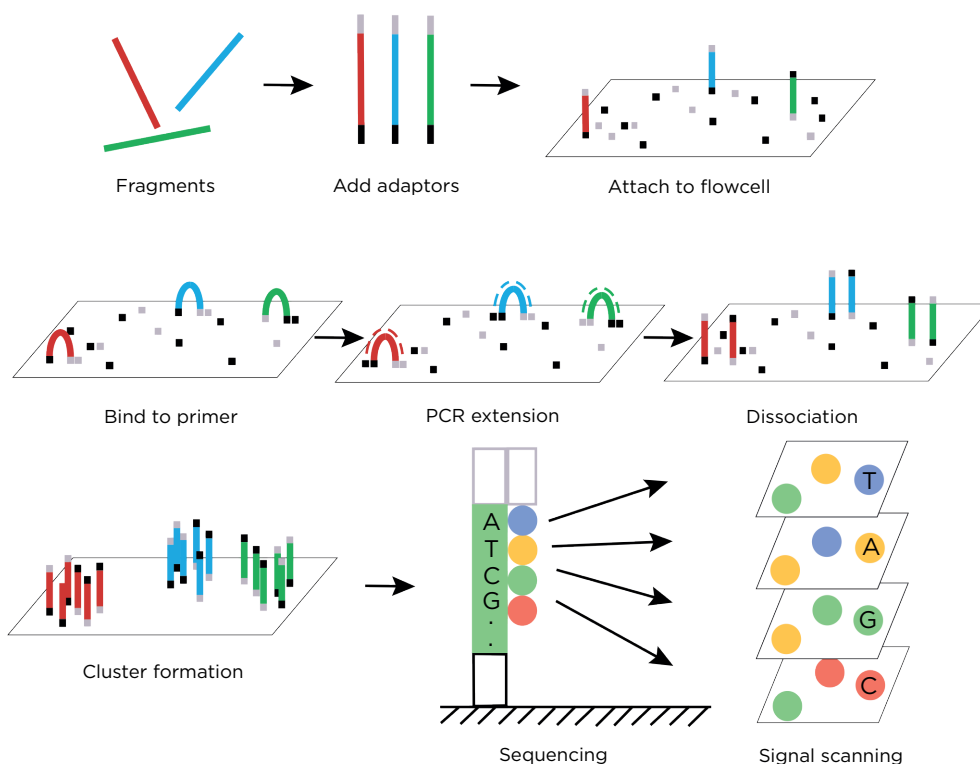


Illustration 2. Next generation sequencing

Prenatal diagnosis of non-chromosomal genetic disorders

Long before DNA sequences could be read, enzymatic tests on amniotic fluid were used to identify certain non-chromosomal inheritable conditions such as Pompe's disease and cystic fibrosis³¹. The direct diagnosis of single gene disorders started with the development of Sanger's dideoxy technique for DNA sequencing in 1977³². In the 1980s the first disease causing single gene mutations were identified, such as the delta f508 mutation in the CTFR gene for cystic fibrosis³³. Scientific progress rapidly allowed for targeted prenatal diagnosis of known familial mutations in most cases. In cases with a familial disease in which the gene was known but the mutation was not yet identified, prenatal diagnosis was established by means of linkage analysis. An example of such a disease was duchenne muscular dystrophy. Sequencing was not applied in prenatal genetic diagnosis for ultrasound anomalies, unless the fetal malformation strongly suggested a certain mutation³⁴, as sequencing required a targeted approach.

The human genome project meant an attention boost for the development of sequencing techniques. Next generation sequencing (NGS), high throughput sequencing or massive parallel sequencing (MPS) was developed from the 2000s onwards and allowed the simultaneous sequencing of numerous genes such as the complete exome (all coding sequences) or genome. In previous years, whole exome sequencing (WES) was implemented in postnatal clinical practice for a variety of indications, such as neurodevelopmental disorders, epilepsy syndromes and congenital malformations^{35, 36}. To date, a handful of pilot studies on prenatal NGS have been published^{37, 38}.

Non-invasive sampling of fetal DNA

Traditionally, fetal DNA for genetic testing could only be obtained by performing invasive sampling through amniocentesis or through the later developed method of chorion villus biopsy. Both hold an additional risk of miscarriage. A safer method to obtain fetal DNA was long-sought for. Initially, fetal cells in the maternal bloodstream seemed to have to be the target. The continuous presence of fetal cells in the maternal body, known as fetal microchimerism, was described by Georg Schmorl in deceased mothers as early as 1893³⁹ and by Herzenberg in healthy mothers in 1976⁴⁰. These fetal cells can be detected as long as 27 years after parturition⁴¹. By 1993, proof for the use of these cells for non-invasive prenatal testing was delivered⁴². However, techniques to harvest fetal cells and analyze their DNA content were extremely time-consuming and labor-intensive. The 1997 discovery of the presence of fragmented (50-200bp) cell-free DNA derived from the pregnancy in the maternal plasma and serum⁴³ meant a breakthrough in the practical feasibility of non-invasive fetal testing. The concentration of pregnancy DNA in maternal plasma and serum proved to be much higher than in the cellular fraction of the maternal blood. Fragmented cell-free DNA from the pregnancy derives from apoptosis of the syncytiotrophoblast⁴⁴ and is then deposited in the maternal bloodstream. Syncytiotrophoblast are cells from the conceptus just as the fetus is. However, the cells that form the syncytiotrophoblast are not always chromosomally concordant with the cells that form the fetus (the inner cell mass) due to a mechanism called mosaicism in early blastocyst development⁴⁵. Therefore, non-invasive prenatal testing (NIPT) is a test of the trophoblast rather than a test of

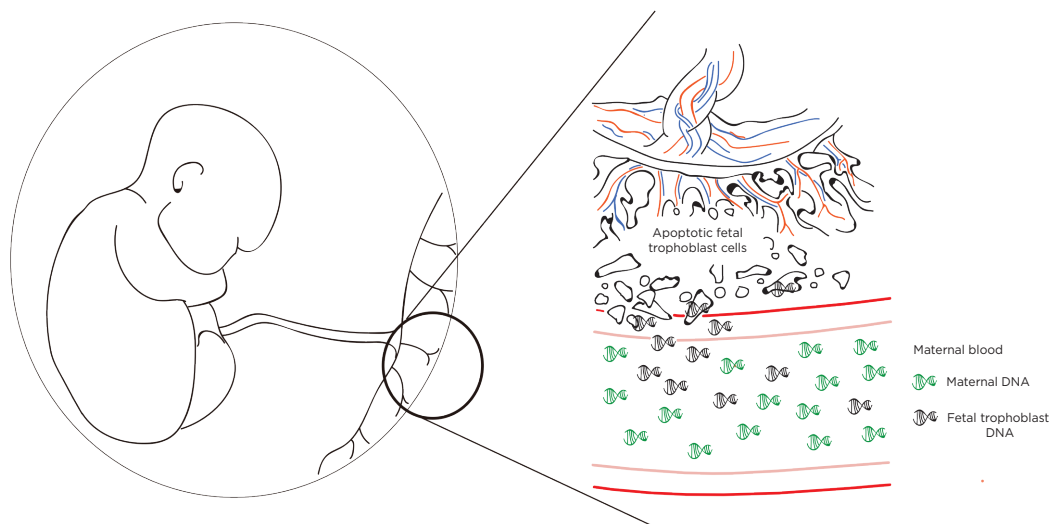


Illustration 3. Free fetal DNA from trophoblast apoptosis entering the maternal plasma

the fetus: a reliable NIPT result relies on the genetic concordance between the two. Clinical applications of NIPT are currently limited to the detection of quantitative chromosomal imbalances⁴⁶ and de novo and paternally inherited autosomal dominant or compound heterozygous monogenic diseases⁴⁷.

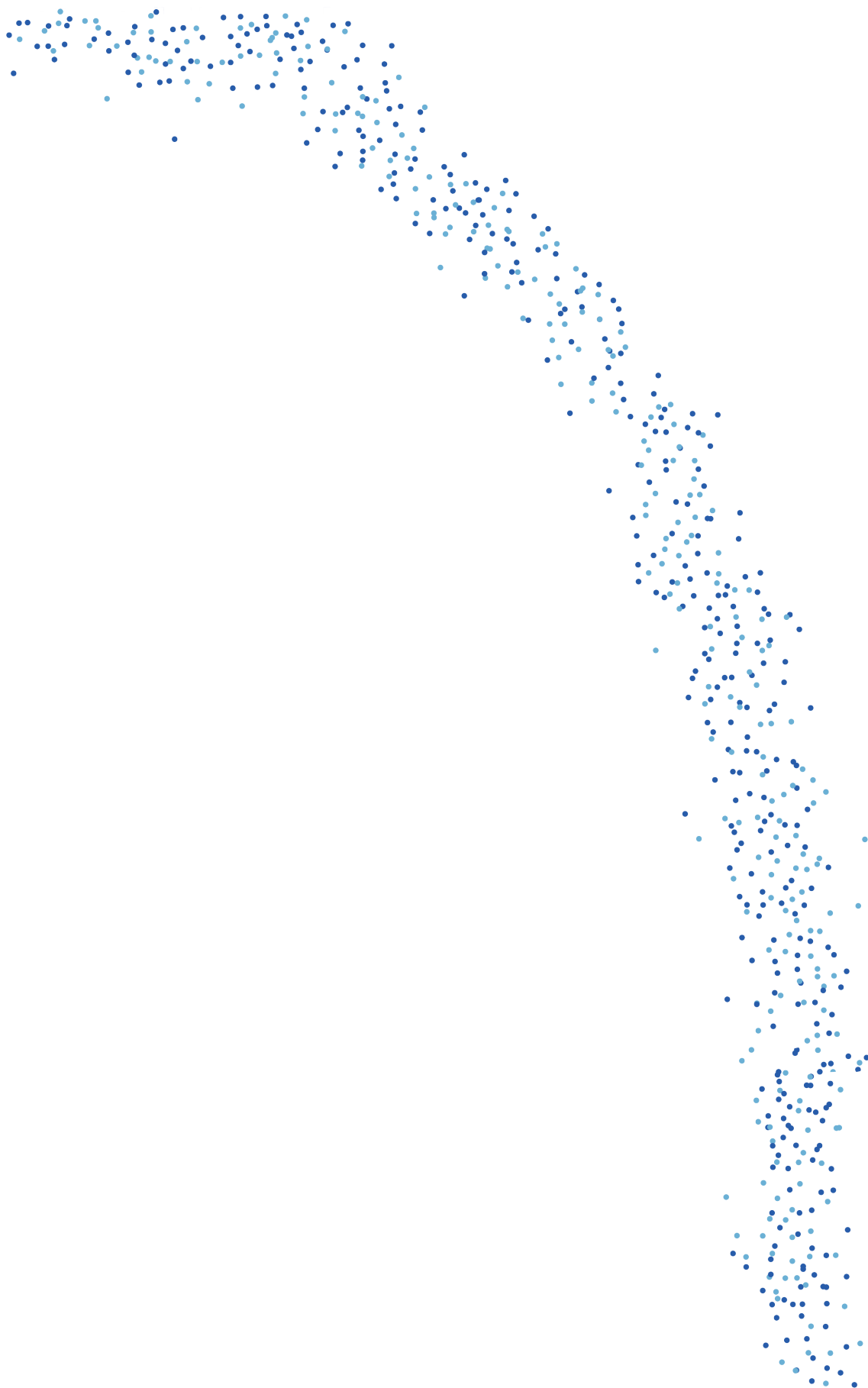
Outline of the thesis

From traditional karyotyping, the first important step in increasing genome wide fetal testing resolution was the development of the chromosomal microarray technology. Microarray can detect chromosomal micro-imbalances, whereas with prenatal karyotyping only imbalances greater than 5-10 megabases (Mb) could be seen. Although it is sheer logic that an increased resolution means the retrieval of more genomic information, only a few studies reported on how much more clinically relevant information could be retrieved in cases with specified abnormalities on ultrasound. **Chapter 2**

describes a systematic review of the literature hitherto on the additional diagnostic value of microarray over karyotyping in structural fetal malformations in certain anatomic systems. The following chapter includes studies on the integral value of microarray in three distinct prenatal ultrasound anomalies: small for gestational age fetal growth, thick nuchal translucency, and isolated agenesis of the corpus callosum. These three entities have in common that they are extremely varied in etiology and prognosis, complicating pretest counseling. Small for gestational age (SGA) fetal growth is a common ultrasound finding. **Chapter 3.1** focuses on the integral value of microarray in the prenatal counseling of and the prenatal care for SGA pregnancies. Another often encountered non-structural ultrasound abnormality is a thick first trimester nuchal translucency (NT). A thick NT has a strong etiological link with fetal aneuploidy. As technical advances made it possible to reliably screen for common aneuploidies through risk-free NIPT, the value of invasive

prenatal testing was questioned. In **Chapter 3.2** the yield of invasive microarray over the yield of the available techniques for non-invasive prenatal diagnosis was tested. In **Chapter 3.3** not only the value of microarray was tested but, by studying the postnatal outcomes of fetuses with isolated agenesis of the corpus callosum (ACC), we also aimed to estimate the diagnostic value of an even more advanced genomic testing technique, whole exome sequencing. In the prenatal follow-up study described in **Chapter 3.4**, the diagnostic potential of fetal WES is further examined in a prenatally ascertained cohort of fetuses affected by the most common congenital malformations of all, those of the heart. The introduction of more advanced genomic techniques, WES in particular, imposes ethical debate. By improving diagnostic yield for clinically relevant, morbidity or intellectual disability causing genomic aberrations, the diagnostic yield for mutations of uncertain clinical significance and mutations for late onset diseases also increases. This uncertainty might harm parents or the fetus that will grow to be a person. Furthermore, with the availability of risk-free NIPT for common aneuploidies, the justification for offering invasive sampling for advanced genomic testing could be questioned. **Chapter 4** introduces our manifesto on the offer of invasive prenatal sampling for advanced fetal genomic testing in pregnancies with congenital malformations.

02



Chapter 2

Additional value of prenatal genomic array testing in fetuses with isolated structural ultrasound abnormalities and a normal karyotype: a systematic review of the literature

M.C. de Wit, M.I. Srebniak, L.C.P. Govaerts, D. van Opstal, R.J.H. Galjaard,
A.T.J.I. Go

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Abstract

Objective

To establish the prevalence of submicroscopic genetic copy number variants (CNVs) in fetuses with a structural ultrasound anomaly (restricted to one anatomical system) and a normal karyotype. The aim was to determine the diagnostic and prognostic value of genomic array testing in these pregnancies.

Methods

Embase and PubMed databases were systematically searched for all relevant articles on prevalence of pathogenic submicroscopic CNVs in fetuses with ultrasound anomalies. Reported cases were sorted into groups according to anatomical site of the detected ultrasound anomaly. The prevalence of causative submicroscopic CNVs was calculated for each group.

Results

Combined data of the reviewed studies (n=18) indicated that fetuses with an ultrasound anomaly restricted to one anatomical system (n=2220) had a 3.1– 7.9% chance of carrying a causative submicroscopic CNV, depending on the anatomical system affected. This chance increased to 9.1% for fetuses with multiple ultrasound anomalies (n=1139).

Conclusion

This review indicates that 3.1– 7.9% of fetuses with a structural ultrasound anomaly restricted to one anatomical system and a normal karyotype will show a submicroscopic CNV, which explains its phenotype and provides information for fetal prognosis. Therefore, we conclude that microarray has considerable diagnostic and prognostic value in these pregnancies.

Introduction

In prenatal medicine, diagnosing the etiology of structural fetal malformations detected by ultrasound is important for determining fetal prognosis and recurrence risk in future pregnancies. Karyotyping has been the cornerstone in prenatal genetic diagnosis in the last decades. Fetuses with multiple congenital abnormalities have a chance of $\geq 18\%$ ⁴⁸ of carrying a microscopic chromosomal abnormality, which justifies the risk of a miscarriage accompanying an invasive procedure necessary for genetic diagnosis. However, the chance of an individual fetus with an isolated malformation on ultrasound having a chromosomal aberration is not well established, with different studies showing variable results^{49, 50}.

Recently, some prenatal centers implemented genomic microarray as a diagnostic tool in the prenatal setting, instead of, or in addition to, karyotyping. Genomic microarray achieves a much higher resolution than does karyotyping, revealing submicroscopic copy number variants (CNVs). These small CNVs are common in the human genome^{51, 52}, but some can be of etiologic importance in congenital malformations and syndromes. The presence of such a causative CNV can be of great importance in estimating pregnancy outcome and prognosis for a fetus with ultrasound anomalies, especially regarding the probability of intellectual disability⁵³.

(Potentially) clinically significant submicroscopic CNVs detected by microarray are found in 5.2–10% of fetuses with ultrasound anomalies and a normal karyotype^{28, 29}. However, there is little information on the prevalence of causative submicroscopic CNVs in fetuses with ultrasound anomalies restricted to one specific anatomical system.

The probability of finding a causative CNV is an important consideration in the decision

as to whether to perform invasive prenatal microarray testing. This literature review aims to provide an overview of the prevalence of causative genomic submicroscopic imbalances in pregnancies with a structural ultrasound anomaly isolated to a single anatomical system and in pregnancies with multiple ultrasound anomalies.

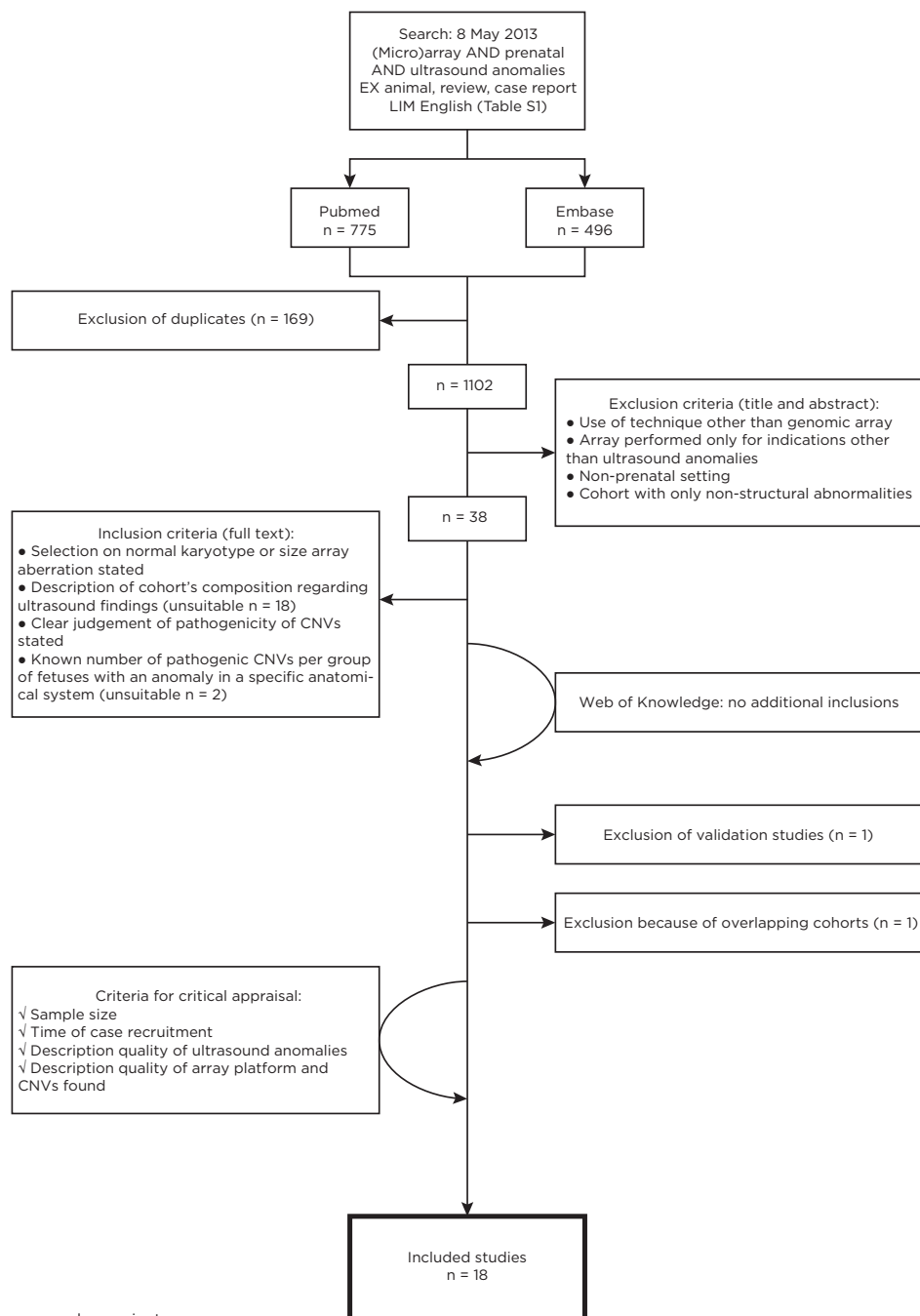
Methods

Search strategy

Embase and PubMed databases were searched in order to collect all relevant articles on the prevalence of pathogenic submicroscopic CNVs in fetuses with ultrasound anomalies, published to date. The search strategy was developed in consultation with a research librarian, with systematic review experience, on 8 May 2013. A string of the following terms and their synonyms was used: 'array', 'prenatal' and 'ultrasound anomalies' (Table S1). The search was restricted to publications in the English language and to human populations involving more than five subjects (Figure 1). Literature reviews were excluded from the search.

Study selection

Four independent reviewers (M.S., L.G., A.G. and M.C.W.) screened the titles and abstracts of all retrieved articles for relevance (Figure 1). Studies that did not use a genomic array technique in the prenatal setting (prospectively or retrospectively) were excluded, as were studies that performed prenatal genomic array only for indications other than ultrasound anomalies (such as advanced maternal age, intrauterine fetal death). As the aim was to describe the prevalence of pathogenic submicroscopic CNVs in fetuses with structural defects, studies of only non-structural fetal ultrasound abnormalities were excluded. The following sonomarkers (single umbilical artery,

Figure 1. Search strategy flow chart.

CNV, copy number variant.

echogenic bowel, echogenic cardiac focus, pyelectasis, short longbones, choroid plexus cysts, clinodactyly and nuchal translucency (NT) < 3.5 mm), as well as undefined fetal hydrops, growth abnormalities, amniotic fluid alterations and fetal akinesia, were considered as non-structural abnormalities. Studies only of abnormalities that cannot be detected by routine ultrasound examination (e.g. dysmorphic facies and brain defects only detected by magnetic resonance imaging) were also not selected for this review.

The full-text versions of 38 articles were obtained and underwent a second trial of selection according to strict inclusion criteria, in order to obtain a homogeneous pooled population from which the prevalence of pathogenic submicroscopic CNVs could be calculated in groups of pregnancies with an ultrasound anomaly (in a specific anatomical system).

Studies were selected that:

- Described fetuses with a known normal karyotype and described their cohort in such a manner that fetuses with large genomic aberrations of > 10 Mb and/or chromosome aberrations detectable by rapid aneuploidy detection (RAD), which would have already been detected if karyotyping was performed, were able to be excluded.
- Described the composition of their cohort regarding the number of fetuses with a structural ultrasound anomaly in a specific anatomical system (e.g. heart defects). This could include a general description of the number of fetuses in which an anomaly was found in a certain anatomical system, or a specific description of each included case. By having this information, all cases with exclusively non-structural anomalies could be excluded.
- Reported a clear judgment of the CNVs

regarding their presumed nature, for example, pathogenic or benign.

- Showed the pathogenic CNVs in detail or gave the number of pathogenic CNVs per group of fetuses with an anomaly in a specific anatomical system.

All disagreements regarding exclusion or inclusion were resolved by discussion between the four reviewers until full agreement was achieved. After the final selection process, Web of Knowledge was used to screen the gray literature. The selected articles were subjected to a critical appraisal designed by the authors. This appraisal included features of validated quality assessment tools and used sample size, cohort selection, study perspective, the description quality of ultrasound findings and CNV classification as the parameters for assessment.

Data extraction

Only cases that met the required criteria (as described above) were included in this review. The cases with structural abnormalities restricted to one anatomical system were categorized according to the system described to be affected. Hereby, general groups were formed (Table S2). Fetuses with a specific structural abnormality and one or more non-structural abnormality were classified according to the structural abnormality. Increased (> 3.5 mm) NT and cystic hygroma were separated into two groups because the two phenomena have a different prognosis^{54, 55}.

Cases with multiple structural abnormalities were sorted in a separate group unless the malformations were all part of a sequence (e.g. clubfeet and a neural tube defect, classified as a central nervous system anomaly).

An array result was assumed to be causative and pathogenic if the reporting author classified it as such. Most often, authors

named such abnormalities as 'pathogenic' or 'clinically significant'. An exception is a study of Tyreman et al.⁵⁶ who classified causative abnormalities as 'likely pathogenic'. 'Incidental findings' (classified as such by the reporting author) were reported separately (Table S3) and were not included in prevalence calculations.

For every group of fetuses with an ultrasound anomaly restricted to one particular anatomical system, the prevalence of pathogenic CNVs and its 95% CI was calculated.

Results

The four reviewers agreed on the relevance and sufficient quality of 20 articles. Screening the gray literature did not yield another publication suitable for inclusion. One of the selected articles was excluded from further analysis because this article was found to be a validation study²⁷ with highly selected cases. Another article⁵⁷ was rejected because the described cases were also included in a more recent paper by the same author⁵⁸. Consequently, the final analysis includes 18 studies (Table S4). One⁵⁹ of these 18 was published online only, yet fully peer reviewed, when this review was submitted.

Prevalence of submicroscopic CNVs in fetuses with congenital abnormalities

The prevalence of clinically significant submicroscopic CNVs in groups of fetuses with a particular anatomical system affected, or with multiple congenital abnormalities is shown in Table 1. The pooled data from all reviewed studies indicate that 5.6% (95% CI, 4.7– 6.6%) of fetuses (n=2220) with an ultrasound anomaly restricted to one anatomical system and 9.1% (95% CI, 7.5– 10.8%) of fetuses (n=1139) with multiple anomalies carried a pathogenic submicroscopic CNV.

Although we aimed to homogenize the cohorts, the differences in prevalence of pathogenic submicroscopic CNVs between the reviewed studies remained (0– 11.1%; Table 1). The most prevalent discrepancies can most probably be explained by the predominantly small sample sizes involved: only four studies describe cohorts of more than 100 fetuses^{29, 60–62}. Furthermore, these discrepancies can partially be explained by differences in cohort selection and by differences between the array platforms applied.

Cohort selection

Studies selected their cases using different criteria, ranging from 'major ultrasound anomalies' to 'any abnormality detected by ultrasound' (Table S4; see 'Inclusion criteria of the original study'). The cohort of Kleeman et al.⁶³ consists of fetuses with isolated (40%) as well as multiple (60%) defects. They classified the fetuses in the latter group according to the most severe malformation present. The same methodology seems to be (partially) used by Lee et al.⁶¹. For example, they classified a fetus with multiple defects (heart anomaly and cleft lip) in their group of isolated heart defects (Table S3; Case 50).

Non-suitable cases

To create a homogeneous case pool of fetuses with structural malformations and a normal karyotype, all cases that did not meet our criteria were excluded from pooling. Cases with non-structural anomalies only (Table S4; 'Nonstructural or unspecified anomalies excluded for pooling') or unspecified ultrasound findings were described in every study reviewed. Although in general these cases were not difficult to identify and exclude, we encountered a few difficulties regarding this process of selection and exclusion. Tyreman et al.⁵⁶ and Hillman et al.²⁹ classified multiple sonomarkers and multiple structural

Table 1. Prevalence of pathogenic submicroscopic copy number variants (CNVs) among fetuses with an ultrasound anomaly in a specific anatomical system

Reference	Isolated anomalies										
	Cardiac	Resp.	CNS	Facial	Musculo-skeletal	GI	Urogenital	NT>3.5 mm	Cystic hygroma	Total	All anomalies
Tyrenan (2009) ⁵⁶	3/34	—	16	—	6	2	—	—	—	3/58 (5.2)	6/24* (11.0)
Vialard (2009) ⁶⁵	—	—	—	—	—	—	—	—	—	—	3/36 [‡] (8.3)
Coppinger: WG (2009) ⁶⁰	13	1	2/15	6	1/6	2	4	—	1/13	4/60 (6.7)	5/122 (4.1)
Coppinger: TA (2009) ⁶⁰	—	1	3	—	—	—	—	—	4	0/8	0/15
Kleeman (2009) ⁶³	23	—	6	2	6	2	3	—	—	0/42	0/1
Evangelidou (2010) ⁸⁵	2	—	1	2	1	—	2	—	—	0/8	—
Leung (2011) ⁶⁷	—	—	—	—	—	—	—	2/38	—	2/38 (5.3)	4/48 (8.3)
D'Amours (2012) ⁶⁶	2	2	1/11	—	—	1	1/3	—	4	2/23 (8.7)	2/24 (8.5)
Lee (2012) ⁹¹	6/49	—	4/22	—	1/22 [‡]	1/7	—	—	—	12/100 (12.0)	15/136** (11.0)
Shaffer (2012) ⁶²	6/237	4/50	25/382	6/88	18/203	6/69	7/115	—	10/232	82/1376 (6.0)	77/808 (7.3)
Faas (2012) ⁸⁸	1/10	4	7	2	—	—	—	27	—	1/50 (2.0)	2/90 (2.2)
Ganesamoorthy (2013) ⁸⁹	—	—	26	—	1/16	3	—	—	—	1/45 (2.2)	4/70** (5.7)
Mademont-Soler (2013) ⁶⁴	28*	—	—	—	—	—	—	—	—	0/28	1/51* (2.0)
Rooryck (2013) ⁹⁶	—	—	—	—	—	—	—	2/56§	—	2/56 (3.6)	—
Schmid (2013) ⁹⁸	18	2	1/8	2	2	1	2	—	—	1/35 (2.9)	4/10 (11.1)
Hillman (2013) ⁹⁹	4/39	20	2/51	7	24	15	20†	—	—	6/176 (3.4)	0/11*
Scott (2013) ⁷⁰	12	—	—	—	—	—	—	1/41	1/9	2/62 (3.2)	2/62** (3.2)
Vestergaard (2013) ⁹⁹	2/9	1/1	15	4	3/19	3	1/4	—	—	7/55 (12.7)	1/22†
Pooled prevalence [95% CI]	22/476 (4.6)	5/81 (6.2)	35/563 (6.2)	6/113 (5.3)	24/305 (7.9)	7/105 (6.7)	9/153 (5.9)	5/162 (3.1)	12/262 (4.6)	125/2220 (5.6)	104/1139 (9.1)
	[2.7– 6.5]	[0.9– 11.4]	[4.2– 8.2]	[1.2– 9.4]	[4.8– 10.9]	[1.9– 11.4]	[2.2– 9.6]	[0.4– 5.7]	[2.0– 7.1]	[4.7– 6.6]	[7.5– 10.8]

Only the first author of each study is given. Data given as number of fetuses with a pathogenic array result (n)/total number of fetuses per study (N), n/N (%) or N unless indicated otherwise.

*Includes also (multiple) sonomarkers; † One case excluded because of only non-structural anomalies on ultrasound; ‡ incidental finding in this group (Table S3); §One case excluded because of cystic hygroma in addition to nuchal translucency (NT) > 3.5 mm; ¶Cases with enlarged NT and cystic hygroma combined in one group, therefore excluded; **An unknown number of rapid aneuploidy detection or karyotype abnormalities included

CNS, Central Nervous System; GI, Gastrointestinal; Resp., Respiratory; TA, Targeted Analysis; WG, Whole Genome analysis

anomalies in one group and therefore we could not exclude all cases with sonomarkers only. Mademont-Soler et al.⁶⁴ described fetuses with both structural and non-structural cardiac anomalies, and 45.1% (n=23) of these cases had extracardiac (non)structural anomalies (Table 1). Unfortunately, this paper also does not allow the identification of cases with non-structural ultrasound malformations only. Vialard et al.⁶⁵ claim that they solely included cases with structural ultrasound anomalies, but nevertheless describe a fetus with isolated intrauterine growth restriction (IUGR) on ultrasound (Case 29, excluded from this review). Lee et al.⁶¹ included a fetus with isolated short femur (Case 45, excluded from this review) and assigned this case to the group of skeletal anomalies. This leads to the presumption that these authors also included fetuses with (multiple) non-structural anomalies only. As Vestergaard et al.⁵⁹ and Hillman et al.²⁹ combine cases with an enlarged NT and cystic hygroma in one group, we could not pool these cases.

Six studies used a preceding normal karyotype as a criterion for inclusion^{56, 63-67}, among which two studies^{56, 64} also selected their cases based on normal fluorescence in-situ hybridization (FISH) results of the 22q11 region. This might explain the relatively low number of 22q11 deletions detected by arrays reported in this review.

In all other reviewed studies, a normal karyotype was not a condition consistently required for inclusion (Table S4; 'Selection on results of preceding tests'). Faas et al.⁶⁸, Hillman et al.²⁹ and Schmid et al.⁵⁸ performed RAD for the 21, 18, 13, X and Y chromosomes preceding inclusion and excluded cases with an abnormal result. Coppinger et al.⁶⁰ excluded all cases with a known abnormal karyotype, but the karyotype was not known in all cases. Only 72% of the cohort of Shaffer et al.⁶² had a normal karyotype. We assumed that the remaining cases underwent at least RAD before

microarray testing, as the detection rate of pathogenic CNVs by array differed only slightly between the total cohort and the cohort with a normal karyotype. Unfortunately, as the distribution of array anomalies > 10 Mb and < 10 Mb is not stated clearly in this article, we could not exclude the chromosome aberrations that would have been detected by karyotyping, as in the case of other publications^{60, 61, 63, 68}. It also seems that Ganesamoorthy et al.⁶⁹ performed RAD before microarray testing, as they do not describe any fetuses with aneuploidies. Scott et al.⁷⁰ and Lee et al.⁶¹ performed RAD and karyotyping, respectively, but we were unable to determine whether the cases with an abnormal result were excluded from these two studies.

Array resolution and CNVs of clinical significance

There is enormous variety in the array platforms used in the reviewed studies regarding array technology (e.g. single nucleotide polymorphisms (SNPs) and bacterial artificial chromosomes (BACs)) and their resolution (Table S4). Five studies even described the use of multiple platforms^{60, 62, 63, 66, 69}. Correction for these forms of heterogeneity is not possible. Unfortunately, there is no consensus regarding minimal resolution of array used in prenatal testing. The resolutions vary from no backbone⁶⁰ to high-density SNP arrays⁵⁶.

All reviewed studies did mention a similar way of interpreting array results. The etiologic role of CNVs was evaluated based on size, inheritance, gene content and previous stated clinical relevance (from DECIPHER and OMIM, or in the published literature from databases such as MEDLINE) and reference populations (public/private). Although it seems that all authors used a similar strategy for array analysis, the interpretation of array results may change as more new syndromes emerge from large postnatal cohort studies⁵³. The interpretation in 2009 may differ from that performed in 2013.

Some of the CNVs presented by the reviewed studies may need reclassification, but in order to be able to present original data we did not do so. Although Shaffer et al.⁶² did not provide the array abnormalities found in particular cases, they reassigned previously classified variants of unknown clinical significance (VOUS) to the normal or abnormal groups as appropriate based on new knowledge gained from the medical literature and from their own experience since the initial reporting of the case. In the paper by Vestergaard et al.⁵⁹, one of the pathogenic CNVs detected in a fetus with a cerebral anomaly is not reported and was therefore excluded. All reported clinically significant CNVs found in fetuses with ultrasound abnormalities are presented in Table S3. Etiologically important losses ($n=54$) of chromosomal material are more frequent than are gains ($n=7$). Most CNVs are located in the interstitial part of the chromosome. Only seven unbalanced translocations were found. Uniparental disomy and low-level mosaicism were both reported once.

In addition to the detection of causal CNVs, genomic array detected two pathogenic CNVs (of 3359) that were not likely to be causal for the fetal phenotype detected by ultrasound ('incidental findings', classified as such by the reporting author). These findings are not included in our prevalence calculations. Although one would expect more incidental findings when a whole genome array platform is applied, one of the incidental findings reported in this review was found by Hillman et al.²⁹, who used a targeted array design with a backbone resolution of > 2 Mb.

Discussion

This review indicates a 5.6% (95% CI, 4.7– 6.6) chance of finding a causal submicroscopic CNV in fetuses with a structural ultrasound anomaly

restricted to one anatomical system and a normal karyotype, leading to the presumption that one in every 20 fetuses shows a submicroscopic CNV that explains its phenotype and will provide prognostic information. The lowest prevalence of pathogenic submicroscopic CNVs is found in the group of first-trimester fetuses with NT ≥ 3.5 mm (3.1%; 95% CI, 0.4– 5.7); this may reflect the aspecific character of this finding⁷¹. Cardiac anomalies are one of the most common fetal malformations. The prevalence of pathogenic submicroscopic CNVs in this group was 4.6% (95% CI, 2.7– 6.5), which agrees with postnatal publications on infants with non-syndromic structural heart defects (0– 4.3%)^{72–74}. Pathogenic genomic micro-imbalances are most frequently found in polymalformed fetuses (9.1%; 95% CI, 7.5– 10.8).

This review shows that 6.8% (95% CI, 6.0– 7.7) of fetuses with isolated or non-isolated ultrasound anomalies and a normal karyotype carry a pathogenic submicroscopic CNV. This prevalence is in accordance with the prevalence reported by Hillman et al. in 2011 (5.2%; 95% CI 1.9– 13.9)²⁸ but lower than that reported by Hillman et al. in 2013 (10%; 95% CI, 8– 13)²⁹. The discrepancy between their last meta-analysis and this review probably reflects the fact that they included a larger number of fetuses with multiple anomalies. Furthermore, they report the prevalence of both pathogenic and potentially pathogenic CNVs, whereas we report pathogenic CNVs only. The difference between the prevalence of pathogenic CNVs only and the prevalence of both VOUS and pathogenic CNVs is likely to increase with the use of high-resolution genome-wide array platforms, which have been applied more often in recent literature.

The clinically relevant submicroscopic CNVs seen in the studied cohort are spread through the whole genome; therefore, screening for subtelomeric abnormalities before array testing is not advised. Screening

with FISH for the 22q11 microdeletions before array testing seems to be of limited value, as Tyreman et al.⁵⁶ missed one atypical deletion nested in the distal DiGeorge region. Two pathogenic CNVs (of 3359) that are not likely to be causal for the fetal phenotype detected by ultrasound were reported by the reviewed studies^{29, 59}. Therefore, we conclude that an 'incidental finding' could be expected in about one of 1500 pregnancies tested by microarray.

The amount of published literature on the prevalence of submicroscopic CNVs among fetuses with specific ultrasound anomalies is small. The main reason for this is the fact that most studies on this subject describe only the cases in which a causative CNV was found and fail to describe their whole cohort^{26, 30, 74-83}. Such studies did not meet our selection criteria because calculation of prevalence is impossible when a cohort's composition is not described. Furthermore, many reports describe ultrasound anomalies in an irreproducible way or combine prenatal and postnatal findings^{25, 84}.

To reinforce the evidence for the value of genomic array in pregnancies with particular isolated fetal malformations detected by ultrasound, additional publications, which give a detailed description of the ultrasound anomalies found just before invasive sampling and the corresponding array result per case, are required. The application of an internationally recognized fetal phenotype coding system would be of great value because such a system would allow better comparison of cases tested by different researchers. The use of such a system would contribute to a more reliable calculation of the prevalence of pathogenic submicroscopic CNVs in specific structural fetal defects (e.g. cleft lip or clubfeet) and the discovery of novel pathogenic CNVs. The International Standards for Cytogenomic Arrays (ISCA) phenotype form (<https://www.iscaconsortium.org>) is a good instrument that uses Human Phenotype Ontology (HPO)

terms. We suggest completing this form by adding more diagnoses (e.g. jugular sacs and the double bubble sign) and further defining and specifying certain codes. Cases with multiple congenital abnormalities should not be classified according to the most severe anomaly, but should be registered as a separate category.

To allow the discovery of new pathogenic CNVs, whole-genome array platforms should be recommended in the prenatal setting and a consensus concerning the minimal requirements for a prenatal array platform would be useful in comparing cohorts. If the local policy does not allow genome-wide analysis, two-step analysis could be performed: targeted analysis with a lower backbone resolution in diagnostic settings and retrospective genome-wide high-resolution analysis in research settings. Both pathogenic CNVs and VOUS should be published and human genome build should always be stated. Furthermore, a clear definition for the term 'incidental finding' should be established.

Postnatal follow-up in cases with specific malformations on ultrasound is essential in drawing conclusions on the impact of the microarray in prenatal diagnosis. There is a gap between the literature focusing on prenatal and postnatal phenotypes, while combining the settings in a continuum would give more information about fetal prognosis. More prenatal cases should be submitted to databases such as ISCA or DECIPHER and the corresponding prenatal phenotype (if known) should also be added if a postnatal case is submitted. In this regard, a well-structured international registry for prenatal cases could also be of great value.

Lastly, a selection bias enters publications when only those pregnancies are included which underwent invasive prenatal genetic testing, because pregnancies with multiple defined or undefined characteristics (the

presence of non-structural anomalies, obstetric history, etc.) will be more likely to be subjected to invasive procedures than will pregnancies with only isolated fetal defects on ultrasound. Analysis of cohorts consisting of fetuses with a specific anomaly with and without prenatal genetic diagnosis would also be informative to estimate true fetal prognosis and the value of prenatal array testing at the time of detecting a fetal structural anomaly with ultrasound.

Conclusions

In conclusion, based on the available literature, we conclude that genomic microarray should be performed in fetuses with both isolated and multiple ultrasound anomalies. It detects causative submicroscopic CNVs in 5.6% of fetuses with isolated anomalies and in 9.1% of fetuses with multiple anomalies. The implementation of an international uniform classification system for ultrasound abnormalities is necessary to contribute to the comparability of cohorts.

Table S1. Search query specifications

PubMed (array* OR microarray*[Title/Abstract]) AND (prenatal OR antenatal[Title/Abstract])) NOT (review[filter]) AND English[filter]
EMBASE (('DNA microarray'/exp OR (((DNA OR gene* OR genom*) NEAR/3 (microchip* OR Chip*)) OR array* OR microarray* OR ((submicroscop* OR chromosom*) NEAR/3 gain*)):ab,ti) AND ('prenatal diagnosis'/exp OR 'prenatal period'/de OR Fetus/de OR (prenat* OR antenat* OR fetus OR fetal OR foetus OR foetal):ab,ti) AND (Ultrasound/de OR echography/exp OR (ultraso* OR echogra* OR echoscop* OR sonogra*):ab,ti) NOT ([animals]/lim NOT [humans]/lim)

Table S2. Classification of ultrasound anomalies

Cardiac anomalies	Anomalies of the heart and great vessels
Respiratory anomalies	Congenital diaphragmatic hernia, anomalies of the lungs
CNS anomalies	Intra-cerebral anomalies, microcephaly, neural tube defects
Facial anomalies	Cleft lip / palate, micrognathia, tumors of the face and ears
Musculoskeletal anomalies	Skeletal deformity (scoliosis, arthrogryposis), skeletal dysplasia, positional abnormalities of the hands and feet (club, rocker bottom)
Gastrointestinal anomalies	Abdominal wall defect, tracheoesophageal fistula, gastrointestinal atresias
Urogenital anomalies	(Cystic) kidney malformations / agenesis, hydronephrosis, echogenic kidneys, bladder malformations, hypo- or epispadias, ambiguous genitalia
Increased NT	First trimester nuchal translucency $\geq 3.5\text{mm}$

Table S3. Pathogenic copy number variations (defined by the authors) reported by the reviewed studies. Chromosome aberrations $\geq 1\text{Mb}$ included only if missed by karyotyping. Human genome build added when specified in the original report

Cardiac anomalies				
Tyreman et al. 2009	Large VSD	arr[hg17] 17q21.31q21.31(41,014,074-41,569,931)x1	LOSS 0.5Mb at 17q21.31: 17q21.31 microdeletion syndrome	
Tyreman et al. 2009	Heart defect	arr[hg17] 22q11.21(9,046,924-19,795,835)x1	LOSS 0.75Mb nested within the typical 3 Mb 22q11.2 microdeletion area	
Tyreman et al. 2009	Hypoplastic left heart	arr[hg17] 15q26.2(92,557,725-96,128,332)x1	LOSS 3.5Mb at 15q26.2	
Lee et al. 2012	Case 33: ToF	arr cgh 4q35.2(RP11-213A19-> RP11-521G19)x1 mat	LOSS 3.6Mb at 4q35.2	
Lee et al. 2012	Case 34: ToF	arr cgh 22q11.2(RP11-690P21-> RP11-116M14)x1 dn	LOSS 2.4Mb at 22q11.21: DiGeorge syndrome	
Lee et al. 2012	Case 35: ToF	arr cgh 22q11.2(RP11-690P21-> RP11-116M14)x1 dn	LOSS 2.4Mb at 22q11.21: DiGeorge syndrome	
Lee et al. 2012	Case 36: ToF	arr cgh 22q11.2(RP11-690P21-> RP11-116M14)x1 dn	LOSS 2.4Mb at 22q11.21: DiGeorge syndrome	
Lee et al. 2012	Case 37: VSD, pulmonary artery stenosis	arr cgh 22q11.2(RP11-690P21-> RP11-116M14)x1 dn	LOSS 2.4Mb at 22q11.21: DiGeorge syndrome	
Lee et al. 2012	Case 50: Cleft lip, VSD*	arr cgh 17pterp13.1(RP11-629C16-> RP11-736G8)x3 dn	GAIN 10.18Mb at 17p	
Faas et al. 2012	ToF	arr[hg19] 22q11.21(8,895,227-21,277,471)x1 dn	LOSS 3.2Mb at 22q11.21	
Hillman et al. 2013	Case 19: VSD, aortic coarctation	arr cgh 22q11.2(RP11-800B02->RP11-330P17)x1 dn	DiGeorge syndrome	
Hillman et al. 2013	Case 20: Double outlet right ventricle	arr cgh 22q11.2(RP11-800B02->RP11-330P17)x1 dn	DiGeorge syndrome	
Hillman et al. 2013	Case 21: ToF	arr cgh 22q11.2(RP11-800B02->RP11-330P17)x1 dn	DiGeorge syndrome	
Hillman et al. 2013	Case 22: Truncus arteriosus	arr cgh 22q11.2(RP11-800B02->RP11-330P17)x1 mat	DiGeorge syndrome	
Vestergaard et al. 2013	Case 982: Steno-Fallot	arr[hg19] 11p15.5(210,100-8,190,071)x3,17p13.3 (86,809-1,724,253)x1 dn	Unbalanced t(11;17): GAIN 7.9Mb (27 morbid OMIM genes) and LOSS 1.6Mb (2 morbid OMIM genes)	
Vestergaard et al. 2013	Case 1516: Steno-Fallot	arr[hg19] 16p13.12p13.11(14,762,239-16,292,235)x1 dn	LOSS 1.5Mb at 16p13 (3 morbid OMIM genes, overlap with 16p13.11 deletion syndrome)	
Respiratory anomalies				
Vestergaard et al. 2013	Case 933: CDH	arr[hg19] 15q26(94,602,969-98,554,319)x1 dn	LOSS 4Mb at 15q26 (NR2F2, MCTP2, SPATA8, ARDDC4 genes)	
CNS anomalies				
Coppinger et al. 2009 (WG)	Case 1: Holoprosencephaly	arr 7q36.1q36.3(151,071,240-158,788,150)x1 pat, 8q24.3(141,793,142-146,236,298)x3 pat	Unbalanced t(7;8): LOSS of 7.6Mb of 7q36.1q36.3 (SHH region) and a GAIN of 4.4Mb of 8q24.3	
Coppinger et al. 2009 (WG)	Case 5: Holoprosencephaly	arr 14q12q21.1(28,168,333-37,866,321)x1 dn	LOSS 9.7Mb at 14q12.211 (HPE8 region)	
D'Amours et al. 2012	Fetus 20: Alobar holoprosencephaly, facial abnormalities (cyclopia, proboscis, arhinia)	arr cgh[hg18] 2p21(44,949,357-45,184,052)x1dn	LOSS 0.23Mb at 2p21 (SIX2 and SIX3 region)	
Lee et al. 2012	Case 39: Lissencephaly, agenesis of corpus callosum	arr cgh 22q11.2(RP11-690P21-> RP11-116M14)x1 dn	LOSS 2.4Mb at 22q11.21: DiGeorge syndrome	
Lee et al. 2012	Case 41: Hydrocephalus, cerebellar hypoplasia	arr cgh 6q27(RP11-841B23-> CTD-201D14)x1 dn	LOSS 2.8Mb at 6q27	
Lee et al. 2012	Case 42: Lissencephaly	arr cgh 17p13.3(RP11-629C16-> CTD-238E6)x1 dn	LOSS 2.1Mb at 17p13.3: Miller-Dieker syndrome	
Lee et al. 2012	Case 40: NT 3.8 mm, cerebellar hypoplasia, tricuspid regurgitation**	arr cgh 6p25.3p25.3(RP11-42D22-> RP11-1103I2)x1 dn	LOSS 0.85Mb at 6p25.3	
Hillman et al. 2013	Case 17: Bilateral ventriculomegaly	arr cgh 1p36.3p36.32(RP5-857k21->RP11-333E3)x1 dn	1p36 microdeletion syndrome	

Hillman et al. 2013	Case 24: Absent corpus callosum and meningocele	arr cgh 5q35.3(RP11-281O15->RP11-99H18)x1mat	Unbalanced (5:17): LOSS 19Mb at 5q35.3 and an additional GAIN 11Mb at 17q25.3; inherited from a mother who had dyspraxia and mild facial dysmorphism
Schmid et al. 2013	Case 3: Myelomeningocele, SUA	arr 2q35q36.3(219,422,799-226,226,656)x1 dn	LOSS at 2q35-36 (WNT10, WNT6, IHH, EPHA4, PAX3 genes)
Musculoskeletal anomalies			
Coppinger et al. 2009 (WG)	Case 4: Rocker-bottom feet, facial dysmorphism	arr 1p36.33p36.22(769,389-9,786,749)x117p13.3(4,453,594-3,505,282)x1	LOSS 9Mb at 1p36.33p36.22: 1p36 deletion syndrome LOSS 0.0517Mb at 17p13.3 (heterozygous deletion of CTNS (homozygous deletions associated with Nephropathic cystinosis))
Lee et al. 2012	Case 44: Polydactyly, camptodactyly, hypoplasia of fifth finger	arr cgh 8q24.13q24.2(RP11-689C11-> RP11-440N18)x1 dn	LOSS 5.69Mb at 8q24
Ganesamoorthy et al. 2013	Case 3: Radial aplasia, ulnar hypoplasia, short bowed humeri, flexed wrists	arr[hg18] 1q21.1(144,095,255- 144,544,340)x1 pat	LOSS 0.5Mb at 1q21.1 associated with thrombocytopenia with absent radius (TAR) syndrome
Vestergaard et al. 2013	Case 769: Micromelia	arr[hg19] 11q22.3 (103,141,484-103,306,930)x1 pat	LOSS 0.17Mb at 11q22.3 (DYNC2H1 gene)
Vestergaard et al. 2013	Case 1444: Limb contractures, absence of fetal movement, polydactyly of the toes, small for gestational age	arr[hg19] Xq26.2(132,996,180-133,087,198)x1 pat	LOSS 0.091Mb at Xq26.2 containing the GPC3 gene associated with Simpson-Golabi-Behmel syndrome type I in a female fetus
Vestergaard et al. 2013	Case 1484: Arthrogryposis multiplex	arr[hg19] 22q11.21(18,894,835-21,464,119)x1	LOSS 2.6Mb at 22q11.21 (overlap with DiGeorge syndrome)
Gastrointestinal anomalies			
Lee et al. 2012	Case 48: Omphalocele	arr cgh 7p22.3p22.1(RP11-90P13-> RP11-936A1)x1.9p24.3p24.3(RP11-1112G24 -> RP11-635C16)x3 dn	LOSS 5.62Mb at 7p22 and a GAIN 2.19Mb at 9p24.3
Urogenital anomalies			
D'Amours et al. 2012	Fetus 53: Hyperechogenic kidneys, bilateral pyelocaliectasis, vesical distension, urethral cyst	arr cgh[hg18] 10q26.13q26.3(124,590,071-132,729,781)x1 dn	LOSS 8.14Mb at 10q26
Vestergaard et al. 2013	Case 1272: Multicystic kidney disease	arr[hg19] 7p13p12.3(45,060,426-47,393,195)x1 dn	LOSS 2.2Mb at 7p12.13 (CCM2, RAMP3, ADCY1, IGFBP1, IGFBP3, NACAD, TBRG4, TNS3 genes)
Increased nuchal translucency > 3.5mm			
Leung et al. 2011	NT 5.3mm	arr[hg18] 5p14.1(27060752-29521748)x1	LOSS 2.5Mb at 5p14
Leung et al. 2011	NT 4.3mm	arr[hg18] 16q22(67676229-68868604)x1	LOSS 1.2Mb at 16q22
Rooryck et al. 2013	NT 3.5mm	arr[hg19]12q21q22x1 dn	LOSS 13.42Mb at 12q21q22*
Rooryck et al. 2013	NT 9mm	arr[hg19] 22q11.21x1 dn	LOSS 2.52Mb at 22q11.21; DiGeorge syndrome
Scott et al. 2013	NT 4.4mm	arr (9p21.1 21q22.3)x1(9p21.13x1)(9q22.2)x1	LOSS 0.2Mb at multiple sites on chromosome 9
Cystic hygroma			
Coppinger et al. 2009 (WG)	Case 2: Cystic hygroma	arr 4p16.3p15.3(13,419-20,558,819)x1.7p22.3p21.3(106,407-794,7720)x3 mat	Unbalanced t(4;7) with a LOSS of 20.6Mb of 4p16.3p15.31* resulting in partial monosomy 4p (deletion of WHSC1, WHSC2) associated with Wolf-Hirschhorn syndrome, and a GAIN of 7.7Mb of 7 22.3p21.31 resulting in partial trisomy 7p. Mother is carrier of a balanced translocation
Scott et al. 2013	Cystic hygroma	arr 22q11.21q11.22x1 dn	LOSS 1Mb at 22q11.21q11.22; DiGeorge syndrome

Multiple anomalies			
Coppinger et al. 2009 (WG)	Case 3: Ventriculomegaly, megacisterna magna, short limbs, 2-vessel cord	arr Xp22.33/Yp11.32(262,578-967,557)x1.2 2q11.21(7,313,884-19,687,285)x3	GAIN 2.5Mb (reciprocal duplication of 22q11.2) and LOSS 0.7Mb (SHOX associated with idiopathic short stature) in a male fetus
Vialard et al. 2009	Fetus 3: IUGR, left heart hypoplasia, aortic hypoplasia, intraventricular communication cerebral ventriculomegaly	arr cgh 8q24qter(RP11-642A1)RP4-686B(6) x3,1qter(RP11-217L21)RP1-26N(8)x1	Cryptic unbalanced translocation of paternal origin
Vialard et al. 2009	Fetus 27: Bilateral club feet, complex heart defect (conotruncal defect)	arr cgh 22q11.2q11.2 (RP11-157E19 ->RP11-316L10)x1 dn	LOSS 3Mb at 22q11
Vialard et al. 2009	Fetus 39: Heart defect, partial corpus callosum, vermiform hypoplasia	arr cgh 1p36.33p36.22 (RP11-340B24->CTB-124M18)x1	LOSS 6Mb at 1p36
Tyrenan et al. 2009	Multiple VSDs and hypospadias	arr[hg17] 22q11.2q11.22(20,247,141-21,300,127)x1	LOSS 1Mb at 22q11.2 (distal)
Tyrenan et al. 2009	Hydrocephalus, cleft lip and palate, absent radii, short forearms, intestinal atresia	arr[hg17] Xp22.2(14,743,675-14,803,466)x0	LOSS 0.06Mb at Xp22.2 (FANCB gene) in a male fetus
Tyrenan et al. 2009	Hypoplastic left heart and dilated renal pelvises	arr[hg17] 16q24.1q24.2(84,286,000-86,275,006)x1	LOSS 2Mb at 16q24
Tyrenan et al. 2009	Posterior ventriculomegaly, echogenic intracardiac focus, and echogenic bowel	arr[hg17] 15q21.1(42,831,204-43,559,740)x3	GAIN 0.73Mb at 15q21.1
Tyrenan et al. 2009	Cleft lip and palate, renal pyelectasis, and bilateral talipes	arr[hg17] 19p13.2(7,628,636-12,388,852)x3	GAIN 4.76Mb at 19p13.2
Tyrenan et al. 2009	Polyhydramnios, micrognathia, abnormal right pinna, and absent stomach bubble	arr[hg17] 19q13.41q13.42(58,932,027-59,461,178)x3	GAIN 0.53Mb at 19q13.4
Leung et al. 2011	NT >3.5 and unspecified MCA	arr[hg18] 6q22.32(125,422,871-133,319,872)x1	LOSS 71Mb at 6q22
Leung et al. 2011	NT >3.5 and unspecified MCA	arr[hg18] 15q12(21,361,586-26,306,683)x3	GAIN 4.9Mb at 15q12
D'Amours et al. 2012	Fetus 47: Cerebral bilateral ventriculomegaly, mega cisterna magna, cerebellum & vermis atrophy, upward rotation of vermis, aortic stenosis, bicuspid aortic valve	arr cgh[hg18] 3q23q25.32(143,856,784-158,118,852) x1 dn	LOSS 14.26Mb at 3q23*
D'Amours et al. 2012	Fetus 48: Severe clubfeet, right polycystic dysplastic kidney, median ureterocele	arr cgh[hg18] 22q11.21(19,064,244-19,790,658)x1 mat	LOSS 0.73Mb at 22q11.21: deletion within the distal part of DiGeorge region
Lee et al. 2012	Case 52: CDH, intrauterine growth retardation**	arr cgh 4p16.3p15.33(RP11-1076P8 -> RP11-34C20)x1 dn	LOSS 10.91Mb at 4p: Wolf Hirschhorn syndrome
Lee et al. 2012	Case 54: Macrosomia, cryptorchidism, hydrocephalus	arr cgh 9q22.3q22.33(95,279,082-98,503,504)x1 dn	LOSS 3.22Mb at 9q22
Lee et al. 2012	Case 51: Small for gestational age, multicystic kidney**	arr cgh 19q12q13.12(RP11-775H11-> RP11-45E23)x1 dn	LOSS 5.84Mb at 19q
Faas et al. 2012	Cystic hygroma, jugular sacs, congenital heart anomaly (atrioventricular septal defect), low-set ears, frontal bossing, abnormal profile	arr[hg19] 4q21.1q21.21(77,514,977-82,004,241)x1 dn	LOSS 4.5Mb at 4q21
Ganesmoorthy et al 2013	Case 1: CDH, unilateral cleft lip/palate, ↑ NT (1:3 risk for T21 on first-trimester screen)	arr[hg18] Xq26.2(131,336,145-132,612,743)x0 mat	LOSS at Xq26.2 including the GPC3 gene associated with Simpson-Golabi-Beckel syndrome in a male fetus
Ganesmoorthy et al 2013	Case 2: Cardiac and abdominal heterotaxy, complex congenital heart disease, severe cerebral ventriculomegaly (NT 9mm)	arr[hg18] 1q43q44(238,168,460-247,169,378)x1 dn	LOSS 9Mb at 1q43-q44
Ganesmoorthy et al 2013	Case 5: Radial club hands, talipes equinovarus, polyhydramnios, micrognathia (1:20 risk for T21 on first-trimester screen)	arr[hg18] 14q11.2q12.33(19,432,659-106,313,072)x2 hnz	Long contiguous stretches of homozygosity (LCSH) involving chromosome 14, was shown to be uniparental isodisomy 14 of paternal origin

Mademont-Soler et al. 2013	ToF, IUGR, bilateral radial aplasia and ectrodactyly of both hands	arr[hg19] 6q21q22.31(106,457,140-121,353,146)x1 dn*	LOSS 14.9Mb at 6q21q22*
Schmid et al. 2013	Case 2: VSD, truncus arteriosus communis, right sided club hand, polyhydramnios	arr 17p11.2p11.2(16,603,639-20,159,414)x3 dn	GAIN at 17p11.2, Potocki-Lupski syndrome
Schmid et al. 2013	Case 54: IUGR, bilateral club foot, coarctation of the aorta, echogenic bowel, echogenic kidneys, SUA	arr 17p13.3p13.2(513,550,797)x1 mat, 19p13.3p13.2(232,483-10,310,392)x3 mat	Unbalanced t(17;13) of maternal origin
Schmid et al. 2013	Case 64: VSD, IUGR, SUA, deformed spine, cerebellar hypoplasia, ventriculomegaly	arr(7)x2-3 dn	Low level mosaicism trisomy 7*
Schmid et al. 2013	Case 69: left sided CDH, IUGR, VSD, hydrothorax, renal hypoplasia	arr 15q26.1q26.3(91,064,818 -> qter)x1 dn	LOSS 9.1Mb at 15q26
Vestergaard et al. 2013	Case 772: CDH, hypoplastic lung, hydrocephalus, hydrops fetalis	arr[hg19] 5p15.33p14.3(51,986-21,524,152)x1pat, 11p15.5p15.1(1613,746-17,957,663)x3 pat	Unbalanced t(5, 11): GAIN 21.5Mb* (10 morbid OMIM genes, overlap with Cri du Chat syndrome) and LOSS 16.3Mb* (30 morbid OMIM genes resp.) of paternal origin
Incidental findings			
Hillman et al. 2013	Case 23: Bladder outlet obstruction	arr cgh 17p12(RP1-27 J12-RP11-385D13)x1 pat	Including gene PMP22: hereditary neuropathy with liability to pressure palsies
Vestergaard et al. 2013	Case 1505: Dandy-Walker malformation, short extremities and ribs, micrognathia	arr[hg19] Xp21.1(32,497,095-32,584,015)x1	LOSS 0.087Mb at Xp21.1(DMD-gene) in a female fetus Parents not analyzed

*Abnormalities ≥ 11 Mb not excluded because they were missed by karyotyping; **According to original classification of ultrasound abnormalities by the corresponding author

CDH, Congenital Diaphragmatic Hernia; IUGR, Intrauterine Growth Restriction; MCA, Middle Cerebral Artery; NT, Nuchal Translucency; SUA, Single Umbilical Artery; ToF, Tetralogy of Fallot; VSD, Ventricular Septal Defect.

Table S4. Characteristic of the reviewed studies and the description of the cohorts

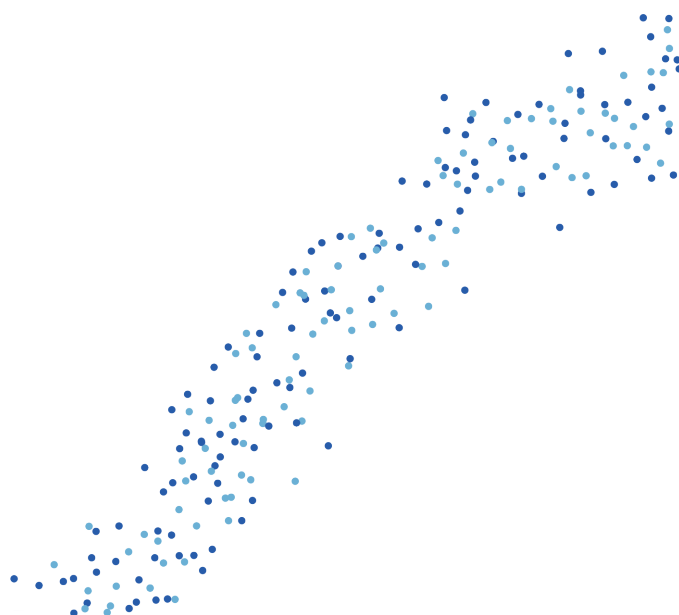
Study	Type	Sample size*	Inclusion criteria of the original study	Selection on results of preceding tests	Non-structural or unspecified anomalies excluded for pooling	Array platform and resolution
Tyrenan et al, 2009 ⁶⁰	R	106 (82)	Major ultrasound anomalies or multiple soft markers	Normal karyotype and FISH 22q11.2	Excluded (n=24): "Very large nuchal mass, cystic hygroma or hydrops foetalis" (n=18) "Other anomalies" (n=6)	6.0 GeneChip (Affymetrix)
Vialard et al, 2009 ⁶¹	R	37 (36)	Fetuses with 2 or more abnormalities in the cardiovascular, urogenital, skeletal, digestive or central nervous system (testing after termination of pregnancy)	Normal karyotype	Excluded (n=1) IUGR	Genosensor array 300 (Abbott) 287 loci - single-copy telomeric sequences, sequences of genes known to be involved in microdeletion syndromes, different loci amplified in various human cancers, and various chromosome locus markers.
Coppinger et al, 2009 ⁶⁰ (Whole genome)	P	155 (122)	Abnormal ultrasound findings	Normal karyotype or FISH result Excluded (n=1): Trisomy 13	Excluded (n=32): Unspecified ultrasound findings (n=10) Non-structural anomalies (akinesia, echogenic bowel, increased nuchal translucency/fold, IUGR, non-immune hydrops, polymicrogyria, short long bones) (n=22)	Signature Chip WG (Signature) 4670 BAC's; 1543 loci, average gap size 1.6Mb - Signature Chip WG 105K (Oligoarray custom design Agilent), average probe spacing 35Kb
Coppinger et al, 2009 ⁶⁰ (Targeted)	P	33 (15)	Abnormal ultrasound findings	Normal karyotype	Excluded (n=18): Unspecified ultrasound findings (n=2) Non-structural anomalies (increased nuchal translucency/fold, IUGR, short long bones) (n=16)	Signature Chip TG v4.0 (Signature) (or Signature Prenatal Chip v1.0) 2100 BAC's covering 100 known syndromes and subcentromeric and subtelomeric regions, no backbone
Kleeman et al, 2009 ⁶³	P	50 (43)	Significant ultrasound malformation and/or IUGR Soft markers for aneuploidy were excluded. Fetuses with multiple anomalies were classified according to most severe malformation present	Normal karyotype Excluded (n=1): mosaic trisomy 22 in amniotic fluid (previously karyotyped in chorion villi)	Excluded (n=6): "Cystic Hygroma/Enlarged NT/Hydrops" (n=3) "Growth disorder" (n=3)	First 26 cases Signature Chip WG (WG) 4.0 (Signature) 1887 clones covering 622 loci Remaining 24 cases: Signature Chip (WG) (Signature) 4685 clones covering 1500 loci Average gap across the genome 1.6Mb and the resolution within targeted regions 80-100Kb
Evangelidou et al, 2010 ⁸⁵	P	25 (8)	Abnormal ultrasound findings or balanced structural chromosome aberration, cases were specially referred for array	Abnormal karyotypes included Excluded (n=10): Abnormal karyotype	Excluded (n=7 incl. 4 with an abnormal karyotype): Non-structural anomalies	Cytochip WG (BlueGnome) BAC array with median resolution of 0.5-1 Mb
Leung et al, 2011 ⁶⁷	R	48 (48)	Increased nuchal translucency (>3.5mm)	Normal karyotype	None excluded	Fetal DNA Chip (Agilent) Targeted 44K oligonucleotide array, including telomeric and pericentromeric regions (resolution up to 100Kb), targeted for 100 known genomic disorders

Study	Type	Sample size*	Inclusion criteria of the original study	Selection on results of preceding tests	Non-structural or unspecified anomalies excluded for pooling	Array platform and resolution
D'Amours et al. 2012 ⁶⁶	R	49 (47)	At least one major ultrasound malformation and the malformation(s) remained unexplained after standard investigations	Normal (n=47) or unknown karyotype (n=2) Normal FISH on different targets (n=14)	Excluded (n=2): Non-structural anomalies	Custom designed arrays 7 fetuses: Signature Chip WG (Signature) BAC array with 4,685 BAC's 16 fetuses: Signature Chip WG OS11 (Signature) Oligo array with 105,000 oligo's 26 fetuses: Signature Chip WG OS 2.0 (Signature) 135,000 oligo's
Lee et al. 2011 ⁶¹	P	194 (136)	Abnormal sonographic findings	No selection Excluded (n=4): Array >10Mb (n=4)	Excluded (n=53): Unspecified findings (n=36) "Increased nuchal translucency" (n=17)	1Mb BAC array CGH 2 nd step (if anomaly detected): 105 K oligonucleotide array (0.1 Mb resolution) last 674 of the whole cohort 60K oligonucleotide array (0.5 Mb resolution)
Shaffer et al. 2012 ⁶²	P	2858 (2184)	Abnormal ultrasound findings	Cases with abnormal karyotype at the time of array testing or family history of a chromosome rearrangement or fetal demise were excluded. Most of the cases had a normal karyotype known before testing (2052/2858, 72%)	Excluded (n=674): Polyhydramnios or oligohydramnios, isolated (n=9) Abnormal growth, isolated (n=76) Single soft marker, isolated (n=59) Multiple soft markers, isolated (n=18) Multiple non-structural anomalies (n=49) Other (n=5) Not specified (n=61) Neck and/or body fluids (other than cystic hygroma) (n=396) Hypoplastic thymus (n=1)	76% of cases: Oligonucleotide-based microarray with 105K-feature whole-genome microarray (SignatureChip Oligo Solution, made for Signature Genomic Laboratories by Agilent Technologies, Santa Clara, CA) with 1 probe every 10 kb in targeted regions – microdeletion/ microduplication syndromes, pericentromeric regions, subtelomeres and genes in important developmental pathways; average probe spacing of 1 probe every 35 kb throughout the genome. 18% of cases: Whole-genome BAC microarray (SignatureChipWG), 4670 BACs representing 1543 loci; subtelomeric and pericentromeric with a higher density; genes in important developmental pathways also covered by contigs of BACs to fill in chromosome arms and provide higher resolution with average gap size between contigs of ~1.6Mb. 7% of cases: Targeted BAC microarray with the Signature PrenatalChip, >2100 BACs representing 100 clinically recognized genetic syndromes; subtelomeric and pericentromeric with a higher density of clones. No backbone coverage between targeted loci.

Faas et al. 2012 ⁶⁸	P	153 (90)	Structural fetal ultrasound anomalies (including NT > 3.5mm);	Normal RAD Excluded (n=39): Abnormal RAD (n=35) Array# >10Mb (n=4)	Excluded (n=24): "Other anomalies" (n=19) "Hydrops / hydrothorax" (n=5)	GeneChip 250 K SNP array (Affymetrix) ≥150kb for losses and ≥250 kb for gains
Mademont-Soler et al. 2013 ⁶⁴	P	51 (51)	Structural and non-structural cardiac anomalies on ultrasound (45.1% also extra-cardiac anomalies)	Normal karyotype and normal or no FISH22q11	None excluded	CytoChip focus Constitutional (BlueGnome) 139 specific loci Resolution 100kb (target), 1Mb (backbone)
Ganesamoorthy et al. 2013 ⁶⁹	P	104 (70)	Fetal anomalies detected by ultrasound	No selection Excluded (n=3): Abnormal karyotype	Excluded (n=31): Non-structural anomalies (n=15) Unspecified (n=10) or unknown abnormalities (n=6)	2.7M SNP array (Affymetrix) (n=93) Average practical resolution 50kb HumanCytoSNP12 v21 (Illumina) (n=11) Average practical resolution 200kb
Rooryck et al. 2013 ⁸⁶	P	160 (56)	Ultrasound anomalies (including NT >3.5mm)	Karyotype known before inclusion Excluded (n=18): Abnormal karyotype	Excluded (n=86): Cystic hygroma (n=1) Unspecified minor ultrasound anomalies (n=36) Unspecified major ultrasound anomalies (n=49)	8 x 60K oligonucleotide arrays (Agilent) Aberrations were only taken into consideration when including >8 consecutive variant points (Mean resolution 400 kb)
Schmid et al. 2013 ⁹⁶	P	75 (45)	Structural fetal defects	No aneuploidies 13,18,21, X or Y Excluded (n=8): Abnormal karyotype	Excluded (n=28 incl. 6 with an abnormal karyotype): First trimester screening (n=20) IUGR (n=1) Soft markers (n=5) Unspecified (n=2)	Genome-wide human SNP array 6.0 (Affymetrix) Resolution 100kb (n=59) or 200-1000kb (n=16, due to poor DNA quality)
Hillman et al. 2013 ²⁹	P	243 (187)	Fetal anomaly on ultrasound (including "multiple markers" and NT>3.5mm)	Normal RAD Karyotype alongside CMA (no selection) Excluded (n=17): Abnormal karyotype	Excluded (n=44, incl. 5 with an abnormal karyotype): Fetal hydrops (n=4) Enlarged NT and cystic hygroma (one group) (n=40, 5 having an abnormal karyotype)	Cytochip Focus Constitutional (BlueGnome) Resolution: >200kb (target); >2Mb (backbone)
Scott et al. 2013 ⁷⁰	P	152 (62)	NT>3.5mm, structural anomalies on ultrasound	QF-PCR performed, but anomalies not excluded Excluded (n=49): CMA aberrations that would have been detected by karyotyping	Excluded (n=41): Unspecified structural anomalies	ISCA 8 x 60K oligonucleotide array (Agilent) Resolution 70kb (backbone) + increased probe density in target regions adjusted for prenatal testing
Vestergaard et al. 2013 ⁹⁹	P	89 (77)	Abnormal ultrasound findings	No selection Excluded (n=3): Abnormal karyotype (n=2) Aberrant array result not stated (n=1)	Excluded (n= 10, incl. 1 with an abnormal karyotype) Nuchal translucency and cystic hygroma/hydrops (n=5) "Other anomalies" (n=5)	SurePrint G3 Human CGH microarray 180K (Agilent) Resolution approximately 80kb

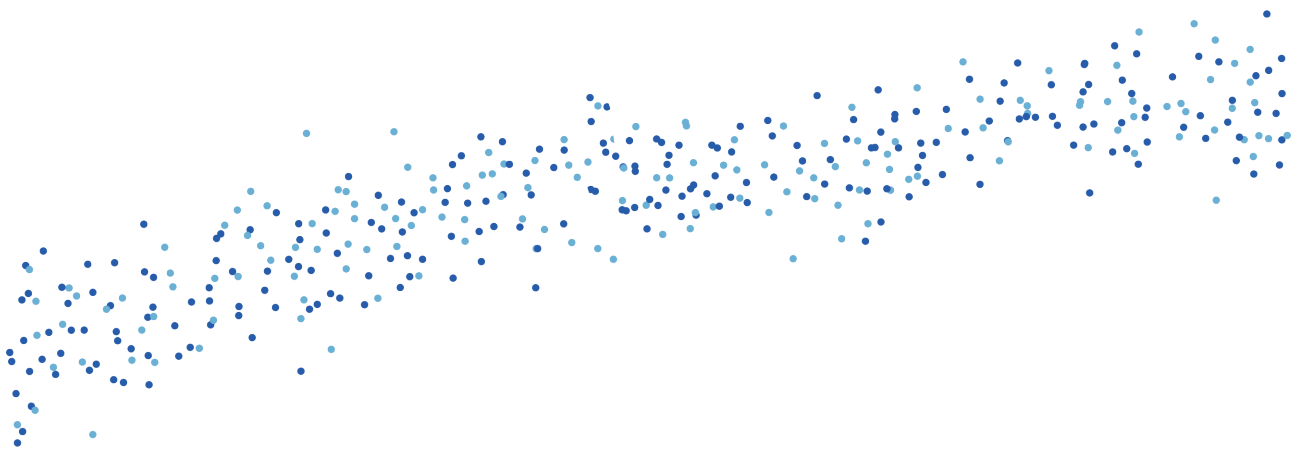
*Total number of cases with ultrasound anomalies in a particular study (number of cases included in this review is given in brackets)
P, Prospective study; R, Retrospective study.

03



Chapter 3.1

Prenatal and postnatal findings in small-for-gestational-age fetuses without structural ultrasound anomalies at 18–24 weeks



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Abstract

Objective

To assess phenotypic and genotypic characteristics of small-for-gestational-age (SGA) fetuses without structural anomalies at 18–24 weeks' gestation.

Methods

This retrospective study included structurally normal singleton fetuses with an abdominal circumference $\leq$ 5th percentile on detailed ultrasound examination between 18 and 24 weeks' gestation. Cases were stratified according to the absence or presence of other abnormal ultrasound findings, such as abnormal amniotic fluid or soft markers. All patients were offered invasive prenatal testing with rapid aneuploidy detection by qualitative fluorescence polymerase chain reaction (QF-PCR) and, if normal, consecutive single nucleotide polymorphism (SNP) array was also offered. Detailed postnatal follow-up ($\geq$ 5 months) was performed. In cases in which a syndromic phenotype became apparent within 5 months after birth and SNP array had not been performed prenatally, it was performed postnatally.

Results

A total of 211 pregnancies were eligible for inclusion. Of the 158 cases with isolated SGA on ultrasound, 36 opted for invasive prenatal testing. One case of trisomy 21 and one case of a submicroscopic abnormality (a susceptibility locus for neurodevelopmental disease) were detected. Postnatal follow-up showed a postnatal apparent syndromic phenotype in 10 cases. In one case this was due to trisomy 21 and the other nine (5.8%; 95% CI, 2.8–10.0%) cases had normal SNP array results. In 32/53 cases with SGA and associated ultrasound abnormalities, parents opted for invasive testing. One case of trisomy 21 and one of

triploidy were found. In 11 cases a syndromic phenotype became apparent after birth. One was due to trisomy 21 and in one case a submicroscopic anomaly (a susceptibility locus) was found. The remaining syndromic cases (17.3%; 95% CI, 8.7–29.0%) had normal SNP array results.

Conclusion

Testing for chromosomal anomalies should be offered in cases of SGA between 18 and 24 weeks' gestation. Whole chromosome anomalies occur in 1.3% (95% CI, 0.2–3.9%) of isolated SGA and 5.8% (95% CI, 1.5–14.0%) of associated SGA. In 0.6% (95% CI, 0.1–2.8%) and 1.9% (95% CI, 0.2–8.2%), respectively, SNP array detected a susceptibility locus for neurodevelopmental disease that would not be detected by karyotyping, QF-PCR or non-invasive prenatal testing. Therefore, and because the genetic causes of SGA are diverse, we suggest SNP array testing in cases of SGA. Thorough postnatal examination and follow-up of infants that presented with reduced fetal growth is important because chromosomally normal syndromic phenotypes occur frequently in SGA fetuses.

Introduction

Prenatal growth assessment is an important part of prenatal care.⁸⁷ Fetal growth impairment can be a consequence of many etiologies. Fetal genetic defects such as chromosomal abnormalities are an important cause. Fetal growth impairment is associated with a chromosomal abnormality detected by karyotyping in 2–19% of cases.^{88–91} Several studies published in the 1990s showed that the strength of this association depends mainly on the gestational age (GA) at which the growth impairment is detected^{88,90} and the coexistence of structural fetal anomalies.^{90,91}

Since the 1990s, the daily practice of prenatal screening has changed dramatically. The introduction of the first-trimester combined test and other first-trimester ultrasound examinations partially enabled early screening for a structurally and/or genetically abnormal fetus⁹². The improvement in ultrasound imaging quality and expertise has contributed to the accuracy of assessment of a fetal phenotype. Also, cytogenetic techniques applied for prenatal genetic testing have changed considerably. Whereas karyotyping was used routinely in the past decades, microarray has now become the recommended technique to detect chromosomal aberrations in fetuses with ultrasound anomalies.⁹³ Microarray reaches higher resolution than karyotyping, enabling the detection of submicroscopic copy number variants (CNVs). Clinically relevant CNVs are detected in up to 9.1% of fetuses with ultrasound anomalies and a normal karyotype, but this frequency depends on the kind of fetal anomalies found.⁹⁴ In this study, we explored the phenotypic and genotypic outcomes in small-for-gestational-age (SGA) fetuses without signs of structural anomalies on ultrasound in mid-pregnancy. Prenatal chromosomal analysis by single nucleotide polymorphism (SNP) array was offered to all patients. In order to avoid

selection bias based on the parental choice for invasive prenatal testing, we included all cases presenting with an abdominal circumference (AC) $\leq$ 5th percentile between 18 and 24 weeks and followed them all until at least 5 months after birth.

Subjects and methods

This was a retrospective study of patients referred to one of three centers for prenatal diagnosis in the South Western part of The Netherlands (Erasmus MC in Rotterdam, Amphia Hospital in Breda and Reinier de Graaf Hospital in Delft) between September 2011 and May 2015. This study period was chosen because, since September 2011, the Erasmus MC cytogenetic laboratory performs SNP array routinely as a first-tier whole genome test for all referrals with fetal ultrasound abnormalities.

We included all singleton pregnancies with a fetal AC $\leq$ 5th percentile⁹⁵ determined on expert ultrasound examination between 18 and 24 weeks. Expert ultrasound examination is offered to patients who have an increased risk of fetal anomalies and to patients who show abnormal or unclear findings on routine ultrasound screening, mainly at the second-trimester fetal anomaly scan (~94.5% uptake in The Netherlands⁹⁶). The ultrasound examination was performed by dedicated obstetric sonographers on high-quality ultrasound equipment (Voluson E8, GE Healthcare, Chicago, IL, USA; or equipment of similar quality), following a protocol described elsewhere⁹⁷. Cases with a structural fetal malformation on ultrasound were not included in this study.

Included cases were stratified according to the presence of associated ultrasound anomalies: (1) cases with isolated SGA (with or without signs of abnormal uterine, placental or fetal circulation); (2) cases with SGA and non-

structural anomalies i.e. soft markers (single umbilical artery, echogenic bowel, pyelectasis, shortened long bones, echogenic cardiac focus, choroid plexus cysts) or abnormal amniotic fluid (index < 2 cm or > 24 cm). GA was based on crown-rump length measurement in the first trimester.

In agreement with the American College of Gynecologists (ACOG) guidelines⁹⁸, all patients with a SGA fetus were offered invasive prenatal testing after they underwent pretest counseling about its risks and benefits. Invasive sampling was carried out by amniocentesis or by chorionic villus sampling in case of anhydramnios. Invasive samples were subjected to rapid aneuploidy detection (RAD) by quantitative fluorescence polymerase chain reaction (QF-PCR) of chromosomes 13, 18, 21, X and Y. Samples with a normal QF-PCR result were tested subsequently by SNP array, as described previously⁸³. We report only SNP array findings that are considered pathogenic. CNVs were classified according to our previously published proposal for subcategorization⁹⁹: causative findings, unexpected diagnoses and susceptibility loci (SLs). A SL is defined as a variant associated with neurodevelopmental disorders and/or congenital abnormalities, but of extreme phenotypic heterogeneity and/or variable expressivity.

For basic cohort characteristics and follow-up, patient files were reviewed for maternal characteristics, reports of fetal ultrasound examinations throughout pregnancy, occurrence of (hypertensive) pregnancy complications, other diagnostic testing in pregnancy (e.g. viral and bacterial serology), mode of delivery, GA at delivery, pregnancy outcome (in terms of survival), birth-weight (BW) percentile¹⁰⁰ and placental pathology. In cases of an intrauterine demise or termination of pregnancy, the postmortem dysmorphology examination report (performed by a clinical geneticist) and the autopsy report were

studied. All liveborn infants were examined physically by a pediatrician. Pediatric patient files were reviewed for any postnatal abnormal findings, either at the examination after birth or at a subsequent visit because of later-onset problems. Postnatal follow-up was performed at least 5 months after delivery for every liveborn infant.

Whenever abnormalities other than prematurity or dysmaturity were found, the child was examined by a clinical geneticist to determine whether it was suspected of having a genetic syndrome (hereinafter referred to as 'syndromic phenotype'). The differentiation between syndromic and non-syndromic, yet anomalous, phenotypes was thus based on the clinical judgment of experienced clinical geneticists¹⁰¹. Phenotypes suspected for a genetic syndrome mainly include (a combination of) the following: major congenital malformations, dysmorphic features, developmental delay and failure to thrive.

In cases in which a syndromic phenotype became apparent postnatally in a live born or stillborn child, and SNP array testing had not been performed prenatally because the patient declined invasive testing, SNP array was performed postnatally. Karyotyping was carried out when the case was suspected of having a common trisomy. Postnatal genetic testing was not performed in children with a normal phenotype.

SPSS Statistics v.17.0 (SPSS Inc., Chicago, IL, USA) and R (<http://www.R-project.org/>) were used for computing statistical analysis. Because of small sample sizes, the package 'binom' was used to compute profile likelihood confidence intervals. The significance level was set at $P < 0.05$. Since multiple comparisons were made, Bonferroni correction was applied. The corrected significance level was $P < 0.008$ ($0.05/6 = 0.008$).

Table 1. Characteristics of 158 pregnancies with isolated small-for-gestational-age (SGA) fetus and 53 with SGA fetus with non-structural anomalies that were offered invasive prenatal testing

Characteristic	Isolated SGA (n = 158)	SGA and non-structural anomalies (n = 53)
Maternal age (years)	30.2 (17.7– 45.2)	30.8 (22.2– 40.7)
Nulliparous	68 (43.0)	29 (54.7)
Non-Caucasian	54 (38.8)*	12 (24.0)*
Smoker	36 (22.8)	12 (22.6)
Combined test	38 (27.9)*	17 (34.0)*
GA at diagnosis (weeks)	21.8 (18– 24)	21.7 (18.4– 23.9)
Fetal AC percentile	2.2 (0– 5)	1.7 (0– 4.6)
Female fetus	106 (67.1)	30 (56.6)
Maternal hypertension	26 (16.5)	12 (22.6)
Maternal diabetes	8 (7.4)*	1 (2.3)*

Data are given as mean (range) or n (%)

*Missing data > 5%

AC, Abdominal Circumference; GA, Gestational Age

Table 2. Pregnancy outcome in 158 pregnancies with isolated small-for-gestational-age (SGA) fetus and 53 with SGA fetus with non-structural anomalies that were offered invasive prenatal testing

Characteristic	Isolated SGA (n=158)	SGA and non-structural anomalies (n=53)
Lost to follow-up	2 (1.3)	1 (1.9)
IUFD or NND	10 (6.3)	17 (32.1)
Termination of pregnancy	7 (4.4)	6 (11.3)
Live birth	139 (88.0)	29 (54.7)
GA at birth (weeks)*	37.5 (24.4– 41.7)	32.6 (24.1– 41.9)
Birth-weight percentile*	6.6	10.2
Placental abnormality on pathological examination	43 (81.0)‡	24 (82.8) ‡
Female neonate	106 (67.1)	30 (56.6)
Prenatal chromosomal anomaly		
Whole chromosome	1 (0.6)	2 (3.8)
SNP array	1 (0.6)	0 (0)
Postnatal chromosomal anomaly†		
Whole chromosome	1 (0.6)	1 (1.9)
SNP array	0 (0)	1 (1.9)
Prenatal and postnatal chromosomal anomaly‡		
Whole chromosome	2 (1.3)	3 (5.7)
SNP array	1 (0.6)	1 (1.9)
Total	3 (1.9)	4 (7.5)
Postnatal (possible) syndromic phenotype, no chromosomal anomaly	9 (5.7)	9 (17.0)
Postnatal non-syndromic phenotype/minor congenital anomaly	5 (3.2)	5 (9.4)
Follow-up of live birth (months)	28.9 (6–46)	31.6 (14–45)

Data are given as mean (range), median or n (%)

*Excluding cases <24 weeks; ‡Missing data >5%; †Not all cases were tested by rapid aneuploidy detection or single-nucleotide polymorphism (SNP) array

GA, Gestational Age; IUFD, Intrauterine Fetal Death; NND, Neonatal Death

Results

Isolated small-for-gestational age

A total of 158 cases with isolated mid-pregnancy SGA visited our prenatal clinic during the study period (Tables 1 and 2). In 36 (22.8%) cases, parents opted for invasive prenatal testing (Figure 1a). These cases were more severely growth impaired than those who declined invasive testing (AC percentile9 was 1.3 vs 2.5; $P < 0.001$). QF-PCR of samples showed trisomy 21 in one case (Table S1, Case 1). SNP array on the remaining 35 samples following a normal QF-PCR result showed one pathogenic CNV (Table S1, Case 2): a 22q11 microduplication (OMIM 608363). This CNV is a susceptibility locus for neurodevelopmental disease and was inherited from the mother.^{102, 103} The child showed normal postnatal growth and psychomotor development at postnatal follow-up at 36 months. In three cases without pathogenic anomalies on prenatal SNP array (Figure 1a; Table S2, Cases 8, 9 and 15), a (possible) syndromic phenotype became apparent after birth.

In those without invasive prenatal testing ($n = 122$), seven cases showed a syndromic or suspected syndromic phenotype that required karyotyping or SNP array testing after birth. In one of these, trisomy 21 was detected (Table S1, Case 3). In the remaining six cases, no pathogenic anomaly was found by SNP array testing.

Consequently, 43 cases underwent SNP array testing for prenatal or postnatal indications. Two cases were lost to postnatal follow-up because they moved away from the area. The remaining 113 cases had no syndromic phenotypic characteristics that required SNP array until at least 5 months after birth. In the total cohort of isolated SGA, two cases (2/156, 1.3%; 95% CI, 0.2– 3.9%) of trisomy 21 and one (1/156, 0.6%; 95% CI, 0.1– 2.8%) of submicroscopic SL were found. In nine

cases (9/156, 5.8%; 95% CI, 2.8– 10.0%) with a syndromic phenotype (Table S2, Cases 8– 14) or suspected syndromic phenotype (Table S2, Cases 15 and 16) that was apparent after birth, no pathogenic anomaly was detected by prenatal or postnatal SNP array. One infant had Fanconi anemia (Table S2, Case 9), and in the others no genetic diagnosis could be established. In a further five cases (5/156, 3.2%; 95% CI, 1.2– 6.8% Table S3, Cases 26– 30), minor (non-syndromic) phenotypic anomalies were detected postnatally.

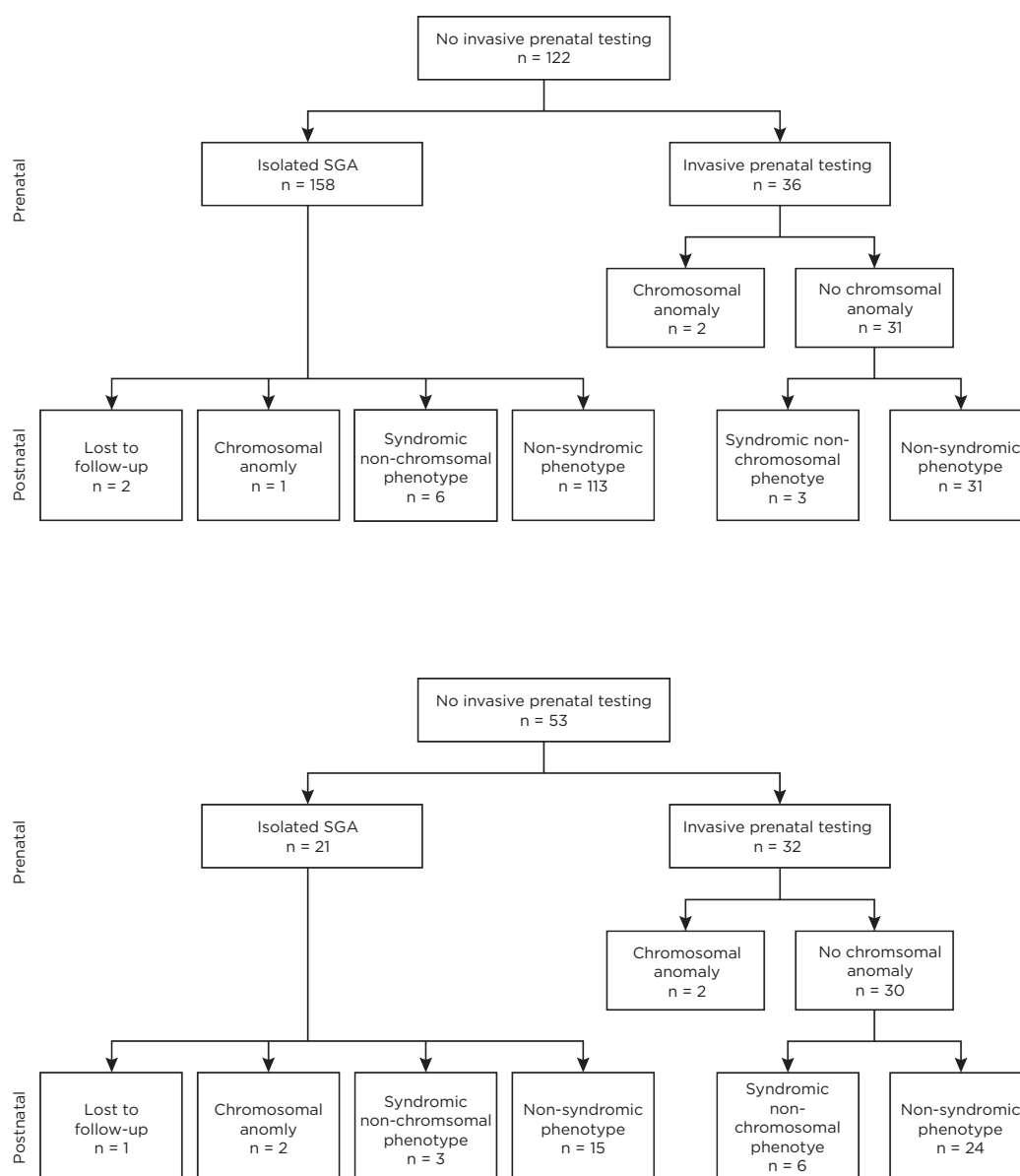
The prevalence of syndromic phenotypes (chromosomally normal or abnormal) was not significantly different between smokers and non-smokers (5.7% vs 7.8%; $P = 0.51$) or hypertensive and non-hypertensive women (3.8% vs 7.7%; $P = 0.42$).

SGA and non-structural ultrasound anomalies

SGA was accompanied by non-structural ultrasound anomalies in 53 cases (Tables 1 and 2). Of these, 32 (60.4%) underwent invasive prenatal testing (Figure 1b). Two cases with whole chromosome abnormalities (trisomy 21 and triploidy) were detected by QF-PCR (Table S1, Cases 4 and 5). Prenatal SNP array on the remaining 30 cases following a normal QF-PCR result showed no pathogenic findings. In six of these cases (Table S2, Cases 17– 19 and 23– 25) a (possible) syndromic phenotype became apparent after birth.

Among those not tested prenatally ($n = 21$), five showed a (possible) syndromic phenotype after birth. One of these had trisomy 21 (Table S1, Case 6) and another had a 15q11 microdeletion (NIPA1/2) (Table S1, Case 7), determined at postmortem examination following intrauterine demise, along with mild anomalies. This CNV is a SL for behavioral problems^{102, 104, 105} that was inherited from a healthy father. In the remaining three cases (Table S2, Cases 20– 22), postnatal SNP array showed no pathogenic abnormalities.

Figure 1. Flowchart of genetic and phenotypic findings in cases of mid-pregnancy isolated small-for-gestational age (SGA) (a) and SGA with non-structural anomalies (b) that were offered invasive prenatal testing.



Consequently, 37 cases were tested for prenatal or postnatal indications. One case was lost to follow-up. The remaining 15 cases had no syndromic phenotypic characteristics that required SNP array until at least 5 months after birth. In the total cohort of SGA with non-structural anomalies, three cases of whole chromosome anomalies (two of trisomy 21 and one of triploidy; 3/52, 5.8%; 95% CI, 1.5– 14.0%) and one of a SL (1/52, 1.9%; 95% CI, 0.2– 8.2%) were detected. In another nine cases (9/52, 17.3%; 95% CI, 8.7– 29.0%), a syndromic (Table S2, Cases 17– 22) or possible syndromic (Table S2, Cases 23– 25) phenotype became apparent after birth. In these nine cases, no pathogenic anomaly was detected by prenatal or postnatal SNP array. One infant showed a rare autosomal recessive disease (trichohepatoenteric syndrome Type 2; OMIM 614602; Table S2, Case 18), and in the other eight cases no genetic diagnosis could be established. In a further five cases (9.6%; 95% CI, 3.6– 20.0%; Table S3, Cases 31– 35), minor (non-syndromic) phenotypic anomalies were found postnatally.

The occurrence of postnatal syndromic phenotypes (chromosomally normal or abnormal) was not statistically significantly different between smokers and non-smokers (23.1% vs 25.6%; $P = 0.59$) or hypertensive and non-hypertensive women (16.7% vs 29.7%; $P = 0.31$).

Discussion

To our knowledge, this is the first follow-up study on the advantages of SNP array in SGA, but structurally normal, fetuses. Our results indicate that whole chromosome anomalies occur in 1.3% (95% CI, 0.2– 3.9%) of isolated SGA and 5.8% (95% CI, 1.5– 14.0%) of SGA with associated non-structural anomalies. In 0.6% (95% CI, 0.1– 2.8%) and 1.9% (95% CI, 0.2– 8.2%), respectively, SNP array detects a SL for

neurodevelopmental disease. Chromosomally normal syndromic phenotypes that become apparent postnatally occur in 5.8% (95% CI, 2.8– 10.0%) of cases with isolated SGA and 17.3% (95% CI, 8.7– 29.0%) of cases with associated SGA.

SNP array

Conventional karyotyping or RAD by QF-PCR would have missed two of the seven chromosomal anomalies that were detected by SNP array testing. These two pathogenic submicroscopic chromosome aberrations were both SLs⁹⁹. SLs are variants associated with neurodevelopmental disorders and/or structural anomalies, but have extreme phenotypic heterogeneity and variable expression¹⁰². The SLs detected in our cohort show incomplete penetrance because they were inherited from an apparently healthy parent. Both SLs identified in our study have been described in SGA cases previously^{103, 105, 106}, but the existing literature is insufficient to conclude that there is a causative association between the SLs and SGA. Bearing these facts in mind, we consider the value of informing parents prenatally about the presence of SLs to be debatable.

As not all submicroscopic CNVs, especially not all SLs, cause a syndromic phenotype within 5 months after birth, their true prevalence in our cohort might be even higher than that reported here. The prevalence of submicroscopic CNVs in our cohort is not statistically significantly different ($P = 0.27$) from the prevalence reported by Shaffer et al.⁶². Using array comparative genomic hybridization, they found two potentially significant CNVs in a group of 74 (2.7%) fetuses with isolated intrauterine growth restriction. We were unable to find any postnatal microarray studies on infants and children with isolated low birth weight. The literature available concerns infants and children with short stature and dysmorphic

features, developmental delay^{94, 107} and/or prolonged postnatal growth problems⁹⁴. The prevalence of pathogenic CNVs in these individuals is presumably much higher than in fetuses with isolated SGA.

Common autosomal aneuploidy and triploidy

Assuming that all cases of non-mosaic autosomal aneuploidy and triploidy have phenotypic expressions within the first 5 months of age, the occurrence of autosomal aneuploidy and triploidy is 1.3% (95% CI, 0.2–3.9%) among fetuses with isolated SGA and 5.8% (95% CI, 1.5–14.0%) among fetuses with associated SGA. In no case with trisomy 21 was a combined test performed because all of these patients had declined the test.

Autosomal aneuploidy was the most common chromosomal anomaly found in our cohort, and therefore non-invasive prenatal testing (NIPT) could be considered in cases of mid-pregnancy SGA. However, certain chromosomal anomalies would not be detected if NIPT were substituted for SNP array. Undetected anomalies could include triploidy, sex chromosomal anomalies and unbalanced microscopic and submicroscopic structural chromosomal anomalies, depending on which non-invasive test is used. Our cohort was small and not all cases were tested by SNP array. Based on the results of our study, we believe that it is too early to conclude that SNP array is redundant as a routine test for fetuses with SGA. On the other hand, NIPT can provide interesting relevant information with respect to confined placental mosaicism. Confined placental mosaicism can cause fetal growth impairment¹⁰⁸ and leads to anomalous cell-free fetal DNA from the cytotrophoblast⁴⁴. When anomalous cell-free fetal DNA is detected, invasive prenatal testing is still necessary to determine the genotype of the fetus.

Other abnormal phenotypes

Prenatal discrimination of a specific single-gene disorder based on prenatally detected SGA has turned out to be very difficult (Tables S1 and S2), and therefore a whole-genome screening approach for genetic conditions is preferable. As mentioned before, only a small subset of postnatally apparent syndromic phenotypes can be explained by a chromosomal abnormality. In order to improve the diagnostic yield, whole-exome sequencing might be of additional value, as suggested in prenatal and postnatal studies.^{36, 109, 110}

Minor, non-syndromic phenotypic anomalies occur frequently in fetuses with apparently isolated SGA on prenatal ultrasound (Table 2). For example, our results show that the prevalence of (mild) hypospadias is higher in male SGA fetuses as compared with the general population of live-born males¹¹¹ (9.5% (95% CI, 4.9–20.4%) vs 0.52%; $P < 0.001$). An association between hypospadias and SGA has been reported previously^{112, 113} and may suggest a common etiology.

Conclusions

In conclusion, the frequent occurrence of syndromic or non-syndromic abnormal findings after birth that were unsuspected prenatally emphasizes the importance of good postnatal follow-up of infants presenting with mid-pregnancy SGA.

The main strength of our study is the systematic diagnostic approach to every case. We included not only pregnancies that were referred for SGA, but we selected our subjects solely on fetal AC. Thereby we included all pregnancies that were diagnosed objectively with SGA between 18 and 24 weeks. By not selecting only patients who opted for invasive prenatal testing, we tried to rule out the selection bias that is often encountered when

analyzing the occurrence of chromosomal anomalies in prenatal cohorts.

Another strength of this study is the minimum of 5 months follow-up after birth. We believe that this covers the period needed to diagnose the main neonatal problems and signs for many genetic syndromes, such as dysmorphic features and failure to thrive. However, certain syndromic phenotypes, such as aberrant cognitive development, do not become apparent within the first 5 months after birth. Therefore, the prevalence of (chromosomally normal) syndromic phenotypes, although already high, might be underreported.

Table S1. Summary of cases with chromosomal anomalies in cohort of isolated small-for-gestational-age (SGA) fetuses and those with SGA and non-structural anomalies

Case	AC percentile ^{as} (gestational weeks at measurement)	Details of prenatal follow-up	Duration of postnatal follow-up (months)	Details of postnatal follow-up	Genetic diagnosis
Isolated mid-pregnancy SGA					
1	2.4 (22+4)	Isolated symmetric SGA. Patient declined combined test	-	TOP at 23+5 weeks. No postmortem of fetus or placenta	Prenatal QF-PCR: trisomy 21 Karyotype: 47,XX+21
2	0.5 (18+4)	Isolated symmetric SGA. Normalization of growth (AC p7 th) from 23+3 weeks. Mother completed primary education. Psychiatric and psychologic problems. Height 1.73m	36	LB at 37+3 weeks; BW, p 0. Postnatal growth (~2.5SD) and psychomotor development within normal range. Child in foster home. Placenta not examined	Prenatal SNP array: 2Mb gain of 22q11.21, susceptibility locus for neurodevelopmental disorders, postnatal growth impairment, muscle weakness, hearing loss and congenital anomaly, inherited from mother
3	3.5 (18+0)	Isolated symmetric SGA, steady growth throughout pregnancy, pyelectasis from 26+0 weeks. Patient had declined combined test.	41	LB at 35+1 weeks; BW, p 0.5. Facial dysmorphias. Patent foramen ovale. Acute lymphatic leukemia. Placenta not examined	No prenatal testing Postnatal karyotype: 47,XX+21
Mid-pregnancy SGA with non-structural anomaly					
4	0 (20+0)	Restriction of growth and abnormal placental circulation from 20+0 weeks, single umbilical artery, unilateral pyelectasis, echogenic cardiac focus, echogenic bowel. Patient had declined combined test.	-	TOP at 24+0 weeks. No postmortem of fetus or placenta	Prenatal QF-PCR: triploidy
5	0 (18+5)	Restriction of growth, abnormal placental circulation and oligohydramnios from 18+5 weeks. Patient had declined combined test.	-	TOP at 20+1 weeks. No postmortem of fetus or placenta	Prenatal QF-PCR: trisomy 21 Karyotype: 47,XX+21
6	3.3 (23+3)	Symmetric SGA with normal placental circulation, choroid plexus cysts. Patient had declined combined test	24	LB at 38+6 weeks. BW, p 3. Facial dysmorphias. Placenta not examined	No prenatal testing Postnatal karyotype: 47,XY+21
7	0.1 (20+4)	Restriction of growth, abnormal placental circulation and oligohydramnios from 20+4, echogenic bowel. TORCH serology negative. Surveillance of fetal condition and glucocorticoids for lung maturation from 27+1 weeks	-	IUFD at 27+4 weeks. BW, p 0. Autopsy showed hypospadias on autopsy and signs of placental insufficiency. Normal babygram. Placenta not examined	No prenatal testing. Postnatal SNP array: 955kb loss of 15q11.2, inherited from father. Susceptibility locus for behavioral problems ^{104, 105} . Father had no medical or behavioral problems

AC, Abdominal Circumference; BW, Birth Weight; GA, Gestational Age; IUFD, Intrauterine Fetal Death; LB, Live Birth; p, percentile; TOP, Termination Of Pregnancy; TORCH, Toxoplasmosis, Other (syphilis, varicella zoster, parvovirus B19), Rubella, Cytomegalovirus and Herpes infections

Table S2. Summary of cases with syndromic phenotypes without pathogenic findings on single-nucleotide polymorphism (SNP) array in cohort of isolated small-for-gestational-age (SGA) fetuses and those with SGA and non-structural anomalies

Case	AC percentile ^{ss} (gestational weeks at measurement)	Details of prenatal follow-up	Duration of postnatal follow-up (months)	Details of postnatal follow-up	Diagnosis
Isolated mid-pregnancy SGA					
8	0.3 (22+0)	Symmetric SGA, steady growth throughout pregnancy. Signs of abnormal placental circulation from 37+4 weeks. Ultrasound image quality impaired due to maternal obesity (BMI 38.2kg/m ²)	40	LB at 38+2 weeks. BW, p1. Small postnatal growth, microcephaly, hyperreflexia of joints, hypospadias, facial dysmorphias (low ear implant, deep nasal bridge), delay in psychomotor development and mild hemiparesis. Placenta: normal, with focal infarction <5%	Prenatal SNP array showed no pathogenic findings; Postnatal testing: no maternal uniparental disomy of chromosome 14; Metabolic testing normal; Genetic diagnosis not yet established
9	0.5 (23+0)	Symmetric SGA, steady growth throughout pregnancy (referred back to non-academic hospital at 32+1weeks)	24	LB at 37+1 weeks. BW, p4. Pre-axial polydactyly of both hands. Microcephaly (-5SD). Failure to thrive. Bilateral 80dB hearing loss. Areas of hyperpigmentation and hypopigmentation of skin. Wilms tumor. Placenta not examined	Prenatal SNP array showed no pathogenic findings; Postnatal diagnosis: Fanconi anemia type FA-D1 (c.2548G>T and c.7875A>T variants in BRCA-2. Parents are both carriers of one pathogenic variant)
10	0.6 (20+0)	Symmetric SGA, steady growth throughout pregnancy	40	LB at 36+6 weeks. BW, p 1. Delayed psychomotor development, mainly in speech development. Failure to thrive. Facial dysmorphias. Small postnatal growth. Brainstem evoked response audiometry normal. Placenta: weight <p10, infarction >5%	No prenatal testing; Postnatal SNP array: no pathogenic findings; Metabolic testing normal; Genetic diagnosis not yet established
11	2.7 (22+0)	Isolated symmetric SGA, steady growth throughout pregnancy	23	LB at 40+3 weeks. BW, p6. Failure to thrive and postnatal growth deprivation as consequence of bowel failure. Delayed psychomotor development. Placenta not examined	No prenatal testing; Postnatal SNP array showed no pathogenic findings; Whole-exome sequencing ongoing; Genetic diagnosis not yet established
12	0.7 (21+0)	Isolated symmetric SGA, steady growth throughout pregnancy	43	LB at 37+1 weeks. BW, p12. Epileptic encephalopathy: delay in psychomotor development, epilepsy, cerebral visual impairment. Placenta: weight p10, immaturity	No prenatal testing; Postnatal SNP array showed no pathogenic findings; Metabolic testing normal; Mutation analysis: MECP2, FOXG1, CDKL5, ST3GAL5 (exon 7): no causal mutations found; Next generation sequencing epilepsy panel (32 genes): no causal mutations found; Whole-exome sequencing ongoing; Genetic diagnosis not yet established
13	2.1 (23+6)	Consanguinity. Previous child suspected for autosomal recessive syndrome. Isolated symmetric SGA, steady growth throughout pregnancy. Gestational diabetes.	5	LB at 37+4 weeks. BW, p2.7. Failure to thrive and postnatal growth deprivation, hypotonia. Delay in psychomotor development, facial dysmorphias. Suspected of having same autosomal recessive disorder as affected sibling. Placenta not examined	No prenatal testing; Postnatal SNP array: Regions of homozygosity; Whole-exome sequencing ongoing; Genetic diagnosis not yet established

14	2.4 (23+6)	Consanguinity. Isolated symmetric SGA, steady growth throughout pregnancy	8	LB at 37+1 weeks. BW, p0.2. Two VSDs, pulmonary stenosis, facial dysmorphias. Placenta: weight p<10, delayed maturation	No prenatal testing; Postnatal SNP array of blood: low grade (10-15%) mosaicism of monosomy 7 (most likely isolated to bone marrow and blood) Differential diagnosis: Fanconi anemia
15	0 (18+5)	Symmetric SGA. Episodes of unexplained bradycardia throughout pregnancy	16	LB at 37+2 weeks; BW, p0. Failure to thrive; facial dysmorphias (triangular facial shape, low set ears and asymmetric ears, high forehead), normal psychomotor development to date. Placenta not examined	Prenatal SNP array showed no pathogenic findings; Genetic diagnosis not yet established
16	5 (18+6)	Pre-eclampsia and isolated growth restriction (time of onset unknown)	19	LB at 34+0 weeks. BW, p0. Congenital laryngoschisis. Feeding difficulties. Placenta not examined	No prenatal testing; Postnatal SNP array: no pathogenic findings; Genetic diagnosis not yet established
Mid-pregnancy SGA with nonstructural anomaly					
17	0 (20+0)	Restriction of growth and abnormal placental circulation from 20+0 weeks; oligohydramnios from 23+2 weeks	37	LB at 30+5 weeks; BW, p0. Hypospadias. Bilateral inguinal herniation. Failure to thrive. Dysmorphia, elf-like appearance, facial dysmorphisms. Normal psychomotor development to date. Placenta: weight p<10, advanced maturation	Prenatal SNP array: no pathogenic findings; Postnatal whole-exome sequencing ongoing; Genetic diagnosis not yet established
18	0 (23+4)	Consanguinity. Symmetric SGA; steady growth throughout pregnancy; echogenic bowel. TORCH serology negative	31	LB at 37+5 weeks. BW, p0. Failure to thrive. Congenital diarrhea. Hypogammaglobulinemia. Delay in psychomotor development. Placenta: normal weight, distal villi immaturity	Prenatal SNP array showed no pathogenic findings; no CF mutations found; NGS: homozygous mutation in SKIVL2L gene (Trichoshepaticentero syndrome Type 2)
19	0 (23+6)	Restriction of growth and abnormal placental circulation from 21+1 weeks, echogenic bowel, oligohydramnios	-	LB at 30+0 weeks. BW, p0. Neonatal death. Autopsy: Facial dysmorphias, anomalous external genitalia, cerebellar hypoplasia, anomalous right ear, ASD. Placenta: MPVFD	Prenatal SNP array showed no pathogenic findings; CF not tested; Genetic diagnosis not yet established
20	3.6 (22+0)	Restriction of growth from 22+0 weeks. Echogenic bowel, subchorial hematoma. TORCH serology: negative. Ultrasound image quality impaired due to echogenicity of amniotic fluid	-	LB at 26+1 weeks. BW, p1. Neonatal death because of pulmonary hypertension. Cerebral atrophy, postaxial polydactyly, high and narrow palate, low and broad nasal bridge, rhizomelia, short neck. Placenta: normal weight and maturation. Subchorial hematoma.	No prenatal testing; Postnatal SNP array showed no pathogenic findings; CF not tested; Postnatal testing: NGS panel ciliopathies (73 genes): no causal mutations found; Metabolic testing: no signs of Smith-Lemli-Opitz syndrome. Genetic diagnosis not yet established
21	1.3 (23+3)	Restriction of growth and abnormal placental circulation from 21+1 weeks; single umbilical artery	-	LB at 25+5 weeks: BW, p0. Neonatal death because of pulmonary hypertension. Cholestasis, hepatosplenomegaly, disturbances in coagulation, short limbs, narrow thorax, block-shaped hands and feet. Placenta: weight <p10, infarction 5%	No prenatal testing; Postnatal SNP array showed no pathogenic findings; NGS panel growth deficiency (90 genes): no causal mutations found; Mother declined further testing; No genetic diagnosis
22	41 (23+5)	Symmetric SGA with normal placental circulation, single umbilical artery, anhydramnios (PPROM) from 23+5 weeks. Impaired ultrasound image quality due to anhydramnios	-	LB at 27+5 weeks: BW, p30. Neonatal death. Multiple congenital anomalies: malalignment VSD, valvular anomaly of pulmonary artery, anal atresia, esophageal atresia with tracheoesophageal fistula, hypospadias. Placenta: weight p75, normal maturation. Active chorioamnionitis	Postnatal SNP array showed no pathogenic findings; Mutation analysis: ZIC3, SALL-1, NKX2.5, JAG1: no causal mutations found; Myotomycin-C testing: no abnormalities; No suggestions for further genetic testing

Case	AC percentile ^{as} (gestational weeks at measurement)	Details of prenatal follow-up	Duration of postnatal follow-up (months)	Details of postnatal follow-up	Diagnosis
23	0.1 (20+0)	Restriction of growth and abnormal placental circulation from 20+0 weeks; echogenic bowel; oligohydramnios from 24+2 weeks. PIH. TORCH serology negative. Wait and see policy	-	IUFD at 27+0 weeks; BW, p0. Autopsy: disproportion, broad hands and feet and short fingers and toes. Suspicious for skeletal dysplasia. Babygram normal. Placenta: weight <p10, ischemia >5%	Prenatal SNP array: no pathogenic findings, no CF mutations found; Genetic diagnosis not yet established
24	1.7 (21+0)	Restriction of growth from 21+0 weeks, abnormal placental circulation from 29+0 weeks, echogenic bowel, mild ventriculomegaly. TORCH serology negative.	25	LB at 33+4 weeks; BW, p0. Stable ventriculomegaly (partial aqueductal stenosis), coronal hypospadias, ASD II, pulmonary artery stenosis. Normal psychomotor development till date. Placenta: weight p3-5, accelerated maturation	Prenatal SNP array showed no pathogenic findings, no CF mutations found; Postnatal testing: Mutation analysis: No mutations in JAG1 (Alagille syndrome) and NOTCH2; No suggestions for further testing; Genetic diagnosis not established
25	0.1 (18+4)	Consanguinity. Symmetric SGA; abnormal placental circulation from 23+5 weeks, echogenic bowel. TORCH serology negative	-	LB at 34+0; BW, p0. Facial dysmorphisms (high anterior hairline, mild frontal bossing, deep nasal bridge, broad nose base, upslant of the eyes, low set ears with a thin helix). Unilateral inguinal hernia. Normal psychomotor development till date. Placenta: weight <p10, cord hyper coiling, ischemic changes in parenchyma	Prenatal SNP array showed no pathogenic findings, no CF mutations found; Postnatal testing: analysis of ROH in SNP array: no candidate genes (facial dysmorphisms); No suggestions for further testing; Genetic diagnosis not established

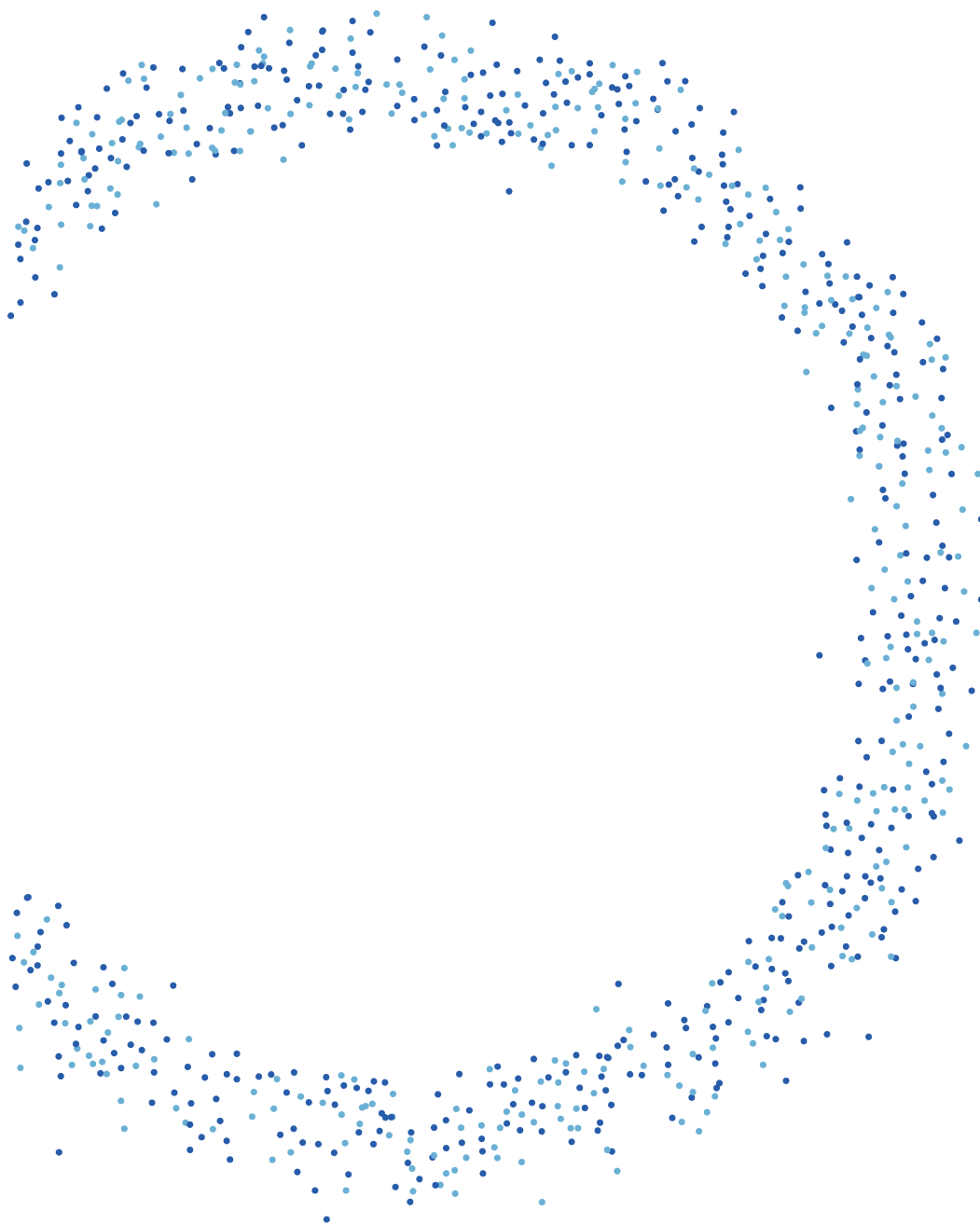
AC, Abdominal Circumference; ASD, Atrial Septum Defect; BMI, Body Mass Index; BW, Birth Weight; CF, Cystic Fibrosis; GA, Gestational Age; IUFD, Intrauterine Fetal Death; LB, Live Birth; MPVFD, Massive Perivillous Fibrin Deposition; NT, Nuchal Translucency; p, percentile; PIH, Pregnancy-Induced Hypertension; PPRM, Preterm Prelabor Rupture Of Membranes; TOP, Termination Of Pregnancy; TORCH, Toxoplasmosis, Other (syphilis, varicella zoster, parvovirus B19), Rubella, Cytomegalovirus and Herpes infections; VSD, Ventricular Septum Defect

Table S3. Summary of cases with minor (non-syndromic) anomalies in cohort of isolated small-for-gestational-age (SGA) fetuses and those with SGA and non-structural anomalies

Case	AC percentile ^{a5} (gestational weeks at measurement)	Details of prenatal follow-up	Duration of postnatal follow-up (months)	Details of postnatal follow-up	Diagnosis
Isolated mid-pregnancy SGA					
26	0.1 (18+0)	Symmetric SGA. Normalization of growth from 26+0 weeks (AC p20-30). Ultrasound image quality impaired due to maternal obesity (BMI: 33.7kg/m ²). Combined test: increased chance of Down syndrome (risk 1:24) with normal NT	35	LB at 35+3 weeks; BW, p7. Perimembranous VSD. No surgical intervention needed. Normal postnatal growth and psychomotor development to date. Placenta not examined	Prenatal SNP array showed no pathogenic findings; No further postnatal genetic testing indicated
27	0.6 (18+1)	Symmetric SGA, steady growth throughout pregnancy. BMI unknown	38	LB at 28+6 weeks; BW, p2.3. Small VSD, ASD II and mild peripheral pulmonary stenosis. Surgical correction not necessary. Normal postnatal growth and psychomotor development to date. Placenta not examined.	Prenatal SNP array showed no pathogenic findings; No further postnatal genetic testing indicated
28	1.0 (20+4)	Symmetric SGA, steady growth throughout pregnancy	8	LB at 37+1 weeks; BW, p4.4. Pes calcaneovarus. Normal postnatal growth and psychomotor development to date. Placenta: weight p<10.	Prenatal SNP array showed no pathogenic findings; No further postnatal genetic testing indicated
29	0.9 (20+3)	PIH. Restriction of growth and abnormal placental circulation from 20+3 weeks	27	LB at 29+5 weeks; BW, p0. Hypospadias; bilateral simian crease, bilateral inguinal herniation. Normal postnatal growth and psychomotor development to date. Placenta: weight p<10	No postnatal genetic testing indicated
30	0.6 (21+4)	Restriction of growth from 27+4 weeks. Abnormal placental circulation from 31+4 weeks	29	LB at 31+6 weeks; BW, p0. Postnatal growth deprivation. Feeding difficulties till date. Placenta: weight p<10. Signs of placental insufficiency	Postnatal maternal uniparental disomy testing of 7q32 (Silver Russell): negative (no Silver Russell)
Mid-pregnancy SGA with nonstructural anomaly					
31	3.7 (22+2)	Symmetric SGA, echogenic bowel. Steady growth throughout pregnancy. TORCH serology negative	29	LB at 37+3 weeks; BW, p0.2. Unexplained neonatal cholestasis/ enzymatic liver disorder. Still mild hepatomegaly. Normal psychomotor development to date	Prenatal SNP array showed no pathogenic findings; no CF mutations found; No postnatal genetic testing indicated
32	1.3 (23+2)	Asymmetric SGA (short long bones (p0.0)), steady growth throughout pregnancy. Cardiac echogenic focus	28	LB at 34+1 weeks; BW, p3. Mild hypospadias, bilateral inguinal herniation. Normal psychomotor development to date. Placenta not examined	Prenatal SNP array showed no pathogenic findings; Not referred to clinical geneticist
33	0.1 (20)	Pre-eclampsia. Echogenic bowel. Restriction of growth from 26+4 and abnormal placental circulation from 27+4 weeks	26	LB at 33+2 weeks; BW, p0. Cyst or bleeding in choroid plexus; jittering, possibly a sign of ADHD. Normal psychomotor development to date. Placenta: weight p<10, accelerated maturation.	Prenatal SNP array showed no pathogenic findings; CF not tested. Not referred to clinical geneticist

34	1:1 (21+3)	Chronic hypertension or PIH. Abnormal placental circulation from 21+3 weeks. Restriction of growth from 23+1 weeks, echogenic bowel, TORCH serology negative. Wait and see policy	-	IUFD at 25+3 weeks; BW, pO. Postmortem Physical Examination: Pre-axial polydactyly on one hand, no other abnormalities. No babygram, MRI or autopsy (patient's mother declined). Placenta: weight p<10	Prenatal SNP array showed no pathogenic findings; no CF mutations found; No postnatal genetic testing indicated (based on postmortem physical examination findings)
35	0 (20+6)	Symmetric SGA; abnormal placental circulation and oligohydramnios from 20+6 weeks	-	TOP at 23+6 weeks. Autopsy: mild facial dysmorphism, cystic dysplastic right kidney. Placenta: low weight; delayed maturation, ischemic changes	Prenatal SNP array showed no pathogenic findings

AC, Abdominal Circumference; ADHD, Attention Deficit Hyperactivity Disorder; ASD, Atrial Septum Defect; BMI, Body Mass Index; BW, Birth Weight; CF, Cystic Fibrosis; GA, Gestational Age; IUFD, IntraUterine Fetal Death; LB, Live Birth; MRI, Magnetic Resonance Imaging; NT, Nuchal Translucency; p, percentile; PIH, Pregnancy-Induced Hypertension; PPROM, Preterm Prelabor Rupture Of Membranes; TOP, Termination Of Pregnancy; TORCH, Toxoplasmosis, Other (syphilis, varicella zoster, parvovirus B19), Rubella, Cytomegalovirus and Herpes infections; VSD, Ventricular Septum Defect



Chapter 3.2

Enlarged NT (≥ 3.5 mm) in the first trimester – not all chromosome aberrations can be detected by NIPT

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Abstract

Objective

Since non-invasive prenatal testing (NIPT) in maternal blood became available, we evaluated which chromosome aberrations found in our cohort of fetuses with an enlarged NT in the first trimester of pregnancy (tested with SNP microarray) could be detected by NIPT as well.

Methods

362 fetuses were referred for cytogenetic testing due to an enlarged NT (≥ 3.5 mm). Chromosome aberrations were investigated using QF-PCR, karyotyping and whole genome SNP array.

Results

After invasive testing a chromosomal abnormality was detected in 137/362 (38 %) fetuses. 100/362 (28 %) cases concerned trisomy 21, 18 or 13, 25/362 (7 %) an aneuploidy of sex chromosomes and 3/362 (0.8 %) triploidy. In 6/362 (1.6 %) a pathogenic structural unbalanced chromosome aberration was seen and in 3/362 (0.8 %) a susceptibility locus for neurodevelopmental disorders was found. We estimated that in 2-10 % of fetuses with enlarged NT a chromosome aberration would be missed by current NIPT approaches.

Conclusion

Based on our cohort of fetuses with enlarged NT we may conclude that NIPT, depending on the approach, will miss chromosome aberrations in a significant percentage of pregnancies. Moreover all abnormal NIPT results require confirmatory studies with invasive testing, which will delay definitive diagnosis in ca. 30 % of patients. These figures are important for pretest counseling enabling pregnant women to make informed choices on the prenatal test. Larger cohorts of fetuses with an enlarged NT should be investigated to assess the additional diagnostic value of high resolution array testing for this indication.

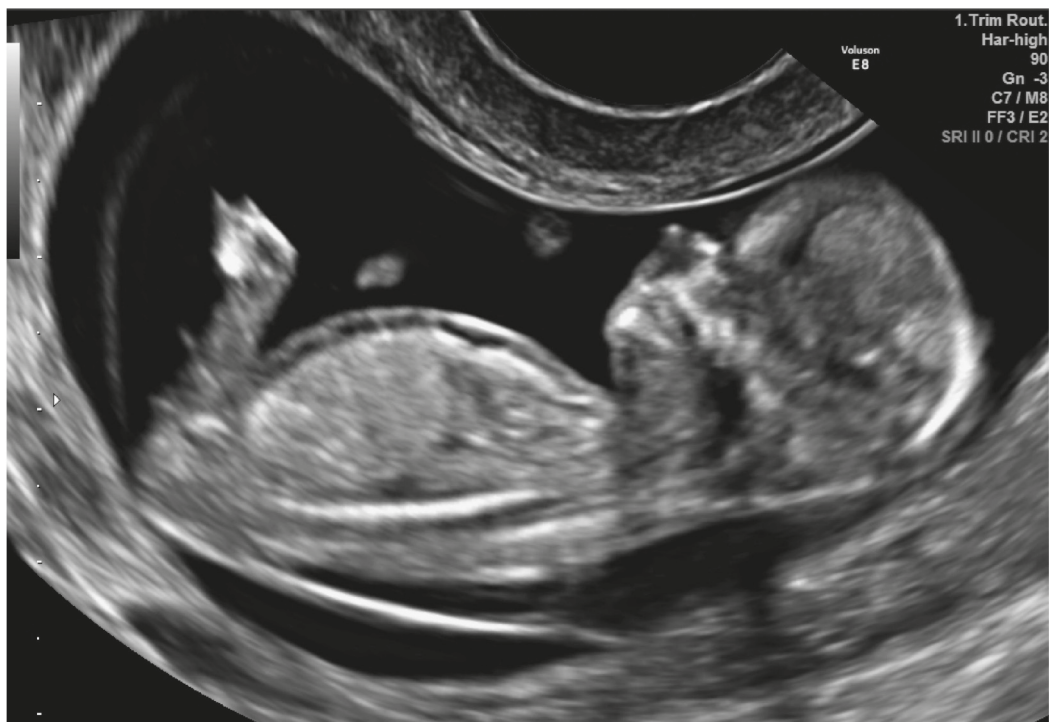


Illustration 1. 2D ultrasound image of increased nuchal translucency

Introduction

Nuchal translucency (NT) measurement is widely used as a marker of fetal abnormalities both of chromosomal and non-chromosomal origin.^{18, 92, 114} A fetal NT > 99th percentile is by definition found in about 1% of pregnancies.¹¹⁵ Enlarged NT is not only associated with aneuploidies and other chromosome abnormalities, but also with a number of genetic syndromes, as well as with structural congenital anomalies, mainly cardiac defects.^{92, 116, 117} The majority of fetuses with NT $\geq$ 3.5 mm have a normal karyotype and the pregnancy outcome is highly dependent on the absence of anomalies on expert fetal ultrasound examination.¹¹⁸ Since non-invasive prenatal testing (NIPT) in maternal blood became available, we evaluated which chromosome aberrations found in our cohort of fetuses with an enlarged NT in the first trimester of pregnancy (tested with SNP microarray) could be detected by NIPT as well. The results of this study can be used in pre-test counseling, which can be helpful for making informed choice between invasive and non-invasive genetic testing.

Subjects and methods

362 women carrying a fetus with an enlarged NT ($\geq$ 3.5 mm) in the first trimester were prospectively referred for Illumina SNP genotyping array as described before.^{26, 83} Fetal material was obtained through chorionic villi sampling (311 cases) or amniocentesis (51 cases) after the NT was measured as a part of the first trimester combined screening or as part of the routine first trimester crown rump length (CRL) measurement for pregnancy dating. Samples collected in our central location and 3 satellite hospitals between 1st September 2011 until 31st March 2016 that were routinely referred for SNP array testing (0.15 Mb resolution) were included

in this cohort. All cytogenetic tests were done in one central laboratory. This cohort overlaps slightly with the cohort published before.¹¹⁹ To create a homogenous cohort as much as possible we excluded the following cases:

- fetuses referred for hydrops foetalis,
- fetuses referred for enlarged NT with co-existing congenital anomalies evident on the CRL scan or the NT scan

All samples were tested with QF-PCR or MLPA to detect common aneuploidies (rapid aneuploidy detection - RAD). When RAD detected trisomy 21 or 13, such samples were karyotyped (GTG banding analysis) to assess the recurrence risk. Cases of triploidy or trisomy 18 were not further tested. All cases showing normal RAD results or sex-chromosomal aneuploidy were tested with Illumina SNP array (HumanCytoSNP-12 or Infinium_CytoSNP_850K with analysis resolution of ca. 0.15 Mb) as described before.⁸³

To answer our research question we have divided chromosome aberrations in the following groups:

1. autosomal aneuploidies
2. sex-chromosome aneuploidies that may be detected by NIPT when sex-chromosomes analysis is included in the test
3. triploidy
4. pathogenic structural unbalanced chromosome aberrations (both microscopic and submicroscopic)
5. susceptibility loci for neurodevelopmental disorders.

Further, we evaluated which aberrations would theoretically be missed by current NIPT approaches (NIPT tests were not routinely performed in this cohort). To be able to make this assessment, for the purpose of this paper, we assumed that all non-mosaic aneuploidies (both autosomal and sex-chromosomal) would be detectable by current NIPT¹²⁰ as well as structural unbalanced aberrations larger than 10 Mb.^{121, 122} We report estimated percentages of abnormal cases missed by particular NIPT

Table 1. Distribution of chromosomal abnormalities according to NT within the study population (n = 362)

NT in mm	Number of cases in the cohort (%)	Number of cases with chromosome aberrations (% within the category)
3.5-4.4	179 (49%)	35 (19%)
4.5-5.4	68 (19%)	32 (47%)
5.5-6.4	42 (11.6%)	30 (71%)
6.5-7.4	24 (6.6%)	14 (58.3%)
7.5-8.4	14 (3.9%)	6 (43%)
≥8.5	10 (2.8%)	6 (60%)
unknown (hygroma colli, where NT measurement was not specified)	25 (7%)	14 (56%)
Total	362	137 (38%)

approaches with Agresti–Coull (adjusted Wald) 95% confidence intervals, which have higher coverage probability than large-sample Wald intervals, in particular for small proportions.^{123, 124}

Results

The distribution of chromosomal abnormalities according to NT within the study population is presented in Table 1. The diagnostic flow was shown in Fig. 1. 38% (137/362) of the cases showed abnormal cytogenetic results, which are presented in Table 2. The most common aberration was trisomy 21 (17% 63/362) and in total in 28% (100/362) of the cases an autosomal aneuploidy was detected (trisomy 21, 18 or 13). In 6/362 (1.6%) a pathogenic unbalanced structural chromosome aberration was found: 5 were microscopically visible (>10 Mb) and 1 was submicroscopic (an atypical 22q11 microdeletion).¹²⁵ There were three cases (3/362, 0.8%) that showed a susceptibility locus for a neurodevelopmental disorder (2 cases of 15q11 microdeletion and one case of 16p11.2 microdeletion), these aberrations are probably not related to the enlarged NT.

Table 2 also shows the estimated percentage of cases that could be missed by current NIPT approaches in each category

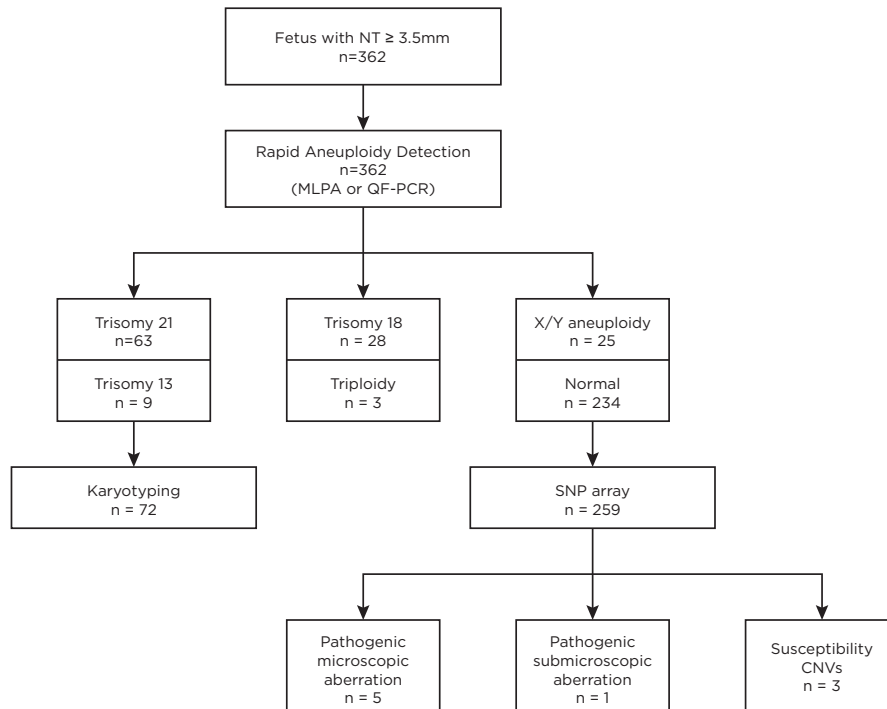
of chromosome aberrations. Based on our estimation a chromosomal anomaly would be undetected by NIPT in about 2–10% of patients, depending on the approach used (Table 3).

Discussion

Nuchal fluid accumulation may be caused by several factors, therefore, diverse genetic abnormalities may be expected in such fetuses. Taking this and numerous advantages of genomic microarrays¹²⁶ into account we have chosen a whole genome SNP array technique for cytogenetic investigations in cases of an enlarged NT.^{26, 83} In the present study, we prospectively investigated the frequency of (sub) microscopic aberrations in this group of fetuses. Since non-invasive prenatal testing (NIPT) in maternal blood became available and the patients can face a choice between an invasive and non-invasive testing, we evaluated how high the risks of missing a pathogenic chromosome finding can be in a cohort of fetuses with enlarged NT measurement.

Autosomal aneuploidies and triploidy

Although most of the anomalous fetuses showed trisomy 13, 18 or 21 (100/137, 73%) and NIPT seems to be an excellent and safe

Figure 1. Diagnostic flow and the cytogenetic findings

test with a high positive predictive value in this selected high risk group, there are some drawbacks that have to be taken into account. One has to be aware of the risk of false-negative NIPT results for the common trisomies.^{127, 128} 3.5% of Down/Patau/Edwards syndrome cases would potentially be missed by NIPT due to generalized mosaicism with discrepant direct results (GMDD).¹²⁹ Therefore, as shown in Table 2, at least 3 trisomic cases in the cohort presented in this paper (3.5% out of 100 cases) would potentially be also missed if this cohort was tested with NIPT as a first-tier test. Additionally, other potential causes for false-negative NIPT results such as a low fetal DNA fraction (e.g. due to a high maternal body mass index) or technical failures, should also be taken into account.¹³⁰ The detection of triploidy

is problematic as well. Although are SNP approaches are able to detect triploidy,^{131, 132} in our knowledge, there are no data showing that this could be achieved in assays based on whole genome shallow sequencing.

Sex-chromosome aneuploidies

The second most common group of aberrations in fetuses with an enlarged NT is monosomy X (5.5% 20/ 362, Table 2). Gil and colleagues showed that cfDNA tests could detect monosomy X in about 90% of the cases,¹³³ so at least 2 cases of monosomy X (10% out of 20 cases) would be missed in this cohort. An accurate non-invasive detection of fetal monosomy X remains problematic due to several reasons. First of all, chromosomal mosaicism is common in sex chromosomal

aneuploidy.¹³⁴ A low percentage of abnormal cells in the cytotrophoblast and co-existence of different abnormal cell lines may mask the actual chromosome aberration (e.g. 45,X/47,XXX) and lead to false negative results. There is also a maternal factor influencing the results as normal adult females show an age-related loss of X-chromosomes.¹³⁵ This mosaicism of chromosome X influences the positive predictive value in case of a monosomy X detection.¹³⁶ Moreover, finding sufficient Y-chromosome loci that are informative for copy number quantification may be difficult,¹³⁷ causing monosomy X detection to be highly dependent on the NIPT method used. These difficulties are reflected in the recent literature that showed very limited follow-up of monosomy X cases diagnosed non-invasively.^{130, 138} So although some authors suggest that there are accurate methods to detect fetal sex chromosomal aneuploidy in maternal plasma,^{139, 140} recent clinical experience shows low positive predictive value for monosomy X. This situation can be expected since the fetal DNA in maternal plasma is derived from the cytotrophoblast of chorionic villi (CV) and cytogenetic studies in CV already showed that sex-chromosomal aneuploidy in the cytotrophoblast of CV (STC-villi, short term cultured villi) is often not representative for the actual fetal karyotype.^{128, 141}

Pathogenic unbalanced structural aberrations

Although an enlarged NT was observed in fetuses with unbalanced translocations, no significant differences were seen in a study that evaluated the role of nuchal translucency (NT) in the prediction of unbalanced translocation in offspring of couples carrying balanced translocations.¹⁴² A recent study of Christiansen and colleagues showed that the distributions for NT measurements in case of an aberration other than trisomy 21, 13 or 18 more closely resembled that of the normal population.¹⁴³ So

the detection of rare unbalanced chromosome anomalies in cohorts with an enlarged NT may be co-incidental. Nevertheless karyotypically visible unbalanced chromosome aberrations are likely to be detected by whole genome profiling NIPT approaches.^{121, 122, 144} Unfortunately it is difficult to assess how many may be missed due to their mitotic origin and absence in the cytotrophoblast.

The incidence of pathogenic submicroscopic chromosomal abnormalities in fetuses with an enlarged NT has been studied by only few groups^{67, 145-149} resulting in conflicting conclusions.^{94, 150} These differences in frequencies of chromosomal aberrations in published cohorts were observed before⁹⁴ and it may be a consequence of both cohort selection and differences in array design. Many of previously reported cohorts were either retrospectively tested and highly selected^{67, 145} or included a heterogeneous group of fetuses with and without additional ultrasound anomalies detected in both first and second trimester.^{67, 86, 148, 151} Our results show that the prevalence of submicroscopic aberrations in fetuses with enlarged NT resembles the prevalence in fetuses without ultrasound anomalies. This confirms previous results published by Huang and colleagues.¹⁴⁵ Therefore, in our opinion larger unselected cohorts with enlarged NT should be published to assess the actual risk of a pathogenic submicroscopic unbalanced chromosome aberration when an enlarged NT is diagnosed in the first trimester.

Susceptibility loci for neurodevelopmental disorders

We did not take the susceptibility loci for neurodevelopmental disorders into account for the calculations shown in Table 3. Susceptibility CNVs are quite often found in prenatal array testing,³⁰ however there is no study that showed any relationship with an enlarged NT. Finding

Table 2. Results of cytogenetic testing in fetuses with enlarged NT (≥ 3.5 mm) or hygroma colli in the first trimester in fetuses referred for cytogenetic testing

Type of chromosome aberration	Number of fetuses n=362	Potential detection by current NIPT approaches	% of anomalies that are going to be missed due to placental mosaicism or current testing resolution (>7 -10Mb)
Autosomal aneuploidy		Yes	3.5% is likely to be missed ^{129, 154, 155} 0.8% (3/362)
Trisomy 21	63 (17%)		
Trisomy 18	28 (7.7%)		
Trisomy 13	9 (2.5%)		
Sex-chromosomal aneuploidy		Yes (if X/Y analysis are included)	10% of monosomy X are likely to be missed ¹³³ 0.5% (2/362) Mosaic samples are most probably missed 0.5% (2/362)
Monosomy X*	20 (5.5%)		
XXX	2 (0.5%)		
XXY	1 (0.3%)		
Mosaic X/XY	2 (0.5%)		
Triploidy	3 (0.8%)	Yes (if SNP approach is applied)	Most of the current approaches cannot detect triploidy, and so far it is not possible to combine targeted SNP analysis with high coverage and whole genome profiling with high resolution 0.8% (3/362)
Pathogenic unbalanced chromosome aberrations: 1. 46,XY,der(9)t(9;13)(q33.1;q12.11),-13 (NT 4.9 mm; 116Mb gain at 9p24-q33) 2. 46,XX,del(8)(p23.1) inv dup(8)(p11.21p23.1) (NT 4.6 mm; 28Mb gain at 8p) 3. 45,XX,der(4)t(4;15)(q32.1;q13.3),-15dn (hygroma colli; 34Mb loss at 4p) 4. arr[hg19] 9p24.3p22.2(46,587-18,277,618)x1 (NT 5.2 mm; 18Mb loss at 9p) 5. arr[hg19] 1q32.1q44(202,542,202-249,218,992)x3,9p24.3p24.1(46,587-7,017,391)x1 (NT 3.6 mm, 47Mb gain at 1q, 7Mb loss 9p) 6. atypical 22q11 microdeletion of paternal origin arr[hg19] 22q11.21(21,111,299-21,463,730)x1 pat (NT 4.3mm, >0.5 Mb)	6 (1.6%)	Larger than 10Mb Yes (if whole genome profiling with resolution of 10Mb is applied)	1/6 cases would be potentially missed: 0.3% (1/362)
Susceptibility loci for neurodevelopmental disorders: 1. 15q11 microdeletion of NIPA1/NIPA2 of paternal origin arr[hg18] 15q11.2(20,191,584-21,025,923)x1pat (NT 4.7mm) 2. 15q11 microdeletion of NIPA1/NIPA2 of maternal origin arr[hg19] 15q11.2 (22,299,434-23,272,733)x1mat (NT 4.4mm) 3. de novo 16q11.2 microdeletion arr[hg19] 16p11.2(29,595,483-30,198,151)x1dn (hygroma colli)	3 (0.8%)	No	So far genome wide detection of submicroscopic aberrations is not feasible. All will be missed 0.8% (3/362)
Total number abnormal cases	137 (38%)		

*One case showed also isochromosome Xp (case published before in¹⁵⁶)

Table 3. Percentage of abnormal cases missed by particular NIPT approaches. Additionally as 3.5% of trisomy 13, 18 and 21 would be missed due to placental mosaicism 3/362 (0.8%) cases were included in all NIPT options. As susceptibility CNVs are not the primary reason for invasive testing they were not included in the number of cases with structural anomalies that would be missed by NIPT

Type of NIPT testing	Percentage of patients in our cohort that would show false negative results with NIPT	95 % CI for percentage LL, UL	Number of cases that would be missed in our cohort
Targeted approach for trisomy 13, 18 and 21	10% (37/362)	7.48, 13.80	34 + 3/362 (20x monosomy X, 5x other sex chromosomal aneuploidy, 3x triploidy, 6x structural anomalies + 3/362 (0.8%) based on placental mosaicism)
Targeted approach for common aneuploidies of chromosomes 13, 18, 21, X/Y	4% (14/362)	2.26, 6.44	11 + 3/362 (2x mosaic sex chromosomal aneuploidy, 3x triploidy, 6x structural anomalies + 3/362 (0.8%) based on placental mosaicism)
Targeted approach for common aneuploidies of chromosomes 13, 18, 21, X/Y and triploidy	3% (11/362)	1.64, 5.42	8 + 3/362 (2x mosaic sex chromosomal aneuploidy, 6x structural anomalies + 3/362 (0.8%) based on placental mosaicism)
Whole genome profiling with a resolution of ca. 10Mb	2% (7/362)	0.09, 4.02	4 + 3/362 (3x triploidy and 1x submicroscopic anomaly will be missed + 3/362 (0.8%) based on placental mosaicism)

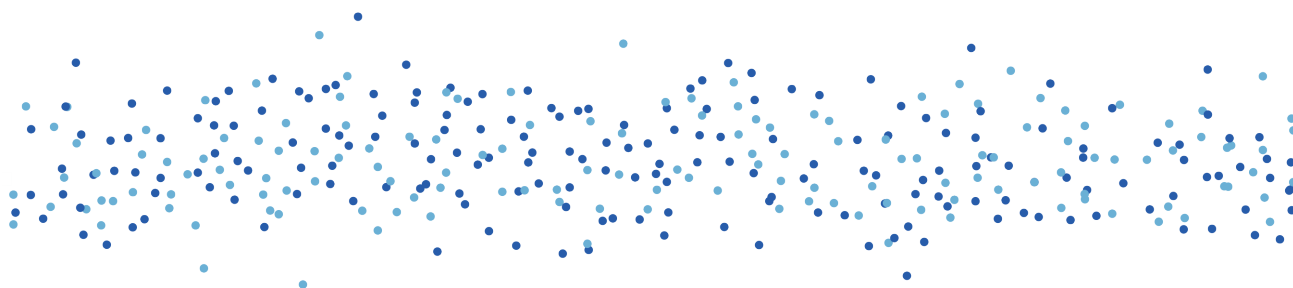
CI, confidence interval; LL, lower limit; UL, upper limit

additional predisposition factors may play a role in decision making in pregnancy, however it is less likely that one would choose invasive testing with a primary aim to investigate these. Therefore we assume that missing these findings is not the most important incentive for deciding on the prenatal test. Moreover the frequency of susceptibility CNVs in this cohort (0.8% 3/362) resembles more the frequency in fetuses without ultrasound anomalies (1.3%)¹⁵² than those with ultrasound anomalies (2.7%).¹¹⁹

Conclusions

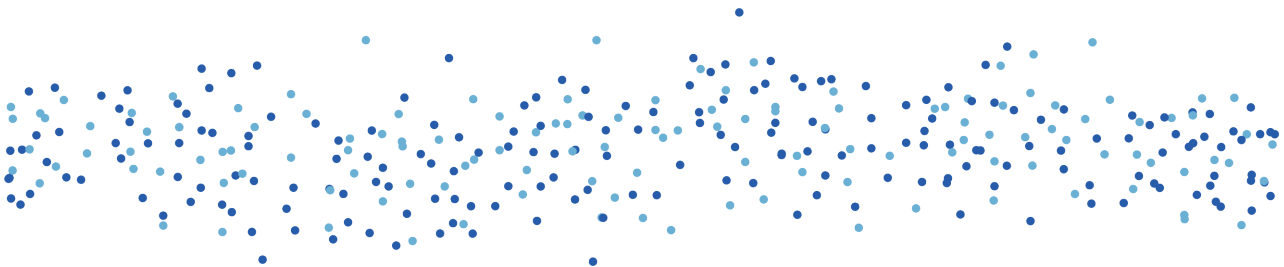
Since most (73%; 100/137) chromosome aberrations in cases of an enlarged NT (≥ 3.5 mm) in the first trimester involved trisomy 21, 18 and 13, NIPT at first sight seems to be an appropriate test. However, our study confirms

the previously published data by Lichtenbelt and colleagues¹⁵³ and shows that because of current limitations of NIPT (depending on the type of analysis) in ca. 2-10% of cases with an enlarged NT a chromosome aberration will be missed by non-invasive testing. Moreover since the risk for a chromosome aberration is high (1:3) and since aberrant NIPT requires confirmatory studies due to the origin of the cfDNA, therefore NIPT as compared to invasive testing will delay a final diagnosis in about 30% of patients. The limitations of NIPT should be clearly addressed in the pre-test counseling: possible diagnostic delay, the risk for false negative and false positive results and the fact that false-positive cases of monosomy X in enlarged NT fetuses may cause additional anxiety.



Chapter 3.3

Advanced genomic testing may aid in counseling of isolated agenesis of the corpus callosum on prenatal ultrasound



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Abstract

Objective

Isolated agenesis of the corpus callosum on fetal ultrasound has a varied prognosis. Microarray and exome sequencing (ES) might aid in prenatal counseling.

Methods

This study includes 25 fetuses with apparently isolated complete corpus callosum (cACC) on ultrasound. All cases were offered single nucleotide polymorphism array. Complementary ES was offered postnatally in selected cases. Clinical physical and neurodevelopmental follow-up was collected.

Results

Eighteen cases opted for single nucleotide polymorphism array testing, which detected a causal anomaly in 2/18 (11.1%; 95% CI 2.0%-31%). Among ongoing pregnancies without a causal anomaly on microarray, 30% (95% CI 8.5%-60%) showed intellectual disability. Postnatal magnetic resonance imaging and physical examination often (64%; 95% CI 38%-85%, and 64%; 95% CI 38%-85%, respectively) revealed additional physical anomalies in cases without a causal anomaly on microarray. Two cases appeared truly isolated after birth. Postnatal sequencing in 4 of 16 cases without a causal anomaly on microarray but with intellectual disability and/or additional postnatal physical anomalies revealed 2 single-gene disorders. Therefore, the estimated diagnostic yield of ES in chromosomally normal cACC fetuses is between 2/4 (50%; 95% CI 11%-89%) and 2/16 (13.3%; 95% CI 2.4%-36%).

Conclusion

In accordance with current guidelines, we conclude that microarray should be offered in case of isolated cACC on ultrasound. ES is likely to be informative for prenatal counseling and should be offered if microarray is normal.



Illustration 1. 3D ultrasound image of cCCA

Introduction

Agenesis of the corpus callosum (ACC) is among the most prevalent brain anomalies seen on prenatal imaging. The condition presumably has a wide clinical spectrum, from asymptomatic in childhood to severe intellectual disability (ID).¹⁵⁷⁻¹⁶⁰ Agenesis of the corpus callosum prevalence estimates range from 0% in healthy children and adults¹⁶¹⁻¹⁶⁷ to 0.08% in apparently normal term neonates^{168, 169} and 2.3% in developmentally disabled individuals.¹⁷⁰ Because of this wide clinical spectrum, counseling in expected isolated ACC on prenatal ultrasound can be difficult. An important review concludes a 24.6% chance of abnormal neurodevelopment if ACC is isolated on ultrasound and if third trimester magnetic resonance imaging (MRI) and karyotyping are normal.¹⁵⁹ This is confirmed by another recent meta-analysis.¹⁷¹ However, ACC is associated with many important genetic syndromes that are not detectable by karyotyping. We therefore anticipate that the use of more advanced genetic testing techniques, such as chromosomal microarray (CMA) and exome sequencing (ES), will allow the physician to be more accurate about the fetal prognosis. Chromosomal microarray is able to detect chromosomal imbalances beyond the microscopic resolution of karyotyping. Therefore, it recently became the recommended primary genomic test in case of fetal structural anomalies.¹⁷² Based on the available literature, we hypothesize that CMA has a 4.8% to 7.5%¹⁷¹ plus 5.7% to 6.2%^{94, 171} diagnostic yield for microscopic and submicroscopic chromosomal aberrations, respectively, in fetuses with anomalies of the brain. Exome sequencing (ES) permits the simultaneous examination of the nucleotide sequence of most expressed genes in the genome. This technique was recently introduced in prenatal diagnosis and presumably has a high diagnostic yield in fetuses

with congenital malformations.^{37, 110} Based on postnatal literature, the diagnostic yield of ES in brain anomalies can be estimated at 31%.³⁵ In the present study, we aim to determine the additional value of single nucleotide polymorphism (SNP) array and ES for prenatal counseling in isolated complete ACC on prenatal ultrasound. This estimate is based on the analysis of the phenotypic, genotypic, and neurodevelopmental outcomes of a prenatally ascertained cohort of 25 patients.

Subjects and methods

This is an observational cohort study of patients attending the Erasmus Medical Center (MC) antenatal clinic for prenatal diagnosis between January 1, 2008 and June 1, 2015. Cases were retrospectively collected from our electronic patient files. Inclusion started a year after the introduction of the 20 weeks anomaly screening in the Netherlands. The study was approved by the Erasmus MC medical ethics committee (April 21; MEC-2015-243). The Erasmus MC is a tertiary referral center for patients in whom fetal ultrasound anomalies are suspected and a primary ultrasound center for high-risk pregnancies. Expert ultrasound examination (EUE) is performed according to protocol by experienced obstetric sonographers using high-quality ultrasound equipment (Voluson E8 and E10, General Electric, Boston, Massachusetts, USA). Our protocol for fetal neurosonography is based on well-established guidelines.^{173, 174} All patients diagnosed with isolated complete agenesis of the fetal corpus callosum (cACC) on EUE at any moment in pregnancy were included. The diagnosis of cACC required complete absence of the ACC on 2D and 3D ultrasounds. Patients with presumed partial ACC were excluded. Agenesis of the corpus callosum was considered isolated if EUE showed no signs of

Table 1. Cohort Characteristics

Maternal age (mean $\pm$ SD)	30 ($\pm$ 5)
Gestational age at diagnosis (median, IQR)	21(21-29)
Diagnosed in second trimester	76% (19/25)
Co-existing midline cyst	16% (4/25)
Second trimester colpocephaly	37% (7/19)
Third trimester colpocephaly	95% (18/19)
Termination of pregnancy	24% (6/25)
Live births	76% (19/25)
Gestational age at birth (median, IQR)	39 weeks (29-41)
Birthweight (mean $\pm$ SD)	3308 grams ($\pm$ 620)
Follow-up (median, min-max)	45 months (9 -98)

CNS, Central Nervous System; ES, Exome Sequencing; IQR, Interquartile range; MRI, Magnetic Resonance Imaging; SD, Standard Deviation; SNP, Single Nucleotide Polymorphism

other fetal anomalies or sonomarkers. Fetuses with coexisting midline cysts and colpocephaly (posterior horn of lateral ventricle ≥ 10 mm) were included in this analysis, because these anomalies are considered to be part of the ACC sequence rather than separate brain anomalies.¹⁷⁵ Fetal MRI was not included in our standard diagnostic work-up, but second or third trimester fetal MRI was performed in certain cases for confirmation of the diagnosis or for detection of additional brain anomalies. Because not all fetuses were assessed with MRI, we did not consult the MRI results to determine a case's eligibility for inclusion. All patients were offered invasive prenatal genetic testing by means of SNP array. Invasive sampling was carried out through amniocentesis. Samples were handled and SNP array was performed as described previously.⁸³ Chromosomal aberrations were classified according to the subcategorization proposed by Srebniak et al.⁹⁹ When parents decided to terminate the pregnancy, a babygram, postmortem MRI, and fetal autopsy were offered. For live births, postnatal follow-up was done by a team of a pediatric neurologist, a neuro-clinical geneticist, and a pediatric radiologist. Follow-

up included physical examination and clinical neurodevelopmental assessment, cranial ultrasound, and MRI. The median follow-up in this cohort was 45 months (min 9, max 98). Because this is a retrospective analysis, neurodevelopmental assessment was not standardized for this study. However, besides specialized clinical follow-up in hospital, all newborns in the Netherlands are admitted in a nationwide standardized screening program for neurodevelopmental problems. Therefore, we assume that all neurodevelopmental abnormalities in this cohort were noticed either in our hospital or in this program. Intellectual disability (ID) was defined as a developmental abnormality that is characterized by deficits in both intellectual functioning and adaptive functioning in the conceptual, social, and practical domains (definition by the American Psychiatric Association¹⁷⁶). If SNP array was not yet performed prenatally through amniocentesis, parents were offered SNP array testing after birth. When SNP array had not shown a causal chromosomal anomaly and a certain specific genetic syndrome was suspected based on the postnatal phenotype, targeted gene testing was the next step in the

Table 2a. Descriptions of all cases with isolated cACC on ultrasound subjected to SNP array

Case No.	MRI	Cysts / colpocephaly	SNP array	Outcome	ID	Additional anomalies on postnatal physical examination and imaging	Postnatal ES
4	+	-	Inverted deletion-duplication of 8p	TOP	-	Mild dysmorphic facies	-
16	-	Second + third trim. colpocephaly	Mosaic tetrasomy 8p	LB	Yes	Valvular aorta stenosis; Periventricular white matter abnormalities.	-
6	-	Second trim. colpocephaly	Paternal deletion Yp11.32 (Leri-Weill dyschondrosteosis)	TOP	-	No postmortem examinations	-
11	+	-	Maternal duplication in 15q13.2q13.3 (SL)	TOP	-	Agenesis of cavum septum pellucidum; Abnormal cerebral falx.	-
17	-	Second + third trim. colpocephaly	Maternal duplication in 17p (CMT 1A)	LB	Yes	Alcardi syndrome	-
12	+	Third trim. colpocephaly	Normal	LB	Yes	Mild dysmorphic facies; Unilateral renal duplicate system, urachus cyst.	Mowat Wilson syndrome
25	-	Colpocephaly	Normal	LB	Yes	Dysmorphic facies; Gracile brain stem	Normal
13	-	Third trim. colpocephaly	Normal	LB	No	Hallus valgus; Brainstem hypoplasia.	Fibro-dysplasia ossificans progressiva
3	-	-	Normal	LB	No	Hypothalamic fever; Reverse Shapiro's Syndrome; Brain-in-brain malformation, heterotopia, unfused thalami, partial agenesis of the cerebral falx	Normal
1	+	2 cysts	Normal	TOP	-	Polymicrogyria	-
2	+	Third trim. colpocephaly	Normal	LB	No	None	-
7	-	Second + third trim. colpocephaly	Normal	LB	No	Impaired stereovision. Large head circumference. Vermin and cerebellar hypoplasia.	-
8	+	Cyst, second + third trim. colpocephaly	Normal	LB	No	Mild hypermetropia. Small head circumference; Focal polymicrogyria.	-
9	-	Cyst	Normal	TOP	-	Mild dysmorphic facies; Thin cortex.	-
14	-	-	Normal	TOP	-	Mild dysmorphic facies; pACC (or pre-optic holoprosencephaly).	-
19	-	Second + third trim. colpocephaly	Normal	LB	LTFU	Heterotopias in lining of posterior horns	-
22	-	Third trim. colpocephaly	Normal	LB	No	None	-
23	+	Third trim. colpocephaly	Normal	LB	No	pACC (posterior)	-

CMT, Charcot Marie Tooth; ES, Exome Sequencing; ID, Intellectual Disability; LB, Livebirth; LTFU, Lost to Follow-Up; pACC, partial Agenesis of the Corpus Callosum; SL, Susceptibility Locus; TOP, Termination of Pregnancy; trim., trimester

diagnostic work flow. Complementary ES was offered in cases with a challenging differential diagnosis based on the judgment of a clinical geneticist and if the previous mentioned techniques had not revealed a diagnosis.

Statistics

Profile likelihood confidence intervals were calculated by using the R package “binom” (<http://www.R-project.org/>) because of small sample sizes. SPSS Statistics v.17.0 (SPSS Inc., Chicago, IL, USA) was used to compute the Fisher exact test and to evaluate the difference between the occurrence of outcomes in different groups.

Results

Between January 1, 2008 and June 1, 2015, isolated cACC was detected by prenatal ultrasound in 25 patients (Table 1). Nineteen were first diagnosed in the second trimester and 6 in the third trimester.

Diagnostic yield of single nucleotide polymorphism array in prenatally isolated complete corpus callosum

In the 25 patients with isolated cACC on ultrasound, 18 patients opted for prenatal ($n = 7$) or postnatal SNP array ($n = 11$). Among these 18 cases, 2 causal abnormalities were found (cases 4 and 16; Tables 2 and S2b in the Supporting Information), both of microscopic size. The diagnostic yield of SNP array therefore is 11.1% (95% CI 2.0%-31%). Three cases showed an incidental finding, non-causal for cACC or ID (cases 6, 11, and 17; Tables 2 and S2b).

Neurodevelopment in fetuses without causal anomalies on single nucleotide polymorphism array

In the 16 cases without a causal anomaly on SNP array, 5 pregnancies were terminated

and 11 resulted in a live birth (Figure 1). After losing 1 case to follow-up (case 19), 10 live births were available for neurodevelopmental follow-up (median 40 months, interquartile range [IQR] 20-63). Three of 10 showed ID (30%; 95% CI 8.5%-60%): one case with Mowat Wilson syndrome (case 12), 1 case with Aicardi syndrome (case 17), and 1 case with a complex syndromic appearance (case 25; Tables 2 and S2b).

Neurodevelopment in fetuses without causal anomalies on single nucleotide polymorphism array or additional anomalies on prenatal magnetic resonance imaging

Six in 16 cases without a causal anomaly on SNP array underwent prenatal MRI (median gestational age 25 weeks, IQR 22-33). Magnetic resonance imaging showed no other malformations (in case 11, an uncertain result was retrieved: a possible migration disorder at 21 weeks). Two cases, including case 11, were terminated. Consequently, 4 cases with a normal result of both SNP array and prenatal MRI were available for neurodevelopmental follow-up (median 37 months, IQR 17-75), revealing ID in 1 case (25%; 95% CI 2.0%-72%). This case had Mowat Wilson syndrome (case 12, Tables 2 and S2b and Figure 2).

Postnatal physical anomalies in fetuses without causal anomalies on single nucleotide polymorphism array

Fourteen of 16 cases without a causal anomaly on SNP array were available for postnatal physical examination. Nine (9/14; 64%; 95% CI 38%-85%) showed additional physical abnormalities, varying from hypermobility to epilepsy. The most common anomalies apparent after birth were facial dysmorphias (Tables 2 and S2b). Postnatal MRI was done in 14 cases with a normal SNP array, showing additional anomalies in 9 (9/14; 64%; 95% CI 38%-85%). Consequently, only 2 in 16 cases (12.5%; 95%

Figure 1. Neurodevelopment in fetuses with isolated complete corpus callosum (cACC) on ultrasound

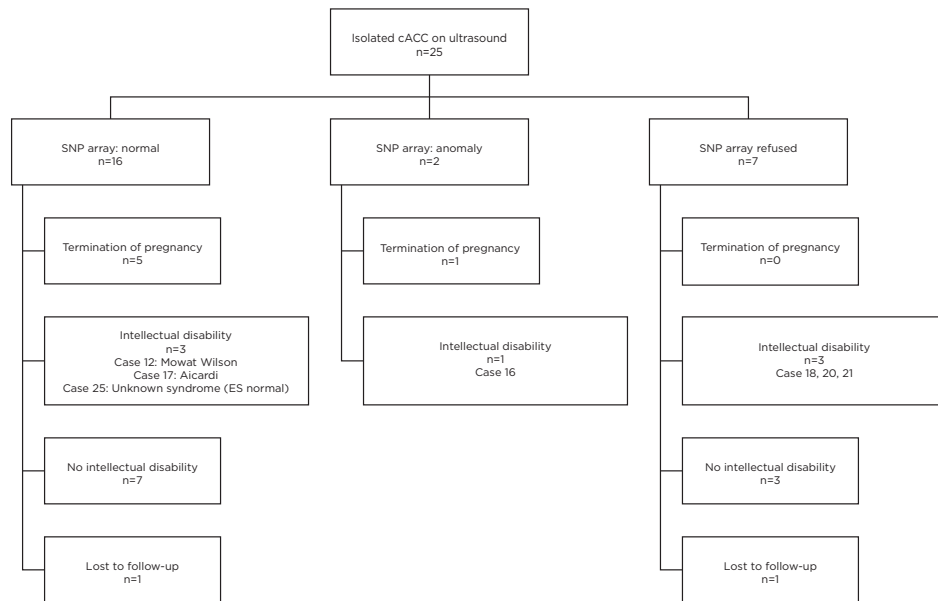
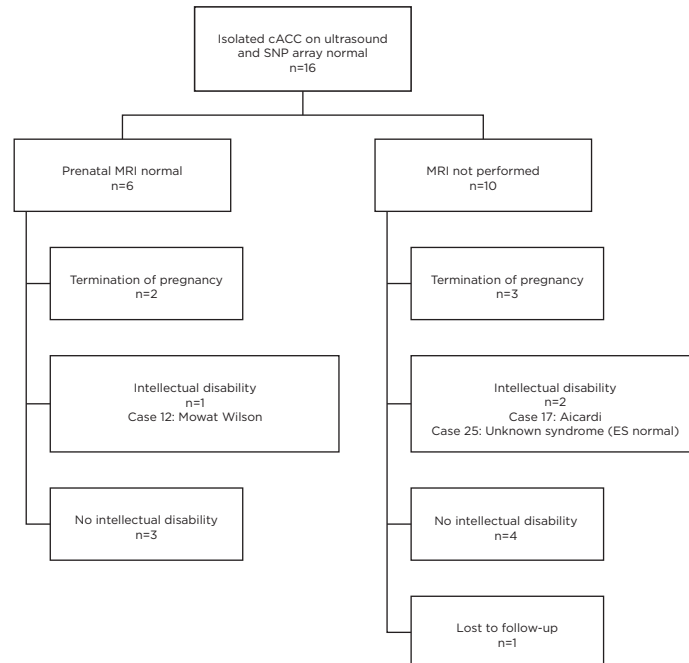


Figure 2. Neurodevelopment in fetuses with isolated complete corpus callosum (cACC) on prenatal ultrasound, prenatal magnetic resonance imaging, and single nucleotide polymorphism array



CI 2.2%-34%; cases 2 and 22; Tables 2 and S2b) with isolated cACC on ultrasound and a normal SNP array proved to be truly isolated after postnatal physical examination and MRI. Both cases showed normal neurodevelopment (Figure 3).

Potential diagnostic yield of exome sequencing in fetuses without causal anomalies on single nucleotide polymorphism array

Among the 3 cases with ID and a normal SNP array result, there was 1 case with Mowat Wilson syndrome (case 12, ZEB2 mutation), 1 case with dysmorphic facies and a normal result of ES (case 25), and 1 case of Aicardi syndrome (case 17). The latter is a clinical diagnosis and ES was not performed. Among the 9 cases with additional physical anomalies on postnatal physical examination and MRI, but no ID, 1 single-gene disorder was found in 2 cases (cases 3 and 13) that consented to ES: a ACVR1 mutation for fibrodysplasia ossificans progressiva (case 13). An etiologic relation between this mutation and cACC remains unclear; brain malformations have only been associated with an atypical ACVR1 mutation.¹⁷⁷ In summary, sequencing in 4 cases revealed 2 single-gene disorders. Therefore, the diagnostic yield of ES in postnatally selected cases is 2/4 (50%; 95% CI 11%-89%). Because this postnatal selection introduces bias, we estimate the true diagnostic yield of ES in fetuses without causal anomalies on SNP array to be between 2/4 and 2/16 (13.3%; 95% CI 2.4%-36%).

Prognostic importance of midline cysts and colpocephaly

We tested whether midline cysts or colpocephalies were of influence on the rate of ID in cACC fetuses without chromosomal anomalies on SNP array (Table 3): There was no difference between cases with (0/1) and without (3/9) midline cysts ($p=0.7$), cases

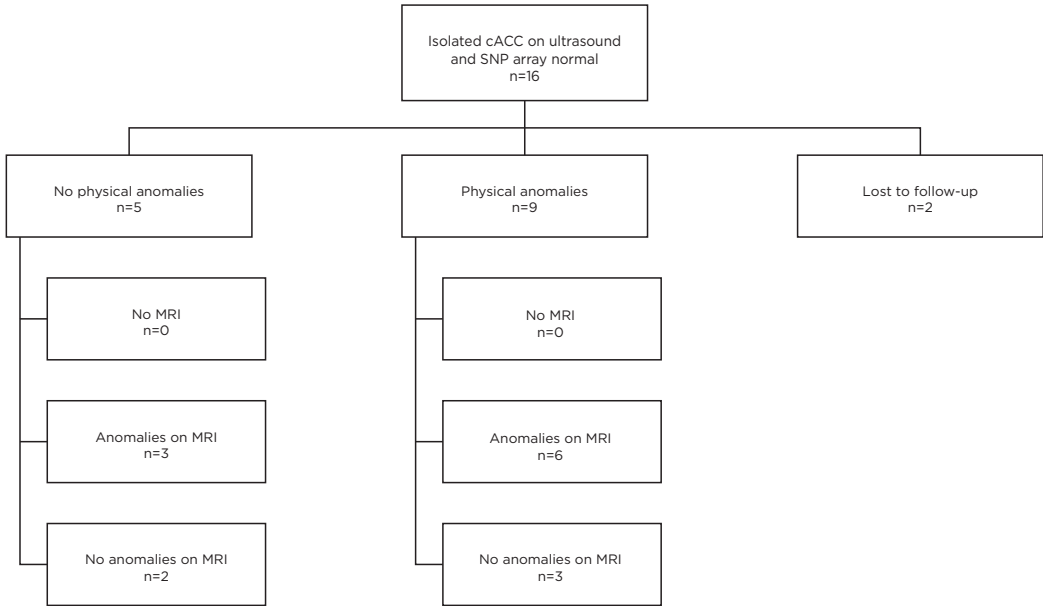
with (1/3) and without (1/4) second trimester colpocephaly ($p=0.71$), and cases with (3/9) and without (0/1) third trimester colpocephaly ($p=0.70$).

Discussion

In 11.1% (2/18; 95% CI 2.0%-31%) of pregnancies with isolated cACC on ultrasound, SNP array detected a causal chromosomal anomaly. In cases without a causal anomaly on SNP array, who were born alive and available for follow-up, 30% (3/10; 95% CI 8.5%-60%) showed ID. In fetuses with isolated cACC on ultrasound and without causal anomalies on SNP array, postnatal MRI and physical examination often (64%; 95% CI 38%-85%, and 64%; 95% CI 38%-85%, respectively) revealed additional physical anomalies. Therefore, only 2 cases showed truly isolated callosal agenesis after birth. Four cases with additional congenital anomalies apparent on postnatal examination and/or ID were postnatally subjected to sequencing. This identified 2 single-gene disorders. The estimated diagnostic yield of ES in prenatally ascertained cACC fetuses without chromosomal anomalies on SNP array therefore is between 2/4 (50%; 95% CI 11%-89%) and 2/16 (13.3%; 95% CI 2.4%-36%).

In this study, we described a relatively large cohort of fetuses with prenatally apparent isolated cACC in extensive detail. Our findings are very useful for prenatal counseling. However, our analysis was complicated by several factors. Because of the rarity of isolated cACC, a long inclusion period was needed to acquire a sample of 25 cases. Over this long course, progressing scientific insights have led to changes in diagnostic approach. Therefore, the diagnostic strategies applied in the cases included in this study are heterogeneous. For example, 6 of 16 cases with a normal result of

Figure 3. Postnatal physical anomalies in fetuses with isolated complete corpus callosum (cACC) on prenatal ultrasound and single nucleotide polymorphism array



SNP array were subjected to prenatal MRI, of which the timing had a wide spread (median 25 weeks, IQR 22-33). The timing of fetal MRI is important as its diagnostic efficacy increases with gestational age.¹⁷⁸ Because the legal termination limit in the Netherlands is set at 24 weeks, MRI is frequently scheduled <24 weeks. However, it should be taken into account that additional brain anomalies might have been missed in cases that underwent MRI in the early second trimester. Moreover, further heterogeneity arose because not all cases opted for maximal diagnostic testing. For example, SNP array was refused by the parents in 7 cases. Although the rate of ID between the cases that were subjected to SNP array (4/11) and were not subjected to SNP array (3/6) was not statistically different ($p=0.6$), selection bias cannot be ruled out completely. Furthermore, this diagnostic heterogeneity led to small

samples available for sub-analysis and this has substantially complicated our analysis. Another limitation of our study is found in the way we could report on neurodevelopment. Ideally, this is tested with a validated, standardized tool at set times throughout childhood. The retrospective design of our study did not allow us to assess and report neurodevelopment in such a manner. However, we did have thorough follow-up of almost all cases during a median of 45 months, with a minimum of 9 months. Children were examined frequently by a specialized pediatric neurology team and in a population screening program. Unfortunately, our follow-up might not be long enough to identify all cases with ID. Furthermore, we only reported the rate of ID, while cases without ID often showed delayed or abnormal motoric development or mild learning difficulties (Table S2b). This agrees with the findings of other

Table 3. Chance of ID in cases with and without midline cyst and colpocephaly

	Present	Absent	p
Midline cyst	0%(95%CI0-86)	33%(95%CI9.6-65)	0.7
Second trimester colpocephaly	33%(95%CI2.6-84)	25%(95%CI2.0-72)	0.71
Third trimester colpocephaly	33%(95%CI9.6-65)	0%(95%CI0-86)	0.7

research groups, including those by Mangione et al.¹⁷⁹ and Moutard et al.¹⁵⁸ Children with prenatally isolated cACC score significantly lower for gross motor and fine motor skills on the Ireton's "Child Developmental Inventory" have more difficulties with visuomotor skills and half of children experience mild to moderate learning difficulties. Therefore, the rate of all neurodevelopmental problems combined in our cohort is presumably higher than the rate of ID we reported.

Only a small proportion of cases with isolated cACC on prenatal ultrasound are truly isolated on postnatal examination. Especially in postnatally non-isolated cases, single-gene mutations are the largest group of genetic causes and the number of known candidate genes is still growing.¹⁸⁰ Because of this genetic heterogeneity, ES might be the designated testing technique in cACC. In our cohort, 2 single-gene mutations (in ZEB2 and ACVR1) were found in 4 postnatally tested cases. Because prenatal ultrasound is less efficient in identifying syndromic cases from truly isolated cases as compared to postnatal examination, the actual diagnostic yield of ES in this prenatally ascertained cohort is estimated to be between 2/25 (50%; 95% CI 11%-89%) and 2/4 (13.3%; 95% CI 2.4%-36%). Despite its high diagnostic yield, the use of ES in the prenatal setting is currently limited, mostly because of lack of time and financial resources. Another major restraint in the prenatal use of ES is the likelihood of unsolicited findings, such as variants of unknown significance, variants unrelated to the phenotype, and variants for late onset diseases.¹⁸¹ These variants can also

be found with microarray, as shown in our cohort (cases 6, 11, and 17), but ES presumably retrieves them at a higher rate. It has been a topic of debate whether or not to disclose aberrations with an uncertain phenotypic outcome. Recent research indicates, however, that most parents who were confronted with the prenatal finding of uncertain phenotypic outcome would want to be informed of such a finding again if offered a choice.¹⁸² Moreover, if this genomic uncertainty is explicitly discussed in pretest counseling, it does not have to be received as problematic per se.¹⁸³ In fact, according to a recent study from the United States, most expecting parents claim that ES should be offered before birth.¹⁸⁴ Next-generation sequencing of known candidate genes for cACC could be considered as an alternative³⁸ in settings in which ES is infeasible or undesirable. However, a customized NGS panel for cACC should include a large number of genes because of its genetic heterogeneity. As prenatal phenotyping of midline structures can be difficult, as illustrated by cases 11, 14, and 23 in our study, the panel should be complemented with genes involved in brain anomalies that can resemble cACC prenatally, such as hypoplasia or partial agenesis of the ACC. Furthermore, such a panel should be updated regularly when new ACC-related genes are discovered.

Yet, although NGS could be very valuable in fetuses with isolated cACC, it will, at present, not be able to identify all important cACC syndromes. This is illustrated by Aicardi syndrome (case 17), a clinically distinct entity which is genetically still unraveled.^{180, 185}

Conclusions

In accordance with current guidelines, we conclude that microarray should be offered in case of isolated cACC on ultrasound. Exome sequencing is likely to be informative for prenatal counseling and should be offered if microarray is normal. However, even a diagnostic work-up of both SNP array and ES will not identify all causes for ID in cACC fetuses.

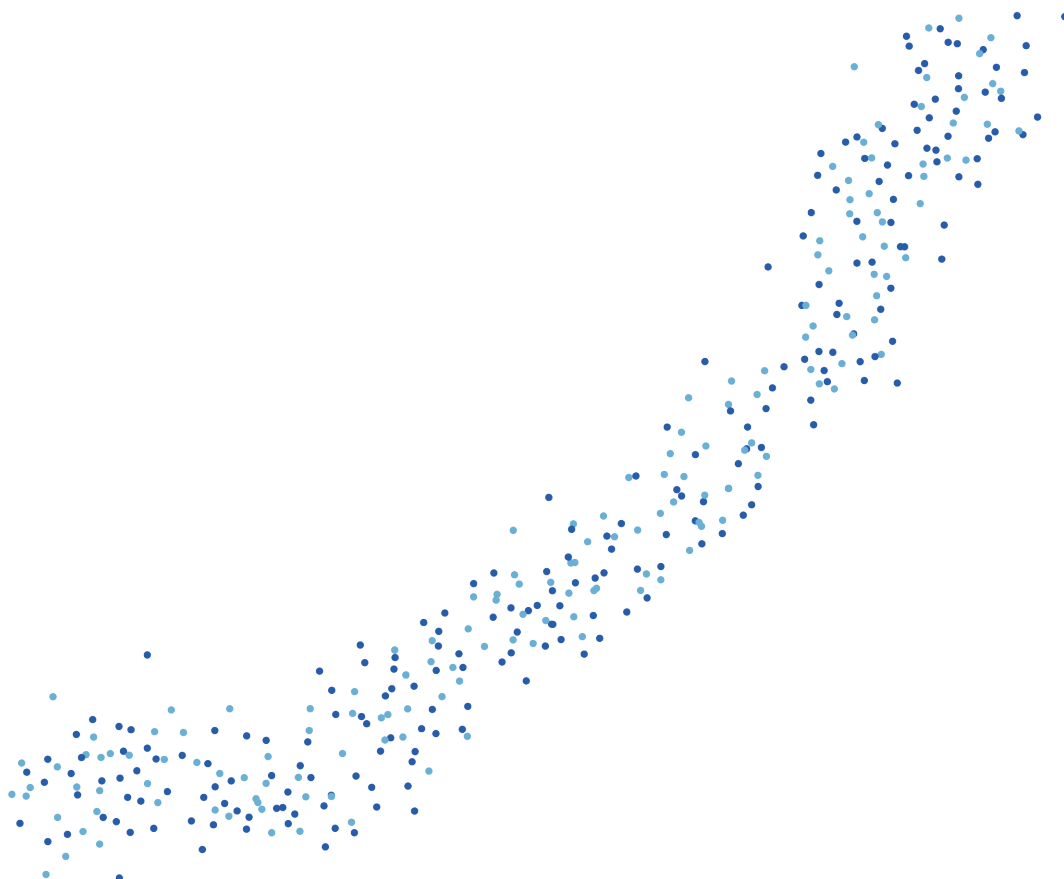
Table S2b. Descriptions of all cases

No. (GA)	Prenatal US and MRI diagnosis	Prenatal genetic diagnosis	Outcome	Follow-up (months)	Postnatal phenotype (incl. MRI)	Postnatal genetic diagnosis	Neurodevelopmental outcome
1 20+3	♂. ACC, two interhemispheric cysts; MRI (21+5): No additional anomalies	SNP array: No pathogenic anomaly	TOP	-	Autopsy: cACC, cysts in central sulcus, polymicrogyria, abnormal migration Other: None	-	-
2 20+3	♂. ACC, 3 rd trimester colpocephaly; MRI (22+2): No additional anomalies	-	LB	61	MRI: cACC with colpocephaly, intraventricular hemorrhage Other: None	SNP array: No pathogenic anomaly	Mildly impaired fine motor skills (handwriting).
3 20+3	♂. ACC without colpocephaly; MRI-	-	LB	22	MRI: cACC, brain-in-brain malformation, heterotopia in left anterior horn, unfused thalami, partial agenesis of the cerebral falk; Other: Hypothalamic fever, Reverse Shapira's Syndrome	SNP array: No pathogenic anomaly ES: No pathogenic mutations	Normal neurodevelopment.
4 20+4	♀. ACC without colpocephaly; MRI (22+3): no additional anomalies	SNP array: 6.9Mb deletion in 8p23.3p23.1 and a 315Mb duplication in 8p23.1p11.1 de novo (inverted deletion-duplication of 8p)	TOP	-	Autopsy: cACC, mild dysmorphic facies Other: None	-	-
5 20+4	♂. ACC, interhemispheric cyst, 3 rd trimester colpocephaly; MRI (21+0): No additional anomalies	-	LB	97	CT: cACC, interhemispheric cyst and colpocephaly; Other: Hypermobility, increased head circumference	Karyotype: normal. No further testing because parents declined.	General mild impaired fine and gross motoric skills and speech development. Follows normal preschool education, but repeated 2 nd class. No behavioral problems.
6 20+5	♂. ACC with colpocephaly; MRI-	SNP array: paternal deletion in Yp11.32 inherited from a father with phenotypic characteristics of Leri-Weill dyschondrosteosis.	TOP	-	No postmortem examinations	-	-

No. (GA)	Prenatal US and MRI diagnosis	Prenatal genetic diagnosis	Outcome	Follow-up (months)	Postnatal phenotype (incl. MRI)	Postnatal genetic diagnosis	Neurodevelopmental outcome
7 20+5	♀. MCDA twin. ACC with colpocephaly, Blake's pouch cyst; MRI-	-	LB	98	MRI: cACC, vermin and cerebellar hypoplasia (symmetric). Other: Impaired stereovision. Increased head circumference compared to homozygous sister.	SNP array: No pathogenic anomaly	Slightly suboptimal motoric and cognitive development as compared to twin sister. No ID.
8 20+5	♀. ACC, interhemispheric cyst; colpocephaly; MRI (32+4); No additional anomalies	SNP array: No pathogenic anomaly	LB	45	MRI: cACC, large interhemispheric cyst left which causes a midline shift and compression of left hemisphere. Focal polymicrogyria around cyst. Colpocephaly. Other: Smaller head circumference, mild hypermetropia.	-	Mild cerebral paresis unilateral right.
9 20+6	♀. ACC without colpocephaly; interhemispheric cyst; MRI-	SNP array: No pathogenic anomaly	TOP	-	Postmortem MRI: cACC. Autopsy: Mild dysmorphic facies. ACC with thin cortex.	-	-
10 20+6	♂. ACC, 3 rd trimester colpocephaly; MRI-	-	LB	21	MRI: cACC with colpocephaly Other: None	-(parents declined)	Mild lateralization in favor of left.
11 21+0	♀. ACC without colpocephaly; MRI (21+2); Uncertain result: Possibly abnormal migration	SNP array: 1.9Mb duplication in 15q13.2q13.3, inherited from a mother without symptoms (Susceptibility locus)	TOP	-	Postmortem MRI: Abnormal cerebral falx Autopsy: Agenesis of cavum septum pellucidum Other: None	-	-
12 21+1	♀. ACC, 3 rd trimester colpocephaly; MRI (28+4); No additional anomalies	-	LB	28	MRI: cACC with colpocephaly Other: Mild dysmorphic facies, unilateral renal duplicate system, urachus cyst.	SNP array: No pathogenic anomaly SS: c.2403C>G (p.(Tyr80T)) nonsense mutation in ZEB2, de novo Associated with Mowat Wilson syndrome	Signs of intellectual disability.
13 21+2	♀. ACC, 3 rd trimester colpocephaly; MRI-	-	LB	34	MRI: cACC with colpocephaly, brainstem hypoplasia Other: Hallus valgus. Mild hypertonia of the legs.	ES: c.617G>A, p.Arg206 His mutation in ACVR1 (exon 7) Associated with Fibrodysplasia ossificans progressiva	Mild delayed motoric skills.
14 21+4	♂. ACC without colpocephaly; MRI-	SNP array: No pathogenic anomaly	TOP	-	Postmortem MRI: pACC (or pre-optic holoprosencephaly). Other: Mild dysmorphic facies.	-	-
15 21+4	♀. ACC with colpocephaly; MRI (22+4); No additional anomalies	-	LB	64	MRI: cACC Other: None	Karyotype: normal. No further testing because parents declined	Normal motoric development. Follows normal preschool education but performs lower than average in class. Short attention span.
16 21+4	♂. ACC with colpocephaly; MRI-	-	LB	55	MRI: cACC. Periventricular white matter abnormalities. Other: Mild valvular stenosis of aorta with mild insufficiency.	SNP array: 43 Mb mosaic amplification in 8p23.3p11, de novo Karyotype: Mosaic tetrasomy 8p	Signs of intellectual disability: Mild cerebral paresis, spastic, delayed speech development.

17 21+6	♀, ACC with colpocephaly (ultrasound image quality low due to maternal BMI); MRI-	-	LB	67	MRI: cACC with colpocephaly, enlarged cisterna magna. Other: Aicardi syndrome	SNP array: Duplication in 17p, maternally inherited Associated with Charcot Marie Tooth 1A.	Signs of intellectual disability, epilepsy.
18 23	♂, ACC with colpocephaly; MRI-	-	LB	56	MRI not performed because parents declined Other: Mild hypertelorism	Karyotype: normal. No further testing because parents declined	Signs of intellectual disability: Speech and language delay, attention disorder, mild epilepsy.
19 26+3	♀, ACC with colpocephaly; MRI-	-	LB	1	MRI: cACC, heterotopias in lining of both posterior horns Other: None	SNP array: No pathogenic anomaly	Lost to follow-up (parents declined neurodevelopmental examination)
20 31+2	♂, ACC with colpocephaly; MRI-	-	LB	35	MRI: cACC with colpocephaly Other: Pierre-Robin sequence like facies	- (parents declined)	Signs of intellectual disability: Delayed speech and language development.
21 31+6	♂, ACC with colpocephaly; MRI-	-	LB	28	MRI: cACC with colpocephaly, heterotopia in lining of left lateral ventricle, arachnoid cyst right infratentorial Other: None	- (parents declined)	Signs of intellectual disability. Cerebral palsy, left spasm.
22 32+1	♂, ACC with colpocephaly; MRI-	-	LB	9	MRI: cACC Other: None	SNP array: No pathogenic anomaly	Mild asymmetry in favor of right
23 32+6	♂, ACC with colpocephaly; MRI (34+2); No additional anomalies	-	LB	13	MRI not performed; Cranial ultrasound: pACC (posterior) Other: None	SNP array: No pathogenic anomaly	Normal neurodevelopment.
24 33+6	♂, ACC with colpocephaly; MRI (33+0); No additional anomalies	-	LB		Lost to follow-up (On the 45 th day after birth the patient's mother informed us that postnatal examination had shown no additional anomalies)	Lost to follow-up (patient moved to another country)	Lost to follow-up
25 37+5	♂, ACC with colpocephaly; MRI-	-	LB	58	MRI: cACC, gracile brain stem Other: Dysmorphic facies	SNP array: No pathogenic anomaly ES: No causal pathogenic mutations.	Signs of intellectual disability.

GA, Gestational age; TOP, Termination of Pregnancy; LB, Live birth; cACC, Complete Agenesis of the Corpus Callosum; pACC, partial Agenesis of the Corpus Callosum; SNP, Single nucleotide polymorphism; SS, Sanger sequencing; ES, Exome sequencing



Chapter 3.4

Prenatal whole exome sequencing
increases diagnostic yield in (non-
isolated) congenital heart malformations

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Submitted

Abstract

Objective

To estimate the potential diagnostic yield of prenatal whole exome sequencing by using the outcomes of postnatal Sanger and next generation sequencing in a prenatally ascertained cohort.

Methods

This is a retrospective cohort study. Pregnancies with an isolated or non-isolated fetal heart malformation on prenatal ultrasound were included. Invasive prenatal testing by single nucleotide polymorphism array was offered. Postnatal follow-up by cardiologists and clinical geneticists was collected for every case. Array was offered again postnatally if parents had renounced prenatal testing. If SNP array showed no pathogenic chromosomal imbalances, but the suspicion of a genetic abnormality remained, patients were offered either Sanger sequencing, small next generation sequencing gene panel sequencing (≤ 41 genes) or whole exome sequencing, based on the postnatal phenotype. To compare the open diagnostic approach of whole exome sequencing with the narrow approach of gene panel sequencing, we drafted a hypothetical panel of 54 cardiac-risk genes from the different diagnostic cardiac-risk panels available in the Netherlands at the time of our study.

Results

445 fetuses were enrolled, including 229 isolated and 216 non-isolated cases on prenatal ultrasound. 358 underwent chromosomal testing, which revealed an anomaly in 28% (95%CI23-33%). Among the chromosomally normal fetuses ($n=258$), subsequent DNA sequencing was done in 128 cases, which revealed a diagnosis in 30 cases. The potential diagnostic yield of prenatal whole exome sequencing in fetuses with a cardiac

malformation on prenatal ultrasound is therefore between 6.7% (30/445; 95%CI4-9%) and 23.4% (30/128; 95%CI16-31%). For prenatally non-isolated cases only, its yield is between 11.6% (25/216; 95%CI7-16%) and 29.8% (25/84; 95%CI20-40). Thirty percent of diagnoses were found in the genes included in our hypothetical cardiac-risk gene panel.

Conclusion

Single gene disorders occur in 6.7-23.4% of all fetuses with a cardiac anomaly on prenatal ultrasound. To diagnose these disorders prenatally, the open diagnostic approach of whole exome sequencing would be more effective compared to the narrow approach of next generation sequencing gene panel testing, as only 30 percent of diagnoses is found in typical cardiac-risk genes. As the prevalence of single gene disorders is the highest in prenatally non-isolated cardiac malformations (11.6-30%), these cases would benefit the most from whole exome sequencing.

Introduction

Congenital heart malformations (CHMs) have an incidence of 9.1 per 1000 newborns¹⁸⁶. Most CHMs are non-syndromic, of which a subset can be explained by de novo¹⁸⁷ and inherited¹⁸⁸ single gene mutations. Syndromic CHMs, however, are frequently associated with distinct genetic conditions of varying nature, such as chromosomal aberrations and single gene disorders. The presence of such a syndrome can influence the surgical outcome of the CHM¹⁸⁹⁻¹⁹² and may be associated with other congenital malformations and intellectual disability. Furthermore, the presence of a syndromic condition can have a high impact on recurrence risk and parental reproductive choices. Prenatal ultrasound screening for CHM and subsequent fetal genetic testing to those affected is offered in most western countries. For fetal genetic testing, the American College of Obstetricians and Gynecologists (ACOG) guidelines advise to offer a chromosomal microarray (CMA). CMA detects quantitative chromosomal aberrations and delivers a genetic diagnosis in approximately 28.1 - 43.7% (microscopic^{193, 194}) plus 4-4.5% (submicroscopic^{94, 195}) of prenatal CHM cases. However, CMA cannot detect monogenic disorders caused by single nucleotide mutations. For decades, the diagnosis of these mutations relied on Sanger sequencing, a labor intensive and time consuming sequencing method that does not allow for upscaling to genome wide sequencing. The use of this technique in prenatal diagnosis is limited, as it requires the predetermination of a few genes of interest, which is difficult based on the limited phenotypic information from prenatal ultrasound. With the introduction of next generation sequencing (NGS) however, large sets of genes can be interrogated simultaneously within a short time frame. NGS of the whole exome or whole genome (WES/WGS) has been a very powerful tool in research

for the identification of genes associated with (syndromic) CHM^{196,197} and it is increasingly used in the postnatal clinical setting for a variety of indications. It has been proposed for prenatal diagnosis too. However, there are ethical and practical restraints against its prenatal use. Inherent to the increase in genomic resolution and its untargeted approach, the likelihood of finding variants of unknown significance (VUS), variants of phenotypic heterogeneity and variants for (untreatable) late onset diseases increases. In prenatal cases the hazard of these findings can be more profound compared to postnatal cases, because of the short time frame available for analysis and the lack of phenotypic information from prenatal ultrasound. Moreover, it is feared that uncertain results might lead to increased pregnancy termination rates.¹⁹⁸

Different authors have joined in the discussion on prenatal large scale NGS. While some argue in favor of WES³⁷ others plea for a more targeted approach³⁸ or no large scale sequencing at all. The present study is undertaken to inform this discussion. We aim to estimate the potential diagnostic yield of prenatal WES by using the outcomes of postnatal Sanger and NGS in a prenatally ascertained cohort.

Subjects and methods

For this cohort study all pregnancies presenting with any fetal CHM on prenatal ultrasound between January 1st 2011 and January 1st 2016 at the Erasmus MC prenatal care clinic were retrospectively collected. The local medical ethics review board approved this study.

Subjects were identified through a query in all obstetric ultrasound patient files of our antenatal outpatient clinic (Astraia software gmbh, Munich, Germany), based on a tick in the box: Abnormality of the heart.

All patient files retrieved from this query were investigated to determine eligibility for inclusion. Patients were included if fetal CHM was detected and confirmed by an experienced prenatal sonographer together with a pediatric cardiologist on high quality equipment (Voluson E8 or E10; General Electric Healthcare, Chicago, Illinois, United States). Both cases with and without other malformations on prenatal ultrasound were included. Cases without other malformations on ultrasound are referred to as prenatally isolated CHM and those in the context of other fetal malformations are considered prenatally non-isolated CHM. Cases were excluded from this analysis if a parent was known to carry a CHM causing genetic anomaly that the fetus could have inherited. The nature of the CHM and the coexisting non-cardiac malformations were retrieved from the prenatal ultrasound files. Anomalies were classified according to the pathogenic classification designed by Edward Clark¹⁹⁹, with the exception of dextro-transposition of the great arteries²⁰⁰ and Ebstein malformation. Follow-up was collected from fetal and pediatric patient files.

Genetic diagnostic work-up

As part of our prenatal CHM diagnostic work-up, all prospective parent-couples were offered invasive testing and prenatal genetic counseling. Invasive testing included first tier quantitative fluorescent polymerase chain reaction (QF-PCR) of the chromosomes 13, 18, 21, X and Y. Illumina 150kb SNP array was done if QF-PCR showed no abnormalities. If and when the presence of malformations was confirmed on postnatal evaluation, parents were offered additional genetic counseling after birth. For clinical genetic consultation physical, radiological, and/or pathological findings were taken into consideration. If parents had waived invasive prenatal genetic testing, they were offered a SNP array again as first line genetic

test. If prenatal or postnatal SNP array showed no pathogenic imbalances, but the suspicion of a genetic abnormality remained, patients were offered different additional genetic tests based on the postnatal phenotype:

- Single/multiple gene Sanger sequencing or NGS gene panel sequencing (≤ 41 genes) in
 - › postnatally isolated “non-syndromic” CHM, when a mutation in a certain gene / certain genes was suspected based on the cardiac phenotype (e.g. NGS panel for left sided heart malformations). Because of the expected low yield of these tests in non-syndromic CHM, these tests were only performed in a subset of the non-syndromic cases.
 - › postnatally non-isolated “syndromic” CHM, when a mutation in a certain gene / certain genes was suspected based on phenotype (e.g. Sanger sequencing of CHD7 in a CHARGE-like phenotype or a NGS Noonan gene panel in a Noonan(-like) phenotype).
- WES in postnatally non-isolated “syndromic” CHM, when the phenotype did not allow a narrow genetic differential diagnosis. WES data were analyzed as a trio with both parents (filtering for de novo, autosomal recessive and X-linked variants). Initially, only genes known to be involved in congenital malformations ($n \approx 2750$) were analyzed. If no pathogenic mutation could be found in these genes, the rest of the exome was analyzed subsequently.

Informed consent for genetic testing was reached after thorough genetic counseling. Parents were free to opt out from any genetic testing offered, at any time.

For the purpose of analyzing the potential diagnostic efficacy of a CHM-risk gene panel instead of WES, we composed a hypothetical

Table 1. Cohort characteristics

Characteristic		
Median maternal age (years, IQR)		30 (27-34)
Median gestational age at diagnosis (weeks, IQR)		21 (20.4-21.9)
Prenatal ultrasound evaluation (n=445)		
	Isolated CHD	229 (51.5%)
	Co-existing non-CHD anomalies	216 (48.5%)
Outcome (n=445)		
	Termination of pregnancy	117 (26.3%)
	Spontaneous fetal demise	30 (6.7%)
	Neonatal demise	30 (6.7%)
	Livebirth	268 (60.2%)
	Median gestational age at birth (in weeks, IQR)	38 (37-39)
	Median duration of follow-up(months, IQR)	14 (3-31)
Genetic evaluation (n=445)		
	No genomic testing	87
	Prenatal or postnatal CMA testing	358
	Pathogenic chromosomal anomaly	100
	No pathogenic chromosomal anomaly	258
	Postnatal no gene testing	130
	Postnatal gene testing	128
	Pathogenic mutation	30
	No pathogenic mutation	98

CHM-risk gene panel (table S4). This panel combines the 57 genes from all clinically available CHM NGS panels in the Netherlands between 2011 and 2016.

Statistical analysis

SPSS Statistics v17.0 (SPSS Inc., Chicago, IL, USA) and R (<http://www.R-project.org/>) was used for computing statistical analysis. In analyses with very small sample sizes ($n < 5$), the R package 'binom' was used to compute profile likelihood confidence intervals. In order to compute odds ratios for CMA subtypes with zero occurrences of genetic anomalies, 0.5 was added to all cells in the cross tabulation. The Pearson's Chi Square test or the Fishers exact test (in case of small sample sizes) were used to explore differences between subgroups. The significance level was set at $P < 0.05$.

Results

445 fetuses were enrolled, of whom 229 showed isolated CHM and 216 non-isolated CHM on prenatal ultrasound. Cohort characteristics can be found in table 1. The CHMs detected on prenatal ultrasound are listed in table 2.

Diagnostic yield in all cases (n=445)

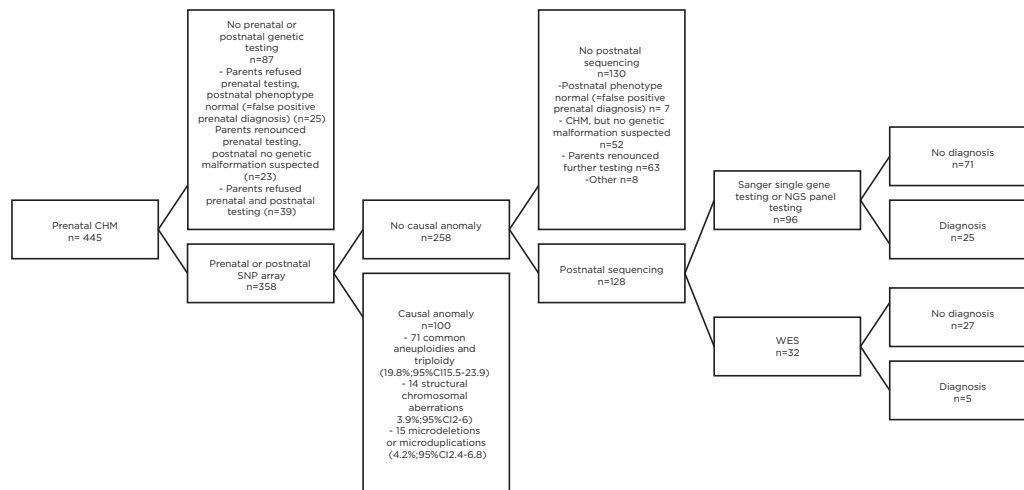
See figure 1a and 1b.

SNP array was performed prenatally or postnatally in 358 of 445 cases. It revealed a causative chromosomal aberration in 100/358 cases (28%; 95%CI 23-33%). Among the chromosomally normal fetuses ($n = 258$), subsequent DNA sequencing was done in 128 cases, revealing 30 diagnoses (30/128; 23.4%; 95%CI 16-31%). 96/128 Cases underwent single/multiple gene Sanger sequencing or NGS gene panel sequencing, which revealed a diagnosis in 25 cases (26%; 95% CI 17-35;

Table 2. Prenatal diagnosis of CHM subtypes

	All cases (n=445)	Isolated CHM cases (n=229)	Non-isolated CHM (n=216)
Conotruncal heart malformation (11604)	80 (18%)	48 (20.9%)	32 (14.8%)
Overriding aorta with subaortic VSD (2623)	11	6	5
Double outlet right ventricle (1719)	3	1	2
Tetralogy of Fallot (1636)	44	28	16
Truncus arteriosus (1660)	10	5	5
Interrupted aortic arch (11611)	8	5	3
Right aortic arch (12020)	4	3	1
Dextro-Transposition of the great arteries (D-TGA)	24 (5.4%)	21 (9.2%)	3 (1.4%)
D-TGA with ventricle septum defect (11607)	6	6	-
D-TGA without ventricle septum defect (11606)	18	15	3
Left sided heart malformation	55 (12.4%)	29 (12.7%)	27 (12.5%)
Hypoplastic left heart syndrome (4383) incl. aortic atresia or mitral valve atresia	32	18	14
Small left ventricle	13	8	6
Aortic (valve) stenosis (1650)	8	1	7
Coarctatio Aortae (12305)	2	2	-
Right sided heart malformation	23 (5.2%)	17 (7.4%)	5 (2.3%)
Hypoplastic right heart syndrome (10954) incl. pulmonary atresia	11	9	2
Ebsteins anomaly (10316)	2	2	0
Small right ventricle	2	0	1
(valvular) Pulmonary artery stenosis (1642)	8	6	2
Atrioventricular (AV) canal defect (6695)	38 (8.5%)	14 (6.1%)	24 (11.1%)
Complete AV canal defect(1674)	26	11	15
Unbalanced AV canal defect(11579)	10	2	8
Ostium primum atrium septum defect (10445)	2	1	1
Ventricular septal defects (VSD; 1671)	46 (10.3%)	16 (7.0%)	30 (13.9%)
Neoplasms of the heart (100544)	5 (1.1%)	5 (2.2%)	-
Rhabdomyoma(9729)	4	4	-
Other	1	1	-
Cardiomegaly	14 (3.1%)	4 (1.7%)	10 (4.6%)
Cardiomyopathy (1637)	3	1	2
Cardiomegaly e.c.i. (1640)	11	3	8
Abnormal anatomic location (4307)	15 (3.4%)	7 (3.1%)	8 (3.7%)
Mesocardia (11599)	6	5	1
Dextrocardia (1615)	5	-	5
Sinistrocardia	4	2	2
Anomaly of the great veins	14 (3.1%)	10 (4.4%)	4 (1.9%)
Persistent left superior cava vein	14	10	4
Heterotaxy (left and right isomerism; 11534)	13 (2.9%)	0	13 (6%)
Complex	105 (23.6%)	48 (21%)	57 (26.4%)
Other Aneurysms, tricuspid / mitral insufficiency, cysts, ectopia cordis	13 (2.9%)	10 (4.4%)	3 (1.4%)

(Human phenotype ontology code)

Figure 1a. Genetic diagnostic flowchart for all cases

105

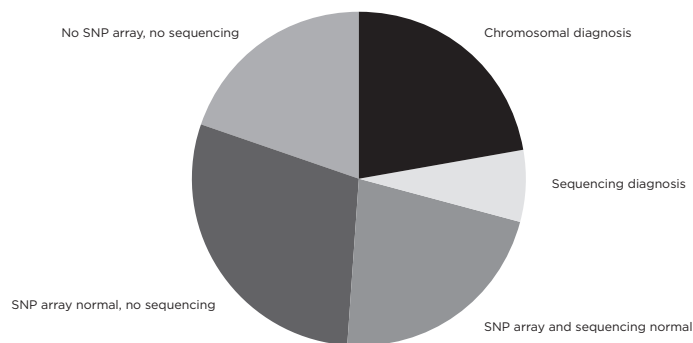
Figure 1b. Pie chart of genetic diagnoses in all cases

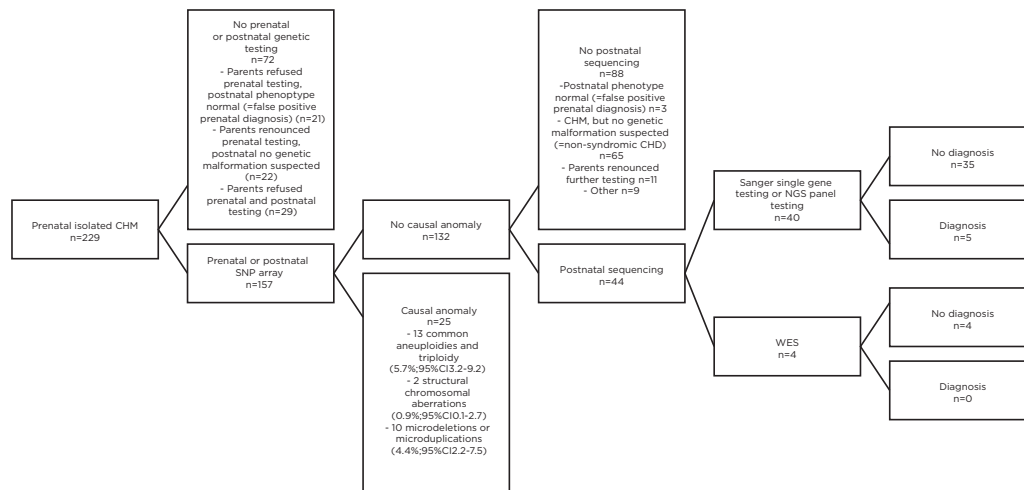
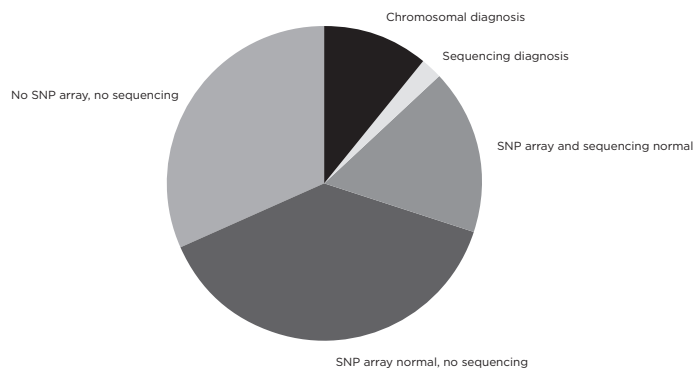
Figure 2a. Genetic diagnostic flowchart for prenatal isolated CHM cases**Figure 2b.** Pie chart of genetic diagnoses in isolated CHM cases

Figure 3a. Genetic diagnostic flowchart for prenatal non-isolated CHM cases

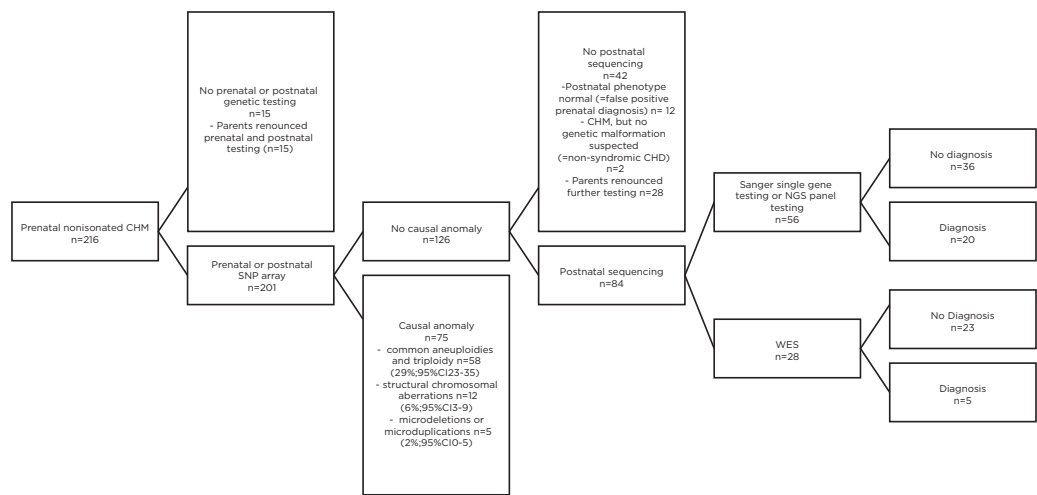


Figure 3b. Pie chart of genetic diagnoses in non-isolated CHM cases

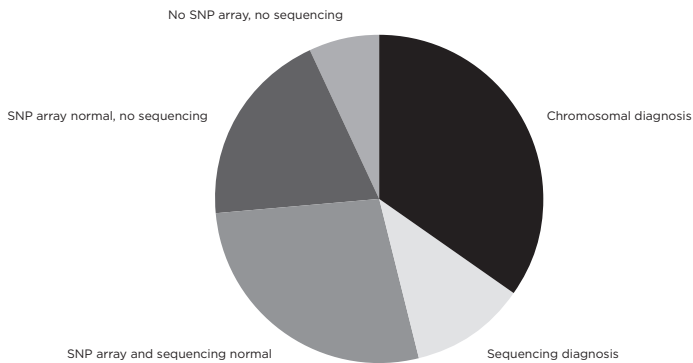


table S5). 32/128 cases underwent WES which delivered a diagnosis in 5 cases (15.6%; 95%CI5.9-30.6;table S5). 9/30 sequencing diagnoses were found in typical CHM-risk genes (table S4 & S5). All mutations were associated with an increased risk of co-morbidity and/or intellectual impairment (table S5).

Diagnostic yield in cases with isolated CHM on prenatal ultrasound (n=229)

See figure 2a and 2b.

229 cases had apparent isolated CHM on prenatal ultrasound. SNP array was done in 157 cases, revealing a chromosomal anomaly in 25 cases (15.9%;95%CI11-23). Among the 132 chromosomally normal cases, 44 cases were postnatally subjected to Sanger sequencing or NGS panel sequencing (n=40) or WES (n=4). This resulted in a diagnosis in 5/44 cases (11.4%;95%CI4.3-23.3;table S5). 3/5 of these mutations were in CHM-risk genes (table S4 & S5).

Diagnostic yield in cases with non-isolated CHM on prenatal ultrasound (n=216)

See figure 3a and 3b.

SNP array was performed in 201/216 non-isolated CHM cases and revealed a diagnosis in 75 cases (37.3%;95%CI31-44). Among the 126 chromosomally normal cases, 84 were subsequently tested by means of Sanger sequencing or NGS panels (n=56) or WES (n=28). A sequencing diagnosis could be made in 25/84 cases (29.8%;95%CI20-40;table S5). 6/25 of these mutations were in CHM-risk genes (table S4 & S5).

Diagnostic yield in specific prenatal CHM subtypes

Table 3 shows the diagnostic yield of SNP array and sequencing in specific prenatal CHM subtypes. To determine whether specific prenatal CHM subtypes were more likely to receive a chromosomal or sequencing diagnosis, the diagnostic yield of postnatal

sequencing for each prenatal CHM subtype was compared to its yield in all prenatal CHM collectively (figure 4a and 4b respectively). Confirming what is known from the literature²⁰¹, atrioventricular canal defects and ventricular septal defects are at a higher risk of a chromosomal disorder relative to other CHM subtypes. As shown in figure 4b, no single prenatally detected CHM subtype was more at risk for a postnatal single gene diagnosis as compared to all CHM subtypes collectively.

Discussion

This study was undertaken to estimate the potential diagnostic yield of prenatal WES in fetuses with CHM on ultrasound. In the current era of new DNA sequencing tests, including NGS gene panels and WES, our aim was to inform the discussion on applying these techniques in the prenatal setting. In our prenatally ascertained cohort of 445 CHM fetuses, 128 postnatally selected cases underwent DNA sequencing. The diagnostic approach was either (semi)targeted (single/multiple gene Sanger sequencing or NGS panel sequencing) or untargeted (WES), based on postnatal phenotype. Thirty diagnoses were made by sequencing. We therefore estimate the true prevalence of monogenic disorders in our prenatal cohort to be at least 30/445 (6.7%;95%CI4-9%) and at the most 30/128 (23.4%;95%CI16-31%). The majority of diagnoses were found by single/multiple gene Sanger sequencing or NGS panel sequencing, because these techniques were applied in phenotypes highly suspect of a specific genetic condition, while WES was only applied in cases with complex phenotypes that did not allow for a (narrow) genetic differential diagnosis. However, we conclude that for prenatal diagnosis, WES would be diagnostically more effective than single/multiple gene Sanger

Figure 4a. Odds ratios for a chromosomal diagnosis by SNP array per CHM subtype (as compared to all CHM collectively)

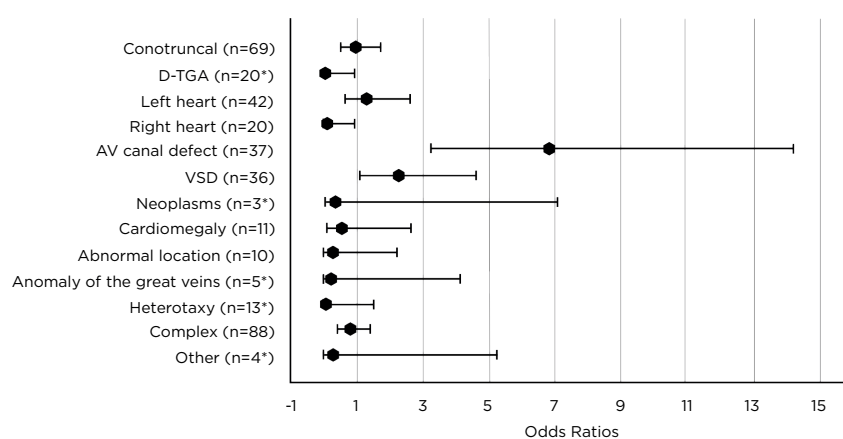
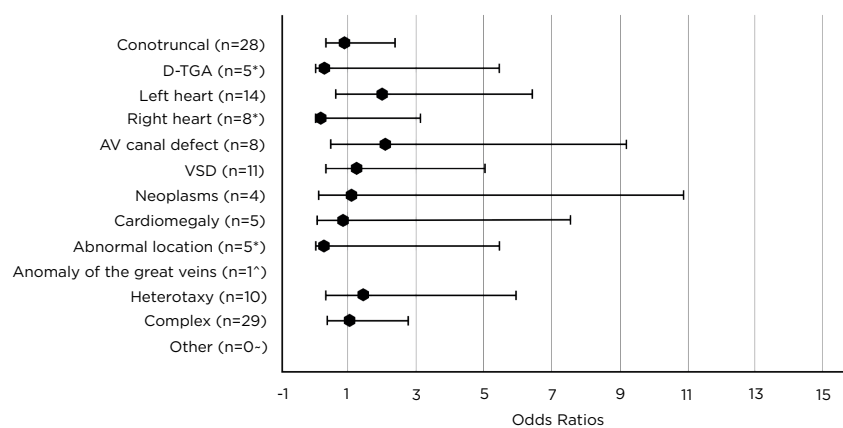


Figure 4b. Odds ratios for a sequencing diagnosis per CHM subtype (as compared to all CHM collectively)



-No cases; *Yield 0% (no occurrences in tested cases); ^Yield 100% (all tested cases were affected)

sequencing or NGS panel testing, for three reasons. Firstly, prenatal ultrasound does not allow for the identification of the full phenotype, especially not of dysmorphic facial features, which are often the most valuable clues for identifying gene targets. Secondly, we found that only 9/30 (30%) diagnoses were found in our hypothetical CHM-risk panel. Thus, a combined panel including all of the 57 CHM-risk genes from the NGS CHM panels available for clinical diagnosis in the Netherlands between 2011 and 2016, would not have detected the majority of single gene disorders in our cohort. Thirdly, given sufficient sequence coverage and depth, all diagnoses found with single gene or panel sequencing can also be found with WES.

In order to identify which prenatal phenotypes would benefit most from WES, we compared isolated and non-isolated CHM cases. In prenatally non-isolated cases the diagnostic yield of the different sequencing approaches combined is higher (between 25/216;11.6%;95%CI7-16% and 25/84; 29.8%;95%CI20-40) than in prenatally isolated cases (between 5/229;2.2%;95%CI0.8-4.6%; $p<0.01$ and 5/44; 11.4%;95%CI4.2-22.9%; $p0.02$). We also compared different types of CHM, but none appeared to be more at risk for a sequencing diagnosis as compared to the all CHM collectively.

To our knowledge, this is the first genetic follow-up study of CHM fetuses aiming to estimate the potential diagnostic yield of prenatal sequencing. We analyzed a large prenatally ascertained CHM cohort with dedicatedly examined phenotypes, both prenatally and postnatally. A majority (82.5%) underwent first line genotyping by means of SNP array and about a third (28.8%) was tested by means of sequencing. A noteworthy complication in our analysis arose from the large diagnostic heterogeneity, as single/multiple gene Sanger sequencing and NGS panel sequencing was targeted at a variety of

genes ($n=181$). Target genes were determined based on postnatal dysmorphology examination by clinical geneticists. Full WES in all of these cases would possibly have been diagnostically more effective. However, this is a retrospective analysis and dysmorphology-free genetic testing is not (yet) the preferred diagnostic practice in our center. Furthermore, although all CHM types collectively have an incidence of 9.1/1000¹⁸⁶, the individual subtypes are rare. Consequently, our subgroup analyses are underpowered. Also, the median follow-up time for livebirths in our analysis was 14 months (IQR 3-31). We assume that if our follow-up was longer, more cases would have been subjected to sequencing, as certain phenotypes such as intellectual impairment take time to become apparent. And lastly, we compared the diagnostic efficacy of a hypothetical CHM-risk panel with WES. This panel includes all of the genes from the CHM panels available for clinical use in the Netherlands between 2011 and 2016. Logically, if we would expand this hypothetical panel, its efficacy (compared to WES) might be different.

We compared the diagnostic yield of WES from our study (5/32) with the findings of several postnatal studies. Lee et al.¹⁰⁹ found a similar ($p0.17$) diagnostic yield of 31% in patients with developmental delay and a heart malformation. Retterer et al.³⁵ analyzed the exomes of 54 cases with cardiovascular malformations and found a definite diagnostic yield of 28%, also similar to our findings ($p0.29$). However, compared to Retterer's multiple congenital anomalies cohort (yield 36%), we found a lower diagnostic yield ($p0.02$). This difference is probably explained by the dominance of isolated CHM in our cohort. Based on the results from our own study and these postnatal studies, we conclude that WES would be valuable in prenatal diagnosis. It could not only identify mutations in known CHM syndromic risk-genes such as PTPN11 and

Table 3. Diagnostic yield per prenatal CHM subtype

	All cases		Isolated CHM only		Non-isolated CHM only	
	SNP array n=358	Sequencing n=128	SNP array n=157	Sequencing n=44	SNP array n=201	Sequencing n=86
Conotruncal	27.5%(17-38) ^{10/69}	21.4%(5-38) ^{6/28}	28.2%(13-43) ^{11/39}	9.1%(0.9-34) ^{1/11}	26.7%(10-43) ^{9/30}	29.4%(5-54) ^{5/17}
D-TGA	0%(0-15) ^{0/20}	0%(0-44) ^{0/5}	0%(0-17) ^{0/17}	0%(0-44) ^{0/5}	0%(0-60) ^{0/3}	-
Left heart	33.3%(18-48) ^{14/42}	35.7%(15-62) ^{5/14}	5.3%(0.5-21) ^{1/19}	20%(1.7-63) ^{1/5}	56.5%(35-78) ^{13/23}	44.4%(17-75) ^{4/9}
Right heart	5%(4.2-20) ^{1/20}	0%(0-3.2) ^{0/8}	0%(0-20) ^{0/14}	0%(0-51) ^{0/4}	16.7%(1.5-55) ^{1/6}	0%(0-51) ^{0/4}
AV canal defect	67.6%(52-83) ^{35/57}	37.5%(11-71) ^{3/8}	46.2%(15-78) ^{6/13}	33.3(2.6-84) ^{1/3}	79.2%(62-97) ^{16/24}	40%(81-80) ^{2/5}
VSD	44.4%(27-61) ^{16/36}	27.3%(4.6-56) ^{3/11}	22.2%(4.1-54) ^{2/9}	0%(0-71) ^{0/2}	51.9%(32-72) ^{14/27}	33.3%(9.6-65) ^{3/9}
Neoplasms	0%(0-60) ^{0/3}	25%(2-72) ^{1/4}	0%(0-60) ^{0/3}	25%(2-72) ^{1/4}	-	-
Cardiomegaly	18.2%(3.3-46) ^{2/11}	20%(1.7-63) ^{1/5}	100%(14-1) ^{1/1}	-	10%(1-37) ^{1/10}	20%(1.7-63) ^{1/5}
Abnormal location	10%(1-37) ^{1/10}	0%(0-44) ^{0/5}	0%(0-71) ^{0/2}	-	12.5(1.2-45) ^{1/8}	0%(0-44) ^{0/5}
Anomaly of great veins	0%(0-44) ^{0/5}	100%(14-1) ^{1/1}	0%(0-71) ^{0/2}	100%(14-100) ^{1/1}	0%(0-60) ^{0/3}	-
Heterotaxy	0%(0-22) ^{0/13}	30%(8.5-61) ^{3/10}	-	-	0%(0-22) ^{0/13}	30%(8.5-61) ^{3/10}
Complex	25%(16-34) ^{22/88}	24.1%(8-41) ^{7/29}	11.1%(3.6-24) ^{4/36}	0%(0-29) ^{0/9}	34.6%(21-48) ^{16/52}	35%(12-58) ^{7/20}
Other	0%(0-51) ^{0/4}	-	0%(0-71) ^{0/2}	-	0%(0-71) ^{0/2}	-
Total	27.9%(23-33)^{100/358}	23.4%(16-31)^{30/128}	15.7%(10-22)^{35/227}	11.4(2-21)^{5/44}	37.3%(31-44)^{75/201}	29.8%(20-40)^{35/84}

Diagnostic yield (95%CI) ^{absolute number diagnoses/absolute total tested}

CHD7, but it could also identify mutations in genes associated with co-morbidities such as intellectual impairment which have not been linked to CHM previously¹⁹⁷. In our cohort this is illustrated by case 151. Prenatal ultrasounds showed cardiac disproportion and subtle brain anomalies. Postnatal WES showed a TUBB mutation associated with intellectual disability. The use of prenatal WES would therefore serve the goals of prenatal diagnosis: inform expecting parents in their reproductive choices and optimize perinatal care. In fact, patients think they should have access to WES¹⁸⁴. Yet, several restraints for its prenatal use remain.

Firstly, a large share of variants found with WES will be of unknown significance²⁰² because their effect on protein formation and function is unclear and/or they have never been extensively studied before in the literature. It is debatable whether parents should have the option to get informed about VUS. Patients representatives argue that VUS cannot be withheld, but professionals indicate that communication about VUS is problematic²⁰³. If VUS were to be fed back to patients, pretest counseling should properly prepare parents for this genomic uncertainty¹⁸³. To increase the knowledge about rare variants, and their corresponding prenatal phenotypes, these data should be published in international databases. Prenatal phenotypes should be described in a standardized manner using a broadly accepted phenotyping ontology, such as the Human Phenotype Ontology.

Secondly, determining the exact prognostic burden of a prenatally found variant can be difficult due to variability of phenotypic expression and reduced penetrance. For example, D'Alessandro et al.²⁰⁴ found enrichment of syndromic genes (e.g. NIPBL associated with Cornelia de Lange syndrome) in infants with clinically isolated AVSD. Chen et al. found in their retrospective study that symptomless healthy adults can harbor autosomal dominant

and autosomal recessive mutations for severe childhood Mendelian conditions, such as Pfeiffer syndrome and Smith-Lemi-Opitz syndrome²⁰⁵. Thus, even if previously assumed highly penetrant mutations for Mendelian conditions are found, the extent of the effect on the postnatal phenotype remains uncertain. This phenotypic variability also complicates the prediction of the presence and the extend of developmental delay and intellectual disability, which is for many women and couples the most important factor in deciding whether or not to continue the pregnancy²⁰⁶.

Thirdly, WES will reveal mutations for late onset diseases unrelated to the fetal phenotype. Identifying these mutations prenatally will harm the fetus anticipatory autonomy rights. The likelihood of these findings can be reduced by analyzing WES in trio by using both parents as a "filter", as many late onset disorders are inherited. However, de novo late onset disease mutations will continue to be identified. Pretest counseling should focus on the possibility of these kind of results and its consequences for the fetal right "not to know". Informed consent should be reached through a layered-staged model²⁰⁷. In such a model, parents can opt out of receiving certain types of findings, such as those for clear-cut late onset diseases.

Lastly, interpretation of big data retrieved from WES will be time consuming but the timeframe for prenatal genomic diagnostics is short. Moreover, Sanger confirmation of found deleterious variants may be mandatory due to the technical limitations of NGS. Furthermore, storing large sets of NGS data will require advanced data infrastructures. Given these restraints of a more practical nature, not every laboratory will be able to provide prenatal WES.

Conclusions

Single gene disorders occur in 6.7-23.4% of all prenatal CHM cases and in 11.6-29.8% of non-isolated prenatal CHM cases. To diagnose these disorders prenatally, the open diagnostic approach of WES would be more effective compared to the narrow approach of gene panel testing, as only 30 percent of diagnoses is found in typical CHM-risk genes. As the prevalence of single gene disorders is the highest in prenatally non-isolated CHM, these cases would benefit the most from WES.

Table S4. CHD-risk Genes

Gene	Associated Syndrome	Associated CHM	Positive (n)/ Total tested (n)
A2ML1	Noonan	-	0 / 7
ACTC1	-	DCM, HDM, NCCM, ASD, VSD	0 / 7
ACVR1/ALK2	Fibrodysplasia ossificans progressiva	ASD, AVSD	0 / 4
ACVR2B	-	Heterotaxy, DORV, D-TGA	0 / 14
ALDH1A2	-	TOF	0 / 3
ANKRD1	-	DCM	0 / 3
ALMS1	Alstrom	DCM	0 / 4
ANKRD1	-	TAPVR	0 / 3
BRAF	Cardiofaciocutaneous, Noonan	PS, ASD, TOF	0 / 12
CBL	Noonan-like	Various Noonan-like	0 / 9
CFC1	-	Heterotaxy	0 / 19
CHD7	CHARGE	TOF, PDA, ASD, HLHS, DORV, AVSD	1 / 6
CITED2	-	VSD, ASD	0 / 3
CRELD1	-	AVSD, dextrocardia	0 / 8
CRKL	VCF	Various	0 / 3
ELN	Curtis laxa	SVAS, PPS, SVPS, PS, AS	0 / 6
FBNI	Marfan	Aortic dilation, MVP, mitral regurgitation	0 / 1
FOG2/ZFPM2	-	TOF, DORV	0
FOXH1	-	TOF, VSD	0 / 11
GATA4	-	ASD, PS, TOF, VSD, DORV	0 / 8
GATA5	-	DORV, VSD	0 / 5
GATA6	-	TA, TOF, AVSD, ASD, PS	0 / 6
GDF1	-	TOF, IAA, DORV, D-TGA	0 / 13
GJC1	-	-	0 / 2
HAND1	-	HLHS	0 / 3
HRAS	Costello	ASD, VSD, PS, HCM	0 / 9
IRX4	-	VSD	0 / 3
JAG1	Alagille	PS, PPS, TOF	1 / 17
KRAS	Noonan, cardiofaciocutaneous	PS, ASD, CoA	0 / 12
LDB3	-	DCM, NCCM	0 / 2
LEFTY2	-	Heterotaxy, L-TGA, AVSD, IAA, CoA	0 / 11
MAP2K1	Cardiofaciocutaneous	Heterotaxy, D-TGA, DORV, TOF	0 / 12
MAP2K2	Cardiofaciocutaneous	PS, ASD, AS	0 / 9
MYBPC3	-	HCM, NCCM	0 / 2

MYH6	-	ASD	0 / 7
NKX2-5	-	ASD, TOF, HLHS, D-TGA, DORV, Ebstein, VSD	0 / 11
NODAL	-	Heterotaxy, D-TGA, DORV	0 / 19
NOTCH1	(Adams Oliver)	AS, CoA, HLHS	1 / 12
NRAS	Noonan	PS, ASD, CoA	0 / 9
PITX2C	-	ASD, VSD	0 / 3
PTPN11	Noonan, LEOPARD	PS, HCM, ASD, CoA	5 / 15
RAFI	Noonan	PS, ASD, CoA	1 / 12
RIT1	Noonan	PS, HCM, VSD	0 / 7
SHOC2	Noonan	PS, ASD, CoA	0 / 9
SMAD6	-	AS, BAV	0 / 3
SOS1	Noonan	PS, ASD, CoA	0 / 9
SPRED1	Legius (NF1-Noonan)	-	0 / 7
TAZ	-	HCM, NCCM	0 / 2
TBX1	DiGeorge	IAA, TA, TOF, PVS, VSD	0 / 6
TBX5	Holt Oram	ASD, VSD, AVSD	0 / 7
TBX20	-	ASD, VSD, LVOTO	0 / 5
TDGF1	-	TOF, VSD	0 / 3
TFAP2B	Char	PDA	0 / 3
TLL1	-	ADS	0 / 3
TNNI3K	-	DCM	0 / 3
ZFPM2	-	TOF	0 / 3
ZIC3	(VACTERL)	Heterotaxy, ASD, AVSD, D-TGA, VSD, TAPVR	0 / 26

ASD, Atrium septal defect; AVSD, Atrioventricular septal defect; AS, Aorta Stenosis; BAV, Bicuspid Aortic Valve; CoA, Coarctation of Aorta; DCM, Dilated Cardiomyopathy; DORV, Double Outlet Right Ventricle; D-TGA, Dextro-Transposition of the great arteries; HCM, Hypertrophic Cardiomyopathy; HLHS, Hypoplastic Left Heart Syndrome; IAA, Interrupted Aortic Arch; L-TGA, Levo-Transposition of the Great Arteries; NCCM, Non-Compaction Cardiomyopathy; PS, Pulmonary Artery Stenosis; TA, Truncus Arteriosus; TAPVR, Total Anomalous Pulmonary Venous Return; TOF, Tetralogy of Fallot; VSD, Ventricular Septal Defect

Table S5. Sequencing diagnoses

Case	Prenatally isolated / non-isolated	Prenatal phenotype	Postnatal phenotype	Mutation (hg19)
CHM-risk genes				
112	Isolated	Cardiac: Persistent VCSS without VCSD Other: None	Livebirth ♂ Cardiac: VSD, VCSS Other: Dysmorphic facies, hypospadias	Alagille syndrome NM_000214.2(JAG1): c.192dup, p.(Lys65fs) de novo
87	Isolated	Cardiac: TOF (overriding aorta, small PA) Other: None	Livebirth ♂ Cardiac: TA type A Other: Dysmorphic facies, glandular hypospadias	NM_0176173(NOTCH1): c.7455dup, p.(Ser2486fs) nat Mutation does explain CHM, but not the rest of the phenotype.
26	Isolated	Cardiac: cAVSD Other: None (1 st trimester NT measurement not done)	Livebirth ♂ Cardiac: cAVSD Other: Dysmorphic facies, feeding difficulties	Noonan syndrome PTPN11 missense mutation NM_002834.3(PTPN11): c.1504T>A, p.(Ser502Thr)
58	Non-isolated	Cardiac: AS Other: 1 st trimester enlarged NT, macrosomia	Livebirth ♂ Cardiac: HCM, PS Other: Dysmorphic facies	Noonan syndrome PTPN11 mutation de novo
417	Non-isolated	Cardiac: DORV and AVSD Other: 1 st trimester enlarged NT; fetal hydrops, hand deformities, lemon shaped skull, small cerebellum	Termination of pregnancy ♀ Cardiac: DORV, VSD Other: Dysmorphic facies, low set ears, skin edema	Noonan syndrome NM_002834.3(PTPN11): c.179G>T, p.(Gly60Val) de novo
434	Non-isolated	Cardiac: RV hypoplasia, small TV, normal PA, myocardial hypertrophy Other: 1 st trimester enlarged NT; dysmorphic facies, enlarged NF, jugular sacs	Termination of pregnancy ♂ Cardiac: HRHS Other: Ear deformities, broad nasal bridge, enlarged NF	Noonan syndrome NM_002834.3(PTPN11): c.214G>T, (p.Ala72Ser) de novo
312	Non-isolated	Cardiac: Unbalanced AVSD Other: 1 st trimester enlarged NT	Neonatal demise ♂ Cardiac: Unbalanced AVSD, AO hypoplasia, AV insufficiency, LV hypoplasia Other: Dysmorphic facies, short neck with nuchal fold	Noonan syndrome NM_002834.3(PTPN11): c.184T>G, p.(Tyr62Asp) Inheritance not investigated
326	Non-isolated	Cardiac: Cardiomegaly, myocardial hypertrophy, PS Other: 1 st trimester enlarged NT, fetal hydrops, dysmorphic facies, brachycephaly, IUGR	Neonatal death ♂ No autopsy Other: Dysmorphic facies, hydrops, toenails hypoplasia	Noonan syndrome NM_002880.3(RAF1): c.778A>C, p.(Thr260Pro) de novo
347	Non-isolated	Cardiac: TOF, PA atresia, right AO arch Other: Giant omphalocele, gallbladder agenesis, blake's pouch cyst, bilateral cleft lip palate, IUGR, SUA	Livebirth, died 3 weeks after birth ♂ Cardiac: TOF, PA atresia, right AO arch Other: Omphalocele, blake's pouch cyst, bilateral CLP, pulmonary hypoplasia, hearing loss, choanal atresia, blindness	CHARGE syndrome NM_017780.3(CHD7): c.7228del, p.(Glu2410fs) Inheritance not investigated

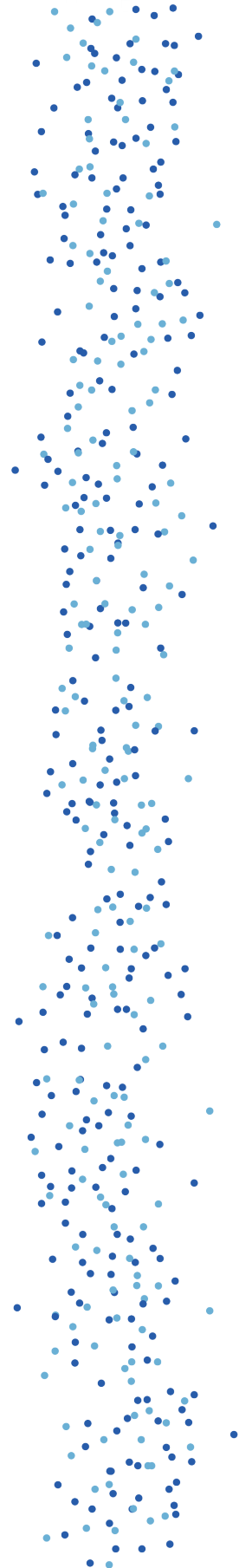
Other genes				
91	Non-isolated	Cardiac: TOF Other: 1 st trimester enlarged NT, prefrontal edema, short long bones, IUGR	Livebirth ♀ Cardiac, autopsy: TOF Other: Dysmorphic facies, microcephaly, choanal atresia, hearing loss, feeding difficulties	Cornelia de Lange syndrome NM_133433.3(NIPBL):c.2622del, p.(Asp876fs) de novo
324	Non-isolated	Cardiac: Persistent VCSS without VCSD, small AO, small LV Other: Lissencephaly, dysmorphic facies incl. retro/micrognathia, echogenic enlarged kidneys, hydronephrosis, PRUV, IUGR	Neonatal death ♂ No postnatal (cardiac) examination	Cornelia de Lange syndrome NM_006306.3(SMC1A): c.1847C>A; p.(Ala616Asp) mat
32	Non-isolated	Cardiac: AS Other: Enlarged cisterna magna, enlarged NF	Livebirth ♂ Cardiac: CoA, MV stenosis Other: Dysmorphic facies, developmental delay	Kabuki syndrome MLL2 mutation
54	Isolated	Cardiac: Multiple rhabdomyomas Other: None	Live birth ♂ Cardiac: Multiple rhabdomyomas Other: Brain MRI: Subependymal nodules and white matter lesions	Tuberous sclerosis complex NM_000368.4(TSC1):c.733C>T, p.(Arg245*) de novo
132	Isolated	Cardiac: Small LV, restrictive FO Other: None	Livebirth ♀ Cardiac: Borderline HLHS, CoA, bicuspid AO valve Other: Dysmorphic facies, hearing loss	3MC syndrome Homozygous NM_001879.5(MASP1):c.649G>T, p.(Glu217*) (consanguineous parents, MASP1 in ROH area)
436	Non-isolated	Cardiac: VSD Other: Skeletal dysplasia	Termination of pregnancy ♂ Cardiac, autopsy: No malformations Other: Skeletal dysplasia	Osteogenesis Imperfecta c.2659G>A p.(Gly887Ser) de novo
194	Non-isolated	Cardiac: Multiple VSDs Other: Sacral agenesis, rocker-bottom foot, contralateral club foot, short long bones	Livebirth ♀ Cardiac: ASD type 2, no VSDs Other: Pierre Robin sequence with cleft palate, skeletal dysplasia, abnormal position of the feet, conductive hearing loss, pulmonary hypoplasia	Spondyloepiphyseal Dysplasia Congenita NM_001844.4(COL2A1):c.3121G>A, p.(Gly1041Ser) de novo
186	Non-isolated	Cardiac: Right AO arch Other: Trichonocephaly	Livebirth ♂ Cardiac: Right AO arch Other: Craniosynostosis, polydactyly, syndactyly, brachydactyly, cryptorchism, hearing loss	Carpenter syndrome (DNA opvragen in AMC)
325	Non-isolated	Cardiac: AS, small LV Other: Agenesis of the corpus callosum, vermis hypoplasia, nasal hypoplasia, prefrontal edema, rocker bottom feet, short long bones, IUGR, SUA	Neonatal death ♂ Cardiac: Small AO, DA aneurysm Other: Dysmorphic facies, feeding difficulties, hyper-echogenic kidneys, hypoplasia of the corpus callosum, vermis hypoplasia, cerebellar hypoplasia, tethered cord, calcaneovalgus deformity, club feet, atrophy of gluteus muscles, nail and finger deformities	Coffin-Siris Syndrome 3 NM_003073.3(SMARCB1):c.1130G>A, p.(Arg377His) de novo
330	Non-isolated	Cardiac: TOF Other: Brachycephaly, echogenic bowel, gallbladder agenesis, scoliosis, short long bones, IUGR, skin edema	Livebirth, died 10 days after birth ♀ Cardiac: cAVSD, overriding AO, deformity of the PA valve Other: Dysmorphic facies, microcephaly, cleft palate, polydactyly, syndactyly, hypotonia	SLO syndrome Homozygous NM_001360.2(DHCR7):c.964-TG>C, p.? Dutch: C=0.30% (non-consanguineous parents)

Case	Prenatally isolated / non-isolated	Prenatal phenotype	Postnatal phenotype	Mutation (hg19)
238	Non-isolated	Male fetus Cardiac: TOF Other: Unilateral cleft lip and palate, enlarged fontanel	Livebirth ♂ Cardiac: TOF Other: Unilateral left lip and palate, hypospadias, laryngotracheal cleft, conductive hearing loss, developmental delay	Opitz GBBB syndrome NM_000381.3(MID1):c.1440_1444dup, p.(Asn482fs) de novo
368	Non-isolated	Cardiac: VSD, AS, PS Other: Skeletal dysplasia, pyelectasis	Termination of pregnancy ♂ Cardiac, autopsy: No malformations Other: Skeletal dysplasia, horseshoe kidney	Tanotaphoric dysplasia NM_000142.4(FGFR3):c.1118A>G, p.(Tyr373Cys) de novo
333	Non-isolated	Cardiac: Small LV, small AO Other: Central position of the stomach, absent gallbladder and spleen, heterotaxy	Neonatal death ♂ Cardiac: HLHS, CoA Other: Pulmonary hypertension, atelectasis	Alveolar capillary dysplasia NM_001451.2(FOXF1):c.253T>A, p.(Phe85Ile) de novo (suspected gonadal mosaicism in mother)
411	Non-isolated	Cardiac: AVSD Other: Central position of stomach, absent gallbladder	Termination of pregnancy ♂ Cardiac, autopsy: ASD, VSD Other: Dysmorphic facies, agenesis of the gallbladder, duodenal atresia, anal atresia, bowel malrotation, enlarged spleen	Alveolar capillary dysplasia NM_001451.2(FOXF1):c.253T>A, p.(Phe85Ile) de novo (suspected gonadal mosaicism in mother)
315	Non-isolated	Cardiac: Cardiomegaly, cardiac hypertrophy, pericardial effusion Other: Agenesis of the corpus callosum, vermis hypoplasia, polycystic kidney disease, ascites, enlarged nuchal fold	Neonatal death ♂ No postnatal (cardiac) examination Fibroblast culture: Cilopathy	Cilopathy syndrome Cilopathy gene panel: CEP290 compound heterozygous mutation NM_025114.3(CEP290): c.1501G>T, p.Glu501*mat NM_025114.3(CEP290): c.4522C>T, p.Arg1508*pat
390	Non-isolated	Cardiac: Cardiomegaly Other: Encephalocèle, microcephaly, sloping forehead, bilateral polycystic kidney disease with oligohydramnios, polydactyly, IUGR	Termination of pregnancy ♀ Cardiac: ASD Other: Dysmorphic facies, encephalocèle, pulmonary hypoplasia, cystic kidneys, liver: ductal plate malformation	Meckel Gruber syndrome Cilopathy gene panel: MKS1 Homozygous mutation (NM_017777.3(MKS1):c.1408-34_1408-6del, p.?) SNP array showed a risk factor (SL): 1.9Mb deletion in 15q11.2 inheritance not investigated, non-consanguineous parents
Whole exome sequencing				
151	Non-isolated	Cardiac: Small LV Other: Blake's pouch cyst, arachnoid cyst, signs of abnormal neuronal migration in 3 rd trimester	Livebirth ♀ Cardiac: No malformations Other: Tubulinopathy, cavernous hemangiomas	Tubulinopathy WES: NM_178014.2(TUBB):c.860C>G, p.(Pro287Arg) de novo
406	Non-isolated	Cardiac: cAVSD, VCSS with VCSD Other: 1 st trimester enlarged NT, agenesis of the corpus callosum, vermis defect, SUA	Termination of pregnancy ♀ Cardiac: cAVSD Other: Dysmorphic facies, Dandy Walker malformation, agenesis of the corpus callosum, micrognathia, anal atresia, 13 sets of ribs	Coffin-Siris Syndrome 2 WES: NM_006015.4 (ARID1A): c.5207dupT, p.(Asp1738*) de novo
264	Non-isolated	Cardiac: VSD Other: Agenesis of the corpus callosum, enlarged cisterna magna, (1 st trimester NT not done)	Live birth ♂ Cardiac: VSD, central ASD Other: Agenesis of the corpus callosum, small germinal matrix bleeding left, feeding difficulties	Coffin-Siris Syndrome 2 WES: M_006015.4(ARID1A):c.3059G>A, p.(Arg1020Lys) de novo

297	Non-isolated	Cardiac: TGA, DORV, VSD, PA stenosis, v. azygos continuation Other: Right sided diaphragmatic hernia, Polyhydramnios Heterotaxy	Neonatal death ♂ No postnatal (cardiac) examination	Visceral heterotaxy WES: Homozygous MMP21 mutation NM_147191.1(MMP21):c.861T>G, p.(His287Gln) (consanguineous parents)
4	Non-isolated	Cardiac: TGA, DORV, VSD, AS, PS Other: Situs inversus abdominalis Heterotaxy	Livebirth ♀ Cardiac: L-TGA, DORV, VSD, MV stenosis, small LV, PS Other: Situs inversus	Visceral heterotaxy WES: Homozygous MMP21 mutation NM_147191.1(MMP21):c.861T>G, p.(His287Gln) (consanguineous parents)

AO, Aorta; PA, Pulmonary Artery; LV, Left Ventricle; RV, Right Ventricle; MV, Mitral Valve; TV, Tricuspid Valve; ASD, Atrial Septal Defect; D- TGA, Dextro-Transposition of the great arteries; VSD, Ventricular Septal Defect; AVSD, Atrioventricular Septum Defect; cAVSD, complete Atrioventricular Septum Defect; TOF, Tetralogy of Fallot; L-TGA, Levo-Transposition of the Great Arteries; DORV, Double Outlet Right Ventricle; VCSS, Vena Cava Superior Sinistra; VCSD, Vena Cava Superior Dextra; NT, Nuchal Translucency; NF, Nuchal Fold; SUA, Single Umbilical Artery; IUGR, Intrauterine Growth Restriction; AS, Aorta Stenosis; CoA, Coarctation of Aorta; DCM, Dilated Cardiomyopathy; HCM, Hypertrophic Cardiomyopathy; HLHS, Hypoplastic Left Heart Syndrome; IAA, Interrupted Aortic Arch, NCCM, Non-Compaction Cardiomyopathy; PS, Pulmonary Artery Stenosis; TA, Truncus Arteriosus; TAPVR, Total Anomalous Pulmonary Venous Return

04



Chapter 4

Amniocentesis is still the best option
for advanced genomic testing in case of
fetal malformations

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With the introduction of non-invasive prenatal testing (NIPT), most health care professionals and patients understandably favor this safe modality over invasive procedures.²⁰⁸⁻²¹⁰ At present, however, NIPT does not allow for the most advanced genomic test available: fetal whole exome sequencing (WES). With WES, the sequence of all coding genes is interrogated, which presumably has a high diagnostic yield in fetuses with congenital malformations.¹⁸¹ In this paper, we argue that invasive testing for WES should be offered to all pregnant women who carry a chromosomally normal fetus with ultrasound anomalies. We believe that the ethical challenges of WES are not new to prenatal diagnosis and they can be faced and solved by dedicated counseling. Moreover, new evidence shows an amniocentesis-related miscarriage risk of 1 in 1000, which is considerably lower than previously thought. By providing adequate counseling and offering women a choice between NIPT and invasive testing, the aim of prenatal testing—reproductive autonomy^{211, 212}—will be best served.

Starting a decade or so ago, the practice of prenatal genetic testing is continually changing. Technical developments revolutionized both the resolution of fetal genomic testing and the ways in which we can obtain fetal DNA. Karyotyping has been replaced by chromosomal microarray (CMA), which surpasses karyotyping in its capacity to detect small quantitative imbalances of chromosomal material and improves the diagnostic yield^{30, 94} by 5% to 10%. At first, the fear that high-resolution CMA would retrieve large amounts of inconclusive information caused health care professionals around the world to hesitate.¹⁹⁸ Subsequent CMA studies however proved that the chance of detecting variants of unknown clinical significance (VUS) decreases as knowledge about rare variants increases, and was estimated³⁰ at 1.5% in 2012. Unsolicited

CMA findings—variants unrelated to the fetal malformation on ultrasound—are found in only²¹³ 0.5% of patients. The uncertainty of CMA results with broad phenotypic variability do not cause harm to patients.¹⁸² Thus, in spite of early ethical concerns, three important medical-ethical principles, that of beneficence and non-maleficence and that of respect for autonomy,²¹⁴ turned out to be upheld through the wider adoption of CMA. This has driven the 2016 recommendation by the American College of Obstetricians and Gynecologists to make CMA available to any patient choosing to undergo invasive diagnostic testing.²¹⁵

Nonetheless, CMA can only detect a minority of severe congenital syndromes as most syndromes are caused by single nucleotide mutations. This diagnostic gap can be overcome by the next step in genomic testing resolution, which is ready to be taken as fetal WES is now timely and feasible in well-developed healthcare systems.³⁷ With WES, the sequence of all coding genes is interrogated simultaneously. In postnatal patients with congenital malformations and a normal result of CMA, WES can establish a genetic diagnosis³⁵ in approximately 36%. A recent review in this journal of prenatal WES studies in fetuses with ultrasound anomalies shows a diagnostic range¹⁸¹ from 6.2% to 80%. In other words, WES can aid in estimating the prognosis of a fetus with congenital abnormalities, satisfying the principle of beneficence.^{181, 214} The American College of Medical Genetics now recommends considering WES as a suitable and proportional test in fetuses with a likely genetic disorder in which other genetic tests, such as CMA and targeted sequencing, have failed to arrive at a diagnosis.²¹⁶

However, restraint in the offer of advanced fetal genomic tests comes from the availability of NIPT or cell free fetal DNA (cffDNA) testing. NIPT can be used to reliably detect large fetal chromosomal aberrations,²¹⁷ targeted or

genome wide. The availability of NIPT as a first line test has logically reduced the incentive for invasive testing as most professionals and patients prefer a safe test.^{208, 210, 218} However in case of fetal ultrasound anomalies, the diagnostic decline—if the currently low resolution of NIPT would be introduced to the exclusion of the high resolution of invasive CMA and WES—should not be neglected in pretest counseling.¹¹⁹ Genome-wide NIPT has become available for the detection of chromosomal micro-imbalances as well (<10 Mb), but at present, its accuracy for these aberrations is not yet established.²¹⁹⁻²²⁴ And although NIPT is available for de novo, paternally dominant or compound heterozygous recessive single gene disorders^{47, 225} reliable large-scale sequencing or fetal WES from cffDNA for clinical purposes seems a distant prospect for most laboratories. Moreover, because cffDNA mainly derives from the apoptosis of the cytotrophoblast, a fetal representative cffDNA result relies on the genetic concordance of fetus and cytotrophoblast. In 1% to 2% of placentas tested through antenatal placental sampling, the chromosomal profile in the placenta is discordant with the fetus,²²⁶ causing false positive results and false negative results.^{129, 154, 227} In conclusion, from a diagnostic point of view, the access to invasive testing in case of fetal anomalies on ultrasound should not be denied because of the availability of the safer NIPT. Moreover, new evidence shows an amniocentesis-related miscarriage risk⁶ of 1 in 1000, which is much lower than previously reported.²²⁸

Other hesitations in the offer of prenatal WES emerge from its possible ethical drawbacks.^{181, 229-233} As the increased resolution holds the potential for detecting more causal genomic abnormalities, it inherently increases the likelihood of unsolicited findings, VUS and mutations for late onset diseases. Although this does not introduce entirely new ethical problems to the practice of prenatal diagnosis,

it does exacerbate certain existing ones. Two major concerns are genomic uncertainty and the fetus' anticipatory autonomy rights.

Any genomic test, from karyotyping to WES, involves 3 types of uncertainty: (1) The absence of a detectable genetic aberration is not a guarantee for health or "normality" (2) Every genetic aberration found has a spectrum of phenotypic expression; therefore, the individual phenotype can be largely unpredictable based on genotype alone.

(3) Aberrations other than the one(s) that were intentionally looked for (unsolicited findings, VUS) can be found. However, by increasing the testing resolution in order to reduce type 1 uncertainty, the likelihood of type 2 and type 3 uncertainty increases. For example, the rarity of many mutations found with WES will complicate phenotype prediction,²³⁴ including the chance of developmental delay, which is for many women or couples the most important factor in deciding whether or not to continue the pregnancy.²⁰⁶ Furthermore, a large share of mutations found by means of WES will be VUS²⁰² because the effect on protein formation is unclear and/or the variant was never described before. Professionals and patient representatives feel that VUS should not be withheld, because this could undermine the patients' autonomy.²³⁵ Professionals do indicate that communication about uncertain results is problematic and may negatively affect the doctor-patient relationship.²³⁵ It is feared that uncertainty may seriously burden expecting parents and may increase termination rates.^{198, 236} We agree with Newson et al, who argue against the premise that uncertainty is always something undesirable. They stress the importance of explicitly discussing uncertainty in genomics during pre-test counseling.¹⁸³ Hereby parents can make an individualized choice about fetal genomic testing, taking into account their own resilience to uncertainty. Moreover, dedicated post-

test counseling can further guide expecting parents in dealing with uncertain genomic results.¹⁸² The interpretation and handling of WES results, as well as counseling, require a multidisciplinary team of molecular geneticists, clinical geneticists and gynecologists⁴ in order to optimize understanding and thereby to enhance the patient's reproductive autonomy, assuring non-maleficence.

Another ethical concern is the potential detriment to one of the fetus's anticipatory autonomy rights: the right not to know about medically relevant adulthood conditions, especially those that are untreatable.^{237, 238} This infringement is not new to the practice of prenatal diagnosis; however, WES does increase the magnitude of this concern as it is able to detect many more late-onset conditions than karyotyping and CMA. Not disclosing information on late onset diseases, which could have clinical implications in the future, is unacceptable.^{239, 240} Several approaches have been proposed to negotiate the problem of these findings, for instance, to exclude preselected known loci for late onset diseases from testing. Given the current limits in genetic knowledge, this approach will be fallible, as lists of preselected loci will be incomplete, and has practical shortcomings, as lists will require regular updating. Alternatively, as many mutations for late onset diseases are inherited rather than de novo, parental genomes can be used as a filter for analyzing the fetal genome. This approach is common practice in postnatal cases³⁵ as it minimizes the retrieval of mutations for late onset diseases considerably. However, de novo mutations for late onset diseases are still detected. Hence, to a certain extent the possible conflict between the parental reproductive autonomy right and the fetal anticipatory right to autonomy will currently resist resolution.²⁴¹ Within certain limits, we agree with Chen and Wasserman²⁴² that allowing parents to decide what information to

obtain and how to manage it for their children is consistent with parental authority. Therefore, we consider the offer of "trio" WES to be acceptable in fetuses with malformations on ultrasound because its high potential value in informing reproductive decision making may outweigh the limited potential detriment to the fetus's anticipatory autonomy rights. Pretest counseling should emphasize that expecting parents should seek information that they wish to have for their reproductive decision making, not for sheer curiosity's sake.²⁴³ It should assist parents in weighing the possible opposing interests of their own right to reproductive autonomy and the fetal right to "an open future".

Studies of patients' expectations of prenatal testing show an increasing demand for information.²⁴⁴ A majority feels that fetal WES should be offered.¹⁸⁴ While many health care professionals prefer a predetermined, limited offer for prenatal testing,²⁴⁵ many patients favor having a choice in what is tested for.^{244, 246} Unfortunately, informed consent for advanced genomic testing is complex and truly informed choice is not always achieved.²⁴⁷ We believe that informed consent for WES can be best pursued through a layered-staged model.²⁰⁷ This model structures the information load in order to keep it comprehensible, providing core information to every patient in the first layer and offering more in-depth information to patients who need more specific information in underlying layers. We agree with Berkman et al that it would be best to have a default package for what is tested for,²⁴⁸ designed by the previously mentioned multidisciplinary team, taking into account local legal rules and regulations. Information about this default would be presented in the first stage. If desired, patients have the option to access information in addition to the default,²⁴² which can be offered in the following stage of the model. To reach informed consent, counseling

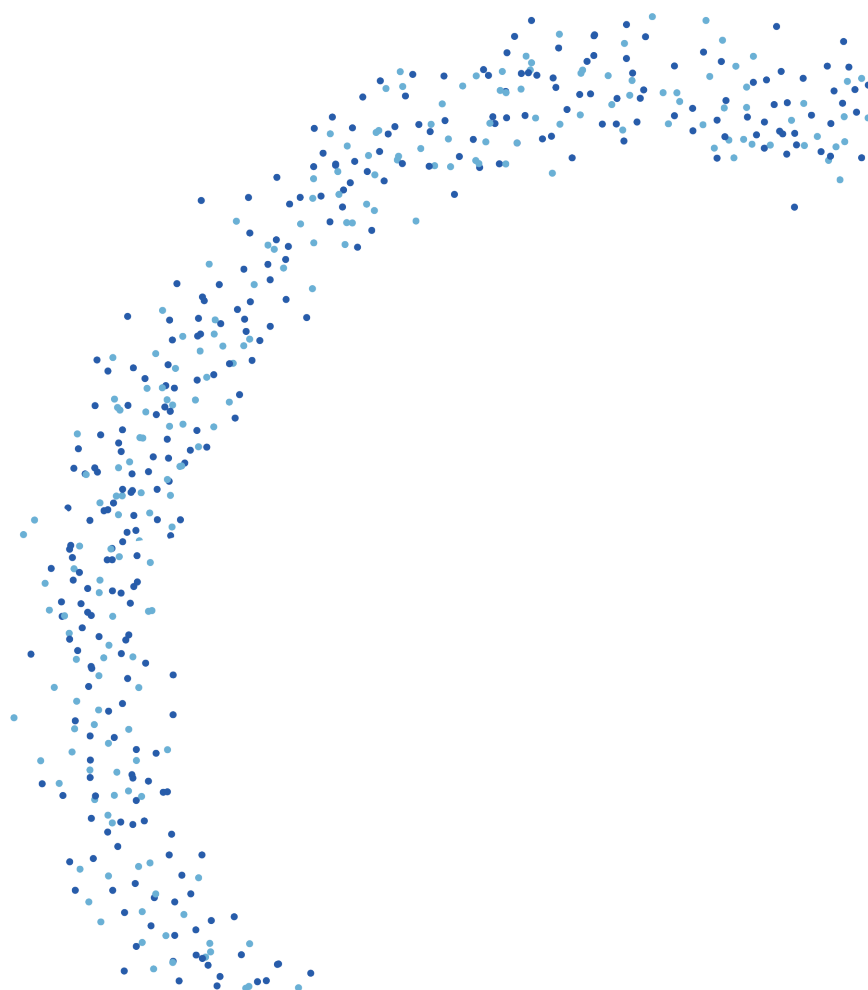
should combine this informative process with a communicative process between counselor and patient to explore the patient's attitude towards testing.^{240, 247, 249} As this process takes time, offering WES will put pressure on health care resources. If it is only offered to those patients with fetal ultrasound, we anticipate that this pressure would be within reason and justified in well-developed health care systems, but this might not apply to all health care systems worldwide.²³⁶

Concluding, if health care resources are sufficient for dedicated counseling, invasive testing for WES should be offered as an option to all patients who carry a chromosomally normal fetus with malformations on ultrasound. With this offer, the aim of prenatal diagnosis, reproductive autonomy, is best served. The ethical issues surrounding WES, notably genomic uncertainty and the anticipatory autonomy rights of the unborn child, can be addressed through the involvement of a dedicated multidisciplinary team in the setup and scope of WES and through pre-test counseling.

05

Chapter 5

General discussion



General discussion

Over the years, technological and scientific developments have revolutionized prenatal genetic diagnostics. These developments allow us to broaden the scope and increase the resolution of routine genetic testing, in general and in the prenatal setting, for all fetuses with congenital malformations, in order to detect substantially more disease causing aberrations that affect the fetal prognosis. In the second Chapter of this thesis, we reviewed the existing literature and found that microarray detects a submicroscopic chromosomal imbalance in 3.1-7.9% of fetuses with an isolated structural anomaly on ultrasound and a normal karyotype. In Chapter 3, we studied the diagnostic yield of single nucleotide polymorphism (SNP) array. We found that this yield is modest in non-specific ultrasound abnormalities, such as small for gestational age (Chapter 3.1) and increased nuchal translucency (Chapter 3.2), and high in congenital heart malformations (Chapter 3.4). Based on postnatal testing, a further increase in diagnostic yield is anticipated with fetal whole exome sequencing (WES), such as in isolated agenesis of the corpus callosum (between 13.3 and 50%) and in congenital heart malformations (between 6.7 and 23.4%). SNP array and WES allow us to genome-wide search for prognostically important genetic anomalies rather than to determine the presence or absence of certain aberrations in preselected loci. Their non-targeted method prevents the discrimination of certain genetic conditions (e.g. trisomies) against others. This approach suits the aims of invasive prenatal testing (Chapter 4), as prospective parents indicate that they wish to have as much information as possible^{184, 246}.

Concurrently with the advances in fetal testing resolution, next generation sequencing based non-invasive prenatal

testing (NIPT) was introduced. Pretest counseling should emphasize that NIPT is diagnostically inferior to invasive SNP array (Chapter 3.3) and WES (Chapter 4). Nonetheless, it is an excellent tool for screening for microscopic chromosomal anomalies, de novo or paternal autosomal dominant mutations and compound heterozygous mutations for recessive disease^{47, 250}. Therefore, the introduction of this modality will enable us to broaden the scope of population-wide screening; from trisomies 21, 13 and 18 to monogenic diseases.

Methodological considerations

The aim of this thesis was to evaluate the integral value of invasive advanced genomic testing compared to karyotyping and non-invasive testing techniques in specific fetal abnormalities detected by ultrasound. We focused on the commonly occurring anomalies small for gestational age fetal growth (SGA, Chapter 3.1) and increased first trimester nuchal translucency (Chapter 3.2) and then on two rare anomalies: isolated agenesis of the corpus callosum (Chapter 3.3) and congenital heart malformations (CHM, Chapter 3.4). We chose to study the first three entities because they are characterized by a variable fetal prognosis, from symptomless to severe disabilities, which substantially complicates antenatal counseling. We hypothesized that, especially in these entities, advanced genomic testing would be of value in helping to estimate the fetal prognosis. The fetal prognosis in case of the last group of anomalies we studied, CHM, is clear in terms that they generally cause postnatal symptoms and generally require surgery. However, genetic causes for cardiac malformations are diverse and usually cause co-morbidity and/or intellectual disabilities. Therefore, we

hypothesized that WES would be of additional value in fetuses with CHM.

Although major congenital malformations have a collective prevalence of 2-4%¹, each malformation individually is rare. When a studied entity has a low prevalence, prospective study designs are difficult. A long timeframe is needed to include a sufficient number of cases. The sample sizes of the presented studies could only be reached thanks to the continuous high quality registration and documentation at the Erasmus MC department of Obstetrics and Gynecology. Due to the rarity of the anomalies the sample sizes are small, yet, given their rarity, the sample sizes can be considered relatively large. Although the tremendous efforts put into high quality registration minimized the occurrence of missing data, it could not be prevented completely. The retrospective observational design used in the studies in Chapter 3 respects the natural clinical situation and therefore allows the application of the results in the clinical practice of pretest counseling. However, the observational approach did cause large inter-cohort heterogeneity in diagnostic workup which substantially complicated analyses. The heterogeneity is mainly caused by the parental free choice to accept or reject prenatal and postnatal genetic testing. But it is also caused by the changes in diagnostic protocols over the years, such as the introduction of fetal MRI. We deliberately chose to enroll our subjects based on the ultrasound phenotype alone, regardless of the subsequent diagnostic choices. This decision was made in order to avoid ascertainment bias, which is often encountered in studies on prenatal testing, as the more severe cases are more likely to be tested. This is illustrated in Chapter 3.1: More severely affected SGA fetuses (smaller abdominal circumference on ultrasound) were more likely to undergo prenatal genetic testing. If we were to include only the cases in which

genetic testing was done, the introduced ascertainment bias would have decreased the external validity of our studies and, therefore, its usefulness for prenatal counseling. On the other hand, untested cases make up for a substantial part of certain cohorts (Chapter 3.1, 3.3 and 3.4). This weakens the power of our conclusions. In the more laboratory-based approach of Chapter 3.2, prenatal genetic testing was required for inclusion. It is possible that fetuses with a slightly or moderately but not seriously increased NT are underrepresented in the studied cohort. Therefore, its validity for counseling in slightly and moderately increased NT could be suboptimal. Lastly, our subjects were selected on their phenotype as examined by means of high quality ultrasound executed by expert ultrasound physicians. Referrals for this kind of examination come from a national, widely adopted program for mid-pregnancy ultrasound screening. Therefore, our results are applicable in clinical settings similar to the Dutch, but not in health care settings in which prenatal testing is only offered to high risk patients (e.g. positive family history of genetic disease).

Future possibilities and challenges in prenatal advanced genomic testing

Unraveling links between genotype and prenatal phenotype

Inherent to the decreasing chance of missing a known disease causing aberration, there is a mounted likelihood of findings with a variable phenotypic expression and variants of unknown clinical significance. Through recent studies, the extend of phenotypic variation associated with one sequence variant becomes more and more obvious²³⁴. This variance is most likely caused by a complex interplay between

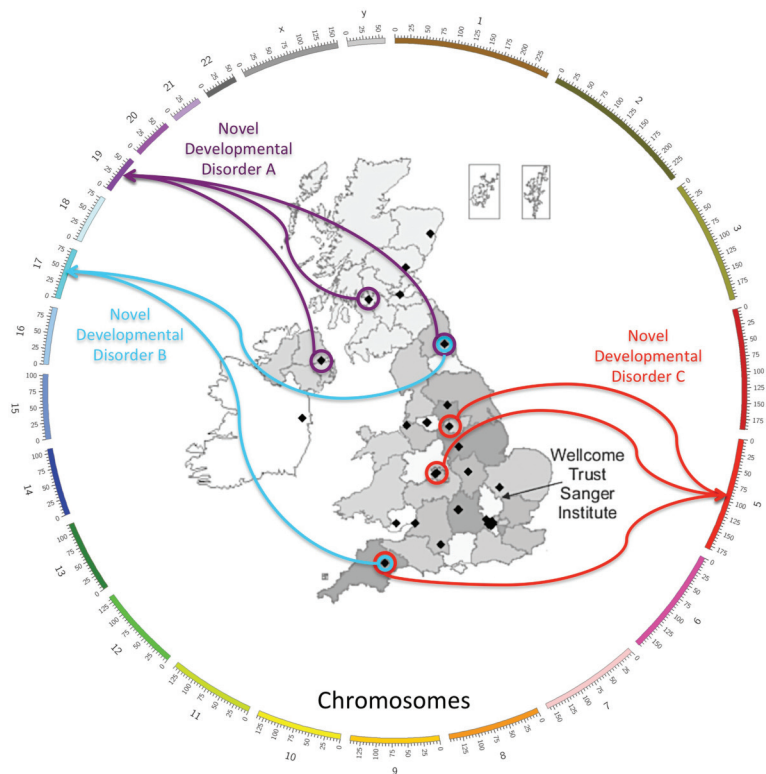


Illustration 1. Collecting geographically scattered cases in (inter)national databases to unravel genotype-phenotype relationships (from the ‘Deciphering Developmental Disorders (DDD) project’ <https://www.ddduk.org/>)

various genetic and non-genetic modulators. An example of effect non-genetic modulation was illustrated by Schulkey et al. in a mouse model: A mouse that carries offspring with a deleterious (NKX2-5) mutation for congenital heart disease can modify her offspring’s risk for CHM by performing exercise²⁵¹. Furthermore, even variants for severe Mendelian disorders, previously thought to be highly penetrant, are found in symptomless healthy adults²⁰⁵. The possibility of uncertain results from microarray and WES has been an important topic in ongoing discussions regarding their implementation in prenatal diagnosis^{198, 230}.

Uncertainty and uncontrollability are key features of stress situations²⁵². Therefore, uncertainty is generally considered potentially harmful and something that should be avoided. However, it seems that patients’ responses to uncertainty in genomic testing depend on their expectations of the information²⁵³. We contend that genomic uncertainty does not have to be problematic, as long as the chance of uncertain results is sufficiently addressed in pretest counseling to appropriately prepare the patient for these results (Chapter 4). Still, continuous efforts should be made to unravel the links between genotype and (prenatal) phenotype

in order to minimize uncertainty as much as possible. As there are so many possible variants in the genome, each of which is so rare, it is impossible for one institution's population to count enough individuals with a certain variant to draw a conclusion on its phenotypic effect. To effectively determine the link between rare genotypes and their phenotype, or phenotypic spectrum, the world must collaborate in open-access international databases for rare genomic variants²⁵⁴ (Illustration 1). Examples are DECIPHER (Database of Genomic variation and Phenotype in humans using Ensembl Resources) and ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>). The success of these databases relies on large scale participation of (clinical) laboratories and on standardized and dedicated registration of genotypic and phenotypic characteristics of entered cases. Phenotypic registration can be standardized by the use of the Human Phenotype Ontology (<http://human-phenotype-ontology.github.io/>), which holds a continuously increasing number of terms describing human phenotypic malformations. Databases for rare variants should pay attention to the moment of ascertainment of entered cases as a certain genotype can have distinct phenotypic expressions before birth (evaluated by ultrasound) that may not have been reported in livebirths. Furthermore, in order to be able to identify the full spectrum of genomic variants, the genotypic and phenotypic investigation of large cohorts of symptom-less control-individuals is just as important as good registration of cases.

Due to the (largely unknown) phenotypic variability associated with many mutations, it is essential to combine information from WES with the phenotypic information from detailed prenatal ultrasound. Both genotypic and phenotypic information is mandatory in providing the most accurate prediction of the fetal prognosis.

Do we need to reconsider whom to offer invasive testing?

The association between advanced maternal age (AMA) and trisomies has been an important motivation for the initial clinical implementation of prenatal genetic testing. Karyotyping was reliable in detecting the conditions AMA mothers were at risk for, trisomies. When prenatal ultrasound screening became feasible in clinical practice, it was only logical to offer prenatal karyotyping to those women carrying a fetus with malformations as trisomies regularly cause congenital malformations. When high resolution microarray technology was introduced to prenatal diagnosis it was initially offered in case of fetal malformations. As pathogenic microdeletions and duplications can cause congenital malformations, the offer agreed with the intent to "test what you screen for". However, it was a subject of debate whether this new modality should also be offered in case of AMA. Women of advanced age compared to younger women, are not at an increased risk of microdeletions or microduplications. Therefore, the offer of narrow scope trisomy testing only in case of AMA has been discussed²⁵⁵. Others argued that any woman taking the risk of invasive testing should have access to the most advanced genomic test available to prevent abnormalities being missed. For this reason, the American College of Obstetricians and Gynecologists (ACOG) recommends to offer microarray testing to all women who decide to perform invasive prenatal testing²¹⁵.

Monogenetic disorders are associated with other risk factors than chromosomal anomalies. Thus, with the introduction of sequencing to prenatal diagnosis, there may be a need to reconsider whom to offer invasive testing. For example, the offspring of fathers of advanced age is at a slightly increased risk for certain mutations, such as FGFR3 mutations and RASopathies, due to selfish mutations in spermatogenesis²⁵⁶ (Illustration 2). This is

The selfish generation

Why some kinds of mutation are surprisingly common in sperm

Sperm are produced when stem cells called spermatogonia divide, to produce sperm and one stem cell

But certain mutations make stem cells divide abnormally, meaning they sometimes produce two stem cells

Over time, the numbers of mutant, or “selfish”, stem cells and sperm increase exponentially

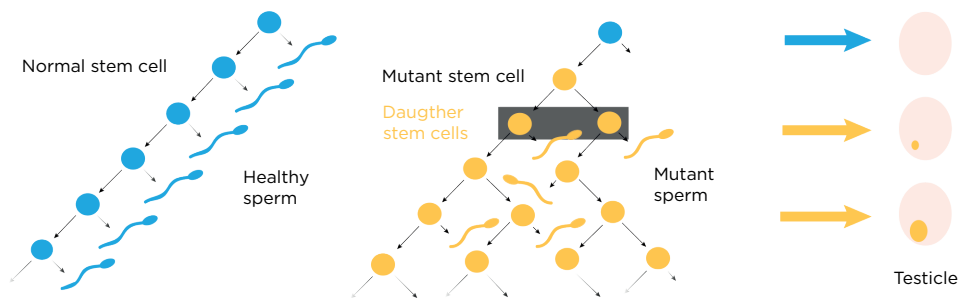


Illustration 2. How selfish spermatogonial selection leads to “Paternal Age Effect” disorders in offspring (image courtesy of NewScientist)

referred to as the “paternal age effect” (PAE). Advanced paternal age occurs increasingly in our society²⁵⁷ and could potentially be a reason to offer invasive testing. On the other hand, PAE mutations are particularly suitable for non-invasive detection and NIPT for these mutations is already clinically available²⁵⁸.

Should we expand our offer for prenatal screening?

Currently in the Netherlands, the offer for prenatal screening entails a “genotype-first” approach with next generation sequencing based NIPT (for trisomy 21, 18 and 13 with optional information on “additional chromosomal findings”) followed by a “phenotype-first” approach with the mid-pregnancy anomaly scan. It would be valuable to include mutation screening in NIPT as the birth prevalence of monogenic disease is

higher than the birth prevalence of aneuploidy (approximately 10:1000 versus 1:1000 for Down’s syndrome)¹⁰⁰ and as monogenic disease often does not cause fetal ultrasound anomalies. As mentioned before, NIPT for de novo and paternally inherited autosomal dominant and compound heterozygous recessive diseases is clinically available. This is referred to as non-invasive prenatal diagnosis (NIPD). We argue that extension of the screenings offer with this type of monogenic disease is a realistic option. For autosomal recessive and maternally inherited autosomal dominant disorders, non-invasive detection is more complex. In this type of inheritance NIPD cannot depend on detecting the presence or absence of an allele not present in the mother. Instead, it should rely on the ratio between the disease-causing allele and the wild type allele because if the fetus is affected there will

be more of the disease-causing allele present in maternal plasma. However, the amount of disease-causing allele will also be determined by the fraction of fetal cfDNA in maternal plasma and therefore, NIPD for maternally inherited mutations will require measurement correction for fetal fraction. This is possible in case of a male fetus (e.g. SRY allele) but yet not clinically possible in female fetuses. With a new technique using targeted haplotyping, which is not clinically available yet, the problem of detection of maternally inherited monogenic disease might be tackled²⁵⁹⁻²⁶³.

Fear of creating “Designer Babies”: Are we practicing neo-eugenetics?

With the introduction of new genomic techniques in fetal diagnosis, there are public concerns that prenatal testing is an emerging practice of neo-eugenetics^{198, 231, 232, 264}. The ethical discussion arises with the interpretation that prenatal testing makes unborn children (genetically) “audition”²⁶⁵ and those that fail (or in case of an uncertain result, those that might fail) would not be allowed to enter the world. With an increasing resolution the genetic audition would become more and more difficult to pass. However, this interpretation is incorrect. A more advanced test does retrieve more information but it does not change the prospective parents’ intrinsic attitude towards pregnancy termination, nor does it change their views on what conditions justify birth or termination. Furthermore, antenatal genetic testing is offered to serve the patient’s right to autonomy in her reproductive choices (Chapter 4). It allows them to gain information to inform their choices but it does not force them to make a particular decision. Furthermore, broadening the scope and resolution of prenatal testing prevents the discrimination of certain genetic conditions against others. When it comes to prenatal testing, prospective parents indicate that they wish to have as much information as

possible on certain outcomes (e.g. intellectual disability). Therefore, it is not logical to offer narrow scope testing for certain specific genetic causes while broad scope testing is possible.

The fear for extermination of the “non-normal” should not be conflated with the intention of the practice to do so. However, patients may feel social pressure to submit to testing, especially when a test is widely adopted and easy²⁶⁶. They may even feel social pressure to terminate a pregnancy in case of a positive test result. This pressure is a societal problem of intolerance not motivated by scientific developments. It is also related to the standard of care for children and adults with mental and/or physical disabilities in their society. I do not think that the scientific society is especially well qualified to tackle and solve this problem by, for example, putting restrictions and regulations on testing. Rather, the problem should be faced by society as a whole.

Nonetheless, medical professionals can and should advise on what is tested for because they are qualified to conclude on test reliability and the fair distribution of health care resources. For example, I think investing in testing for cosmetic and intelligence traits should be avoided. Although most patients reject this kind of testing anyway²⁴⁴, they also admit that if they would have information on these traits, for example through first trimester NIPT, they “would not trust themselves with the information”²³². Apart from the fact that information on beauty or intelligence is medically not useful, professionals should refrain from offering testing for these traits because they are extremely complex. An accurate prediction on someone’s intelligence or beauty based on his/her genetics is not (yet) possible.

However, companies for Direct-To-Consumer (DTC) genetic testing are investing

in the development of testing for non-medical traits to attract consumers. Healthcare professionals or government policies do not regulate these practices. DTC companies can provide misleading or incomplete information to persuade customers to pay for non-informative, useless testing. If they do offer useful testing, it would only be available to those who have the financial resources for it, creating health disparities based on income. Currently, DTC companies refrain from offering invasive prenatal testing but they do sell NIPT and carrier screening. DTC initiatives should be administered by an independent worldwide authority in order to prevent misleading information, uninformed consent and faulty testing. Useful and reliable prenatal testing should be offered within the context of collective healthcare systems in order to avoid health inequities.

Implications for future research

First trimester 2D and 3D virtual reality ultrasound for the early detection of anomalous phenotypes

As stressed previously, both genotype and phenotype are necessary for adequate prediction of fetal prognosis. Ultrasound screening in most countries is limited to the mid-pregnancy anomaly scan. The timeframe between this scan and the legal pregnancy termination limit is currently too short for a diagnostic workflow of chromosomal microarray and subsequent WES. Optimally, ultrasound screening would take place earlier in pregnancy. Many studies reported the detection rate of congenital malformations by first trimester 2D ultrasound. A systematic review and meta-analysis showed an anomaly detection rate of 32.35% in low-risk pregnancies and 61.18% in high-risk pregnancies.²⁶⁷ Further

large prospective studies, such as the Erasmus MC VR fetus study (<http://www.trialregister.nl/trialreg/admin/rctview.asp?TC=6309>), should be undertaken to further assess the feasibility and success of first trimester 2D and 3D virtual reality ultrasound in reliable early fetal phenotyping.

The value of WES in estimating fetal prognosis

Several postnatal studies have reported a high diagnostic yield of WES in individuals with congenital malformations^{35, 36, 109}. Prenatal studies have reported varied results on its diagnostic yield in fetuses with abnormalities on ultrasound¹⁸¹. More large prospective studies should be undertaken to determine the additional diagnostic yield of prenatal WES in fetuses with certain specific ultrasound anomalies. These studies should not only report the prevalence of genomic variants, but they should also explicitly focus on whether the knowledge of these variants helps in estimating the fetal prognosis.

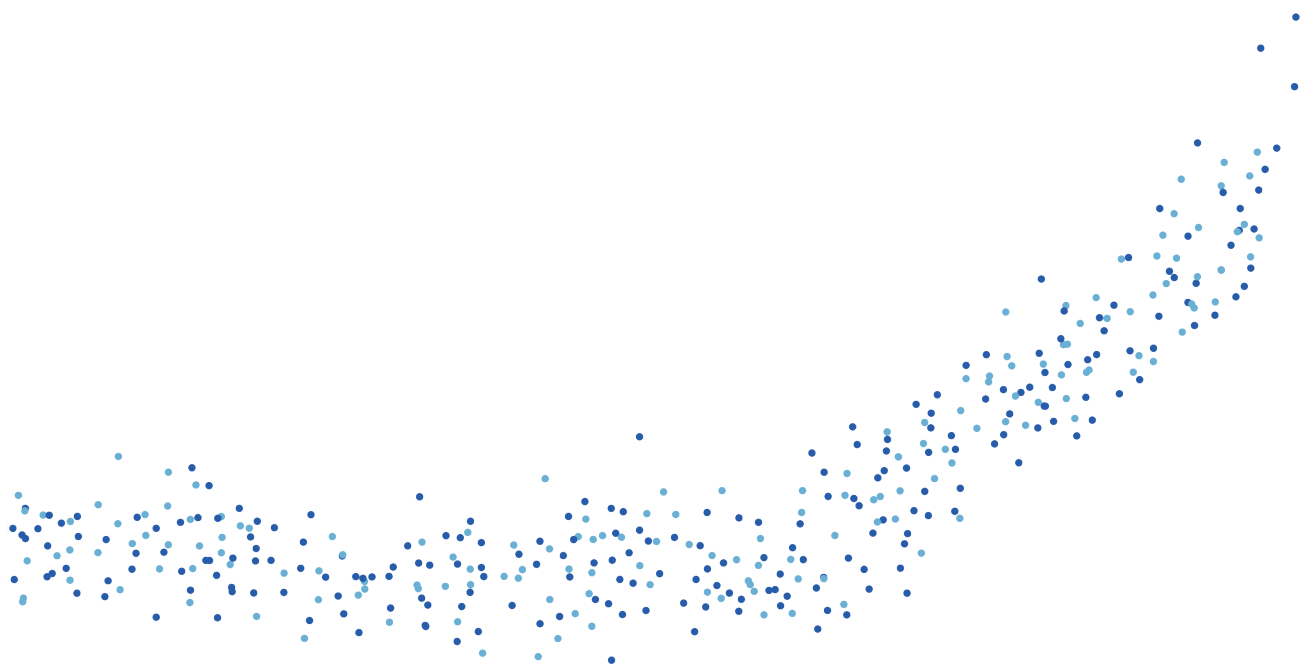
The effects of genomic uncertainty

In Chapter 4 we argued against the premise that uncertain genomic results should always be avoided in order to protect prospective parents from harm. We agree with Newson et al. that as long as genomic uncertainty is explicitly addressed in pretest counseling, genomic uncertainty does not have to be problematic.¹⁸³ In a study on the psychological consequences of genomic uncertainty, specifically after disclosing chromosomal micro-imbalances with variable phenotypic expression, Van der Steen et al. observed no negative psychological impact in prospective parents.¹⁸² However, more prospective studies should follow to examine the impact of clinically uncertain results on prospective parents, their families, their caregivers and, in case of an ongoing pregnancy, the fetus.

General conclusions

The genetic cause of a fetal malformation on ultrasound is an important determinant of the fetal prognosis. Technological and scientific developments have enabled us to routinely test fetuses with malformations for many more clinically relevant genetic aberrations than before. Due to its considerable additional diagnostic yield over karyotyping, invasive microarray testing should be offered in case of any fetal anomaly on ultrasound. When microarray shows no pathogenic chromosomal aberrations parents should be able to opt for WES, as WES will allow for a further major increase in diagnostic yield. Non-invasive prenatal testing is an excellent tool for screening for common aneuploidies and we expect that its scope will broaden in the future. In fetal anomalies on ultrasound however, it is diagnostically inferior to invasive advanced genomic testing. The observations in this thesis emphasize the importance of offering advanced genomic testing in fetal anomalies on ultrasound. These new insights can improve pre-test counseling and perinatal decision-making. The main aim of near-future research on this topic should be to further unravel the (prenatal) phenotypic effects of rare variants. Furthermore, it should also aim to increase the success of first trimester ultrasound screening for anomalous fetal phenotypes, in order to increase the timeframe available for prenatal advanced genomic testing.

06





Chapter 6

Summary

Summary

Congenital malformations are associated with the presence of distinctive genetic abnormalities such as chromosomal anomalies and single gene disorders. The presence of such a genetic anomaly can strongly influence the fetal prognosis as it can cause other entities to arise such as intellectual disability and comorbidities. Prenatal ultrasound is routinely offered to detect congenital malformations and prenatal genomic testing is offered upon the diagnosis of most of these malformations. Invasive karyotyping has long been the standard for prenatal genomic testing. Over the years, technological and scientific developments have resulted in the feasibility of more advanced genomic test techniques, microarray and whole exome sequencing (WES), for prenatal diagnosis. These new techniques allowed for a major increase in fetal genomic testing resolution. Concurrently, next generation sequencing based non-invasive prenatal testing (NIPT) was introduced and posed a safe alternative to invasive prenatal testing. The aims of this thesis were:

1. To evaluate the additional value of invasive single nucleotide polymorphism array over conventional invasive karyotyping or current non-invasive next generation sequencing based testing in specific fetal anomalies detected by ultrasound.
2. To evaluate the potential additional value of invasive fetal WES in specific fetal anomalies detected by ultrasound.
3. To provide an ethical deliberation on the offer of invasive testing for fetal WES in case of fetal anomalies detected by ultrasound.

Chapter 1 provides a general introduction to prenatal advanced genomic testing, an explanation of the genomic techniques used in the studies of this thesis, and a historic timeline

of prenatal diagnosis.

Chromosomal microarray was introduced to prenatal diagnosis between 2005 and 2007. Microarray can detect a (potentially) clinically significant submicroscopic anomaly in approximately 5.2-10% of fetuses with ultrasound anomalies and a normal karyotype. At the start of this dissertation, few studies had focused on the additional value of microarray over karyotyping in case of a certain single fetal malformation on ultrasound. Therefore, we performed a systematic review of the literature in 2013 which is presented in **Chapter 2**. This review demonstrated that in 5.6% (95% CI, 4.7-6.6%) of pregnancies with a fetal malformation restricted to one anatomical system and a normal result of karyotyping, microarray retrieves a (potentially) pathogenic chromosomal aberration. The chance of a pathogenic chromosomal aberration depends on the anatomical system involved: 3.1% (95% CI, 0.4-5.7%) in fetuses with an isolated increased nuchal translucency to 7.9% (95% CI, 4.8-10.9%) in fetuses with an abnormality of the musculo-skeletal system. In fetuses with multiple structural ultrasound anomalies and a normal result of karyotyping, this chance is 9.1% (95% CI, 7.5-10.8%).

This review only considered structural fetal anomalies. Literature on the value of microarray in non-structural ultrasound anomalies, such as small for gestational age fetal growth (SGA) was largely lacking. However, this entity is characterized by a variable fetal prognosis. We hypothesized that, especially in these entities, advanced genomic testing would be of value in estimating the fetal prognosis. To test this hypothesis we studied the phenotypic and genotypic outcomes of SGA fetuses which is presented in **Chapter 3.1**. We included 211 cases with SGA, defined as an abdominal circumference $\leq$ 5th percentile measured on ultrasound between 18 and 24 weeks gestational age. Two subgroups were created:

isolated SGA (n=158) and SGA in coexistence with sonomarkers and/or hydramnios (n=53). The chance of a microscopic chromosomal anomaly, which could be detected by karyotyping, in SGA fetuses with and without coexisting non-structural ultrasound anomalies was 1.3% (95% CI, 0.2-3.9%) and 5.8% (95% CI, 1.5-14.0%), respectively. Submicroscopic chromosomal anomalies, as tested by means of single nucleotide polymorphism (SNP) microarray, were found in 0.6% (95% CI, 0.1-2.8%) and 1.9% (95% CI, 0.2-8.2%), respectively. The occurrence of chromosomally normal, postnatally apparent syndromic phenotypes in SGA fetuses is high: 5.8% (95% CI, 2.8-10.0%) in prenatally isolated SGA fetuses and 17.3% (95% CI, 8.7-29.0%) in SGA fetuses with coexisting non-structural anomalies. These outcomes indicate that chromosomal testing by means of SNP microarray should be offered in SGA fetuses. They also stress the importance of thorough postnatal examination and follow-up by a pediatrician.

Increased first trimester nuchal translucency (NT) is a similarly unspecific but often-encountered non-structural ultrasound abnormality. Although most fetuses with increased NT will have no genotypic or phenotypic anomalies, increased NT can be associated with common aneuploidies and, to a lesser extent, other genetic aberrations. Since the introduction of reliable screening for common aneuploidies by means of NIPT, the offer of an invasive procedure for microarray in case of an increased NT has been questioned. In **Chapter 3.2**, a cohort of 362 pregnancies with increased NT was analyzed to identify to what extent chromosomal anomalies would be missed if NIPT were substituted for SNP array. We showed that the rate of missed chromosomal anomalies would range between 2-10%, depending on the type of NIPT used.

Chapter 3.3 concerns the additional value of SNP array and the potential value of fetal

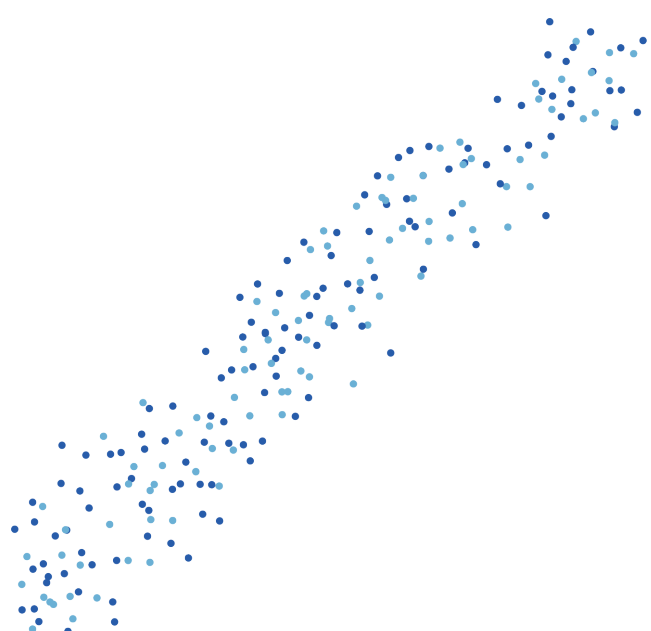
WES in estimating the fetal prognosis in case of isolated complete agenesis of the corpus callosum (cACC). Isolated cACC is a rare congenital malformation that has a variable prognosis, ranging from asymptomatic to severe intellectual disability. This burdens expecting parents with extreme uncertainty. Our analysis of 25 cases with isolated complete ACC on ultrasound showed that they carry a 11.1% (95% CI, 2.0-31%) chance of a pathogenic chromosomal anomaly detected by SNP array. Thirty percent (95% CI, 8.5-60%) of live births without a chromosomal anomaly on microarray showed intellectual disability. Based on postnatal WES results, the estimated diagnostic yield of prenatal WES will be between 13.3% (95% CI, 2.4-36%) and 50% (95% CI, 11-89%).

In the last section of chapter 3, a cohort of fetuses with congenital heart malformations (CHM) was evaluated. CHM are strongly associated with chromosomal aberrations but also with monogenic disorders such as Noonan syndrome. The feasibility of WES in prenatal diagnosis allows us to routinely and broadly test for these latter disorders, but WES can also retrieve variants of unknown significance (VUS), variants of phenotypic heterogeneity, and mutations for (untreatable) late onset diseases increases. There is an ongoing debate about whether WES should be offered prenatally. To inform this discussion, the aim of the study presented in **Chapter 3.4** was to evaluate the potential diagnostic value of fetal WES in prenatally detected CHM. The estimated yield of fetal WES was compared with the estimated yield of a hypothetical sequencing panel targeted on known CHM-risk genes. We enrolled 445 CHM fetuses, including 229 isolated and 216 non-isolated cases on prenatal ultrasound. The potential diagnostic yield of prenatal WES in fetuses with a CHM on prenatal ultrasound is between 6.7% (30/445; 95% CI, 4-9%) and 23.4% (30/128; 95% CI, 16-31%). For prenatally non-isolated cases only,

its yield is between 11.6% (25/216; 95% CI, 7-16%) and 29.8% (25/84; 95% CI, 20-40%). To diagnose these disorders prenatally, the open diagnostic approach of WES would be more effective than the targeted approach of our hypothetical CHM-risk gene panel, as only 30 percent of diagnoses were found in the genes of this panel.

As demonstrated in the chapters of this thesis, fetal microarray and WES can provide expecting parents with valuable information about their fetus' prognosis. In **Chapter 4** we argue for offering invasive fetal WES in case of fetal anomalies on ultrasound without chromosomal anomalies. We believe that the option of access to the most extensive genomic information possible serves the prospective parents' right in reproductive autonomy best. This offer requires the availability of sufficient resources for dedicated pretest counseling. The ethical issues surrounding WES, notably genomic uncertainty and the anticipatory autonomy rights of the unborn child, can be addressed through the involvement of a dedicated multidisciplinary team in the set-up and scope of WES and through pretest counseling.

In **Chapter 5**, the general discussion, we review the previous chapters, consider the methodological issues raised and explain the clinical applications of our results. We consider the future challenges and possibilities of fetal advanced genomic testing and we present our views on the aims of future research.



Chapter 6

Samenvatting



Samenvatting

Aangeboren afwijkingen kunnen het gevolg zijn van een specifieke genetische aandoening bij de foetus, zoals een chromosomale of monogene afwijking. De aanwezigheid van een dergelijke genetische afwijking kan de foetale prognose sterk beïnvloeden, omdat het naast de aangeboren afwijking ook een verstandelijke beperking en/of co-morbiditeiten kan veroorzaken. Foetale echoscopie wordt aan aanstaande ouders aangeboden om foetale afwijkingen voor de geboorte op te sporen. Prenatale genetische diagnostiek wordt aangeboden indien foetale echoscopie een aangeboren afwijking laat zien. Invasieve karyotypering is lang de standaard geweest voor prenatale genomische diagnostiek. In de afgelopen jaren hebben technologische en wetenschappelijke ontwikkelingen het echter mogelijk gemaakt om meer geavanceerde genomische testtechnieken in de prenatale setting toe te passen, zoals microarray en whole exome sequencing (WES). Deze nieuwe technieken hebben een veel hogere resolutie. Tegelijkertijd werd de op next generation sequencing gebaseerde niet-invasieve prenatale test (NIPT) geïntroduceerd. NIPT heeft in tegenstelling tot invasieve diagnostiek, wat nodig is voor microarray en WES, geen miskraamrisico. Het doel van dit proefschrift was om:

1. De aanvullende waarde van invasieve single nucleotide polymorfism (SNP) microarray ten opzichte van invasieve karyotypering en NIPT bij verschillende specifieke foetale echoafwijkingen te evalueren.
2. De potentiële toegevoegde waarde van invasieve foetale WES bij specifieke foetale echoafwijkingen in te schatten.
3. Een ethische afweging te maken in het aanbieden van invasieve WES bij foetale echoafwijkingen.

De resultaten van de studies in dit proefschrift kunnen worden gebruikt om pretest counseling te verbeteren en zo om aanstaande ouders te helpen in het maken van een geïnformeerde keuze ten aanzien van prenatale genomische diagnostiek. Professionals kunnen de resultaten gebruiken om een afweging te maken in het wel of niet aanbieden van prenatale genomische testen bij verschillende foetale aandoeningen.

Hoofdstuk 1 omvat een algemene inleiding tot de verschillende genomische testtechnieken die zijn gebruikt in dit proefschrift alsook een historische tijdlijn van de geschiedenis van de prenatale diagnostiek. Microarray werd voor het eerst geïntroduceerd in de prenatale diagnostiek tussen 2005 en 2007. Microarray detecteert een klinisch relevante submicroscopische chromosoomafwijking in 5,2-10% (2-4) van de foetussen met echoafwijkingen en een normaal karyogram. Bij de start van dit promotie onderzoek was er nog weinig bekend over de toegevoegde waarde van microarray ten opzichte van karyotypering in het geval van een specifieke geïsoleerde foetale echoafwijking. De systematische review van de literatuur gepresenteerd in **hoofdstuk 2**, uitgevoerd in 2013, focust zich op de additionele waarde van microarray bij foetussen met specifieke geïsoleerde echoafwijkingen en een normaal karyogram. Deze review laat zien dat bij 5.6% (95% CI, 4.7-6.6%) van de zwangerschappen met een specifieke foetale echoafwijking beperkt tot één orgaansysteem en een normaal karyogram, microarray een (potentieel) pathogene chromosomale afwijking detecteert. De kans op een pathogene chromosomale afwijking hangt af van het betrokken orgaansysteem: 3,1% (95% CI, 0,4-5,7%) bij foetussen met een verdikte nekplooï in het eerste trimester tot 7,9% (95% CI, 4,8-10,9%) bij foetussen met een afwijking van het musculoskeletale systeem. Bij foetussen met structurele echoafwijkingen in meerdere orgaansystemen en een normaal

karyogram, is de kans op een chromosomale afwijking gedetecteerd met microarray 9,1% (95% CI, 7,5-10,8%).

Deze systematische review van de literatuur omvatte alleen structurele foetale echoafwijkingen. Literatuur over de waarde van microarray bij niet-structurele foetale afwijkingen, zoals beperkte foetale groei voor de zwangerschapsduur, ofwel small for gestational age (SGA) foetale groei, ontbrak grotendeels. Dit fenotype kent een diverse etiologie en daarom een zeer variabele foetale prognose. We veronderstelden daarom dat geavanceerde genomische diagnostiek juist hierbij van waarde zou zijn bij het inschatten van de foetale prognose. Om deze hypothese te testen, hebben we de fenotypische en genotypische uitkomsten van SGA foetussen onderzocht (**hoofdstuk 3.1**). We includeerden 211 zwangerschappen met SGA, gedefinieerd als een abdominale omtrek $\leq$ 5e percentiel gemeten bij foetale echoscopie tussen 18 en 24 weken zwangerschapsduur. Het cohort werd onderverdeeld in twee subgroepen: geïsoleerde SGA (n = 158) en SGA samen met sonomarkers en/of oligo-/polyhydramnion (n = 53). De kansen op een chromosomale afwijking die kon worden gedetecteerd in het karyogram bij geïsoleerde SGA en SGA met niet-structurele echoafwijkingen waren respectievelijk 1,3% (95% CI, 0,2-3,9%) en 5,8% (95% CI, 1,5-14,0%). Submicroscopische chromosomale afwijkingen die enkel konden worden gevonden met SNP array kwamen voor bij 0,6% (95% CI, 0,1-2,8%) en 1,9% (95% CI, 0,2-8,2%), respectievelijk. Postnataal evidente syndromale fenotypen zonder chromosomale afwijking komen vaak voor bij SGA: Bij 5,8% (95% CI, 2,8-10,0%) van de foetussen met antenataal geïsoleerde SGA en bij 17,3% (95% CI, 8,7-29,0%) van de foetussen met SGA in combinatie met niet-structurele echoafwijkingen. Wij concluderen dat invasieve microarray moet worden aangeboden bij SGA. Onze resultaten benadrukken ook het belang

van postnataal onderzoek en follow-up door een kinderarts.

Een verdikte foetale nekplooi (nuchal translucency, NT) in het eerste trimester is een ook een niet-specifieke, maar vaak voorkomende non-structurele echoafwijking. Hoewel de meeste foetussen met een verdikte nekplooi postnataal geen genotypische of fenotypische afwijkingen zullen hebben, is een verhoogde NT geassocieerd met aneuploidie en, in mindere mate, met andere genetische afwijkingen. Sinds de introductie van betrouwbare screening voor aneuploidie van chromosomen 13, 18 en 21 door middel van NIPT, is het aanbieden van invasieve diagnostiek voor genoom-brede testen bij een verdikte nekplooi ter discussie komen te staan. In **hoofdstuk 3.2** onderzochten wij een cohort van 362 zwangerschappen met een verdikte foetale nekplooi om vast te stellen in hoeverre genetische afwijkingen zouden worden gemist als NIPT invasieve diagnostiek middels SNP array zou vervangen. We zagen dat dan de kans op het missen van een chromosomale afwijking tussen 2-10% zal zijn, afhankelijk van het type NIPT dat wordt gebruikt.

Hoofdstuk 3.3 omvat het onderzoek naar de aanvullende waarde van microarray en de potentiële aanvullende waarde van prenatale WES bij het inschatten van de foetale prognose in geval van geïsoleerde complete agenesie van het corpus callosum (cACC). Geïsoleerde cACC is een zeldzame aangeboren afwijking met een variabele prognose, variërend van asymptomatisch tot een ernstige verstandelijke beperking. Dit brengt aanstaande ouders in extreme onzekerheid. Wij onderzochten 25 casus met geïsoleerde cACC op echo. Bij 11,1% (95% CI, 2,0-31%) werd een pathogene chromosomale afwijking gedetecteerd middels SNP-array. Dertig procent (95% CI, 8,5-60%) van de levendgeborenen zonder een chromosomale afwijking toonde een verstandelijke beperking. Op basis van de

postnatale WES-resultaten is de geschatte diagnostische opbrengst van prenatale WES tussen 13,3% (95% CI, 11-89%) en 50% (95% CI, 2,4-36%) .

In het laatste deel van hoofdstuk 3 omvat een onderzoek van een cohort van foetussen met aangeboren hartafwijkingen (congenital heart malformations, CHM). CHM zijn sterk geassocieerd met chromosomale afwijkingen, maar ook met monogene aandoeningen zoals het syndroom van Noonan. Het gebruik van WES in de prenatale setting stelt ons in staat om routinematig en exoom-breed op de laatst genoemde aandoeningen te testen, maar WES kan ook varianten van onbekende klinische betekenis (variants of unknown significance, VUS), varianten met variabele fenotypische expressie en mutaties voor (niet-behandelbare) late-onset aandoeningen aan het licht brengen. Er is een voortdurende discussie over de vraag of WES in de prenatale setting moet worden aangeboden. Om informatie aan te leveren voor deze discussie, was ons doel van de in **hoofdstuk 3.4** gepresenteerde studie om de potentiële diagnostische waarde van foetale WES in prenataal gedetecteerde hartafwijkingen te evalueren. We vergeleken ook de potentiële waarde van foetale WES met de potentiële waarde van een hypothetisch panel van bekende risicogenen voor hartafwijkingen. We onderzochten 445 foetussen met een prenataal gedetecteerde hartafwijking: 229 prenataal geïsoleerde casus en 216 prenataal niet-geïsoleerde casus. De potentiële diagnostische opbrengst van prenataal WES bij foetussen met een hartafwijking op prenatale echo is minstens 6,7% (30/445; 95% CI, 4-9%) en hoogstens 23,4% (30/128; 95% CI, 16-31%). Voor prenataal niet-geïsoleerde casus is de opbrengst tussen 11,6% (25/216; 95% CI, 7-16%) en 29,8% (25/84; 95% CI, 20-40%). Om deze monogene aandoeningen prenataal te diagnosticeren, zou de brede diagnostische benadering van WES

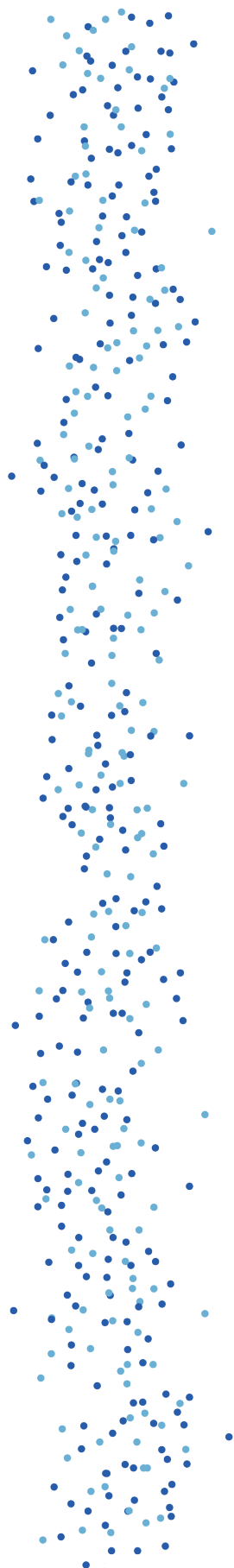
effectiever zijn dan de gerichte benadering van een cardio-risico gen panel, aangezien slechts 30 procent van de diagnoses werd gevonden in de genen van dit panel.

Zoals de resultaten van de studies in dit proefschrift laten zien, kunnen prenatale arrayen WES aanstaande ouders frequent waardevolle informatie geven over de prognose van hun foetus. **Hoofdstuk 4** beschrijft een ethische afweging in het aanbieden van amniocentese voor geavanceerde genomische diagnostiek bij foetale echoafwijkingen. Wij pleiten voor het aanbieden van invasieve foetale WES in het geval van foetale echoafwijkingen zonder een chromosomale afwijking, omdat het aanbieden van de meest geavanceerde genetische test beschikbaar het recht op reproductieve autonomie van de toekomstige ouders het beste dient. Het aanbieden van foetale WES vereist wel de beschikbaarheid van voldoende middelen voor volledige en toegewijde pre-test counseling. De ethische kwesties rond foetale WES, in het bijzonder genomische onzekerheid en de geanticipeerde rechten op autonomie van het ongeboren kind, kunnen worden aangegaan zolang er een toegewijd multidisciplinair team betrokken blijft bij het behandelen en communiceren van WES resultaten en bij pre-test counseling.

In de algemene discussie van **hoofdstuk 5** behandelen we de methodologische aspecten van voorgaande hoofdstukken en beschrijven we de klinische toepassingen van onze resultaten. We blikken vooruit op toekomstige uitdagingen en mogelijkheden van foetale geavanceerde genomische diagnostiek en we sluiten af met onze visie op de doelen van toekomstig onderzoek.



ADDENDUM



Addendum

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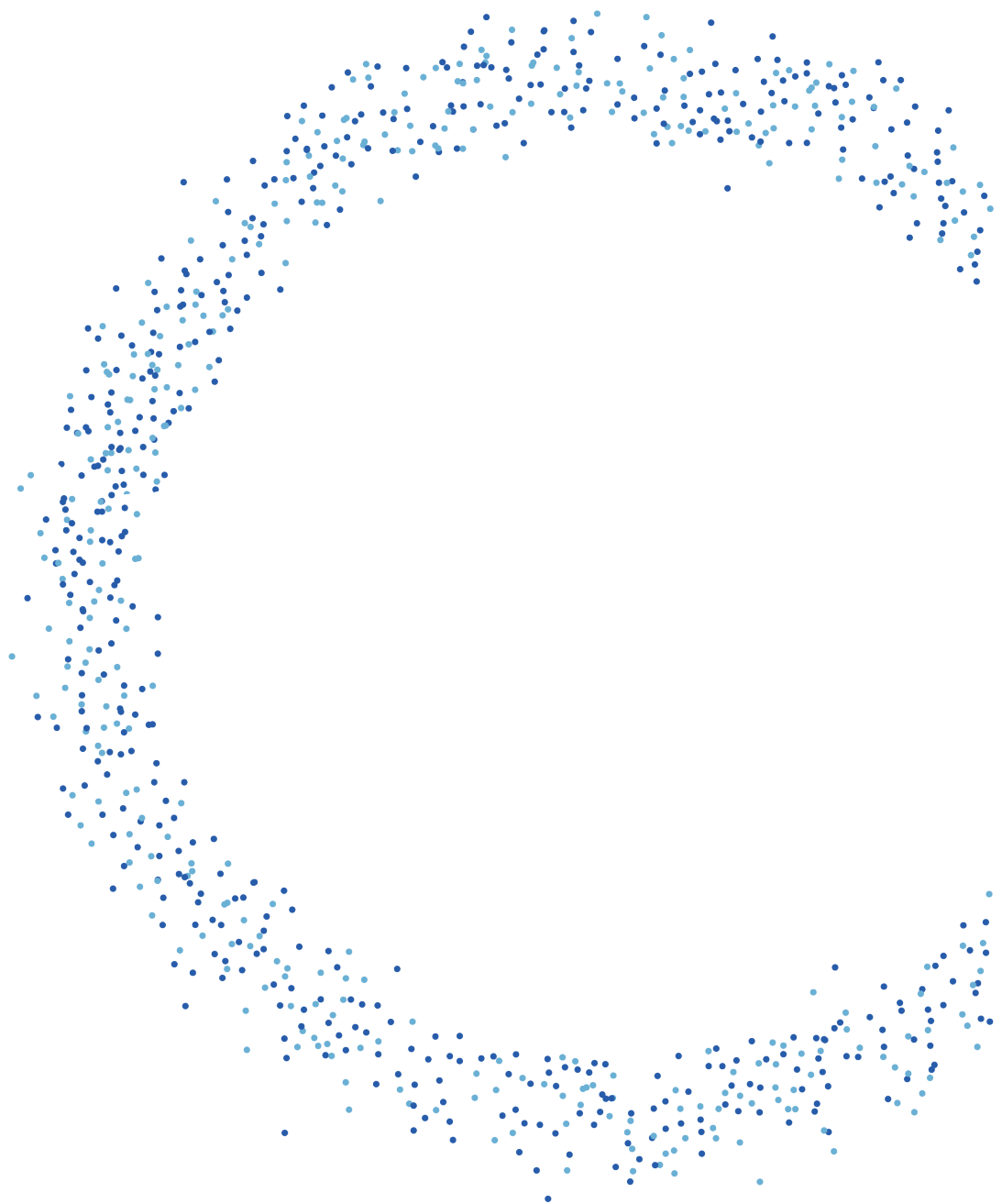
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Addendum

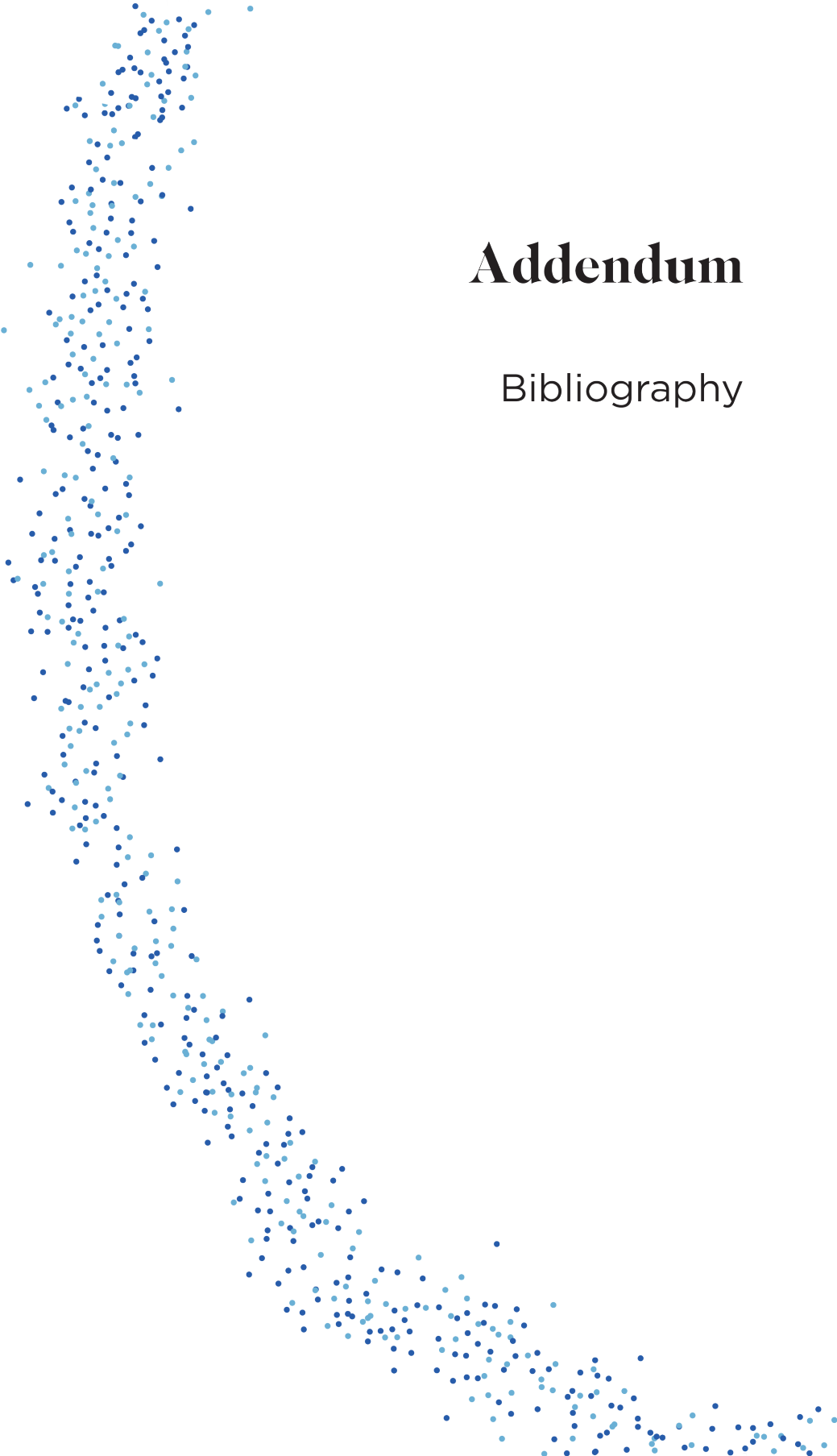
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Addendum

Bibliography

Bibliography

Effect of malaria in pregnancy on foetal cortical brain development: a longitudinal observational study.

M. J. Rijken, M. C. de Wit, E. J. Mulder, S. Kiricharoen, N. Karunkonkowitz, T. Paw, G. H. Visser, R. McGready, F. H. Nosten and L. R. Pistorius
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Ultrasound evaluation of cortical brain development in fetuses with intrauterine growth restriction

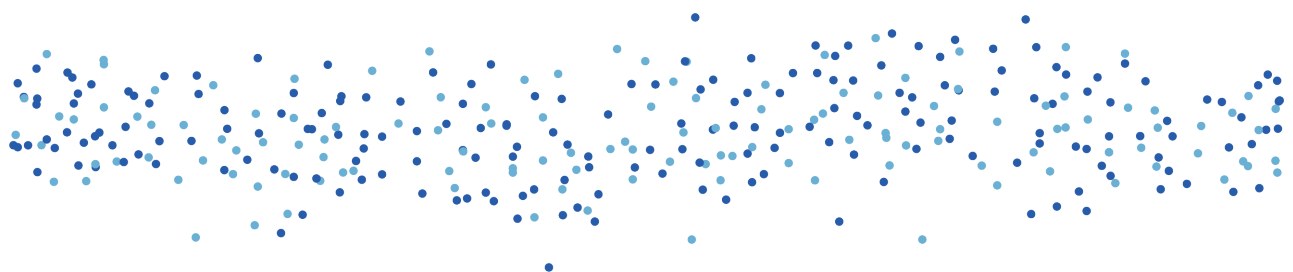
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Prenatal SNP array testing in 1000 fetuses with ultrasound anomalies: causative, unexpected and susceptibility CNVs

M.I. Srebniak, K.E.M. Diderich, M. Joosten, L.C.P. Govaerts, J. Knijnenburg, F.A.T. de Vries, M. Boter, D. Lont, M.F.C.M. Knapen, M.C. de Wit, A.T.J.I. Go, R.J.H. Galjaard, D. van Opstal
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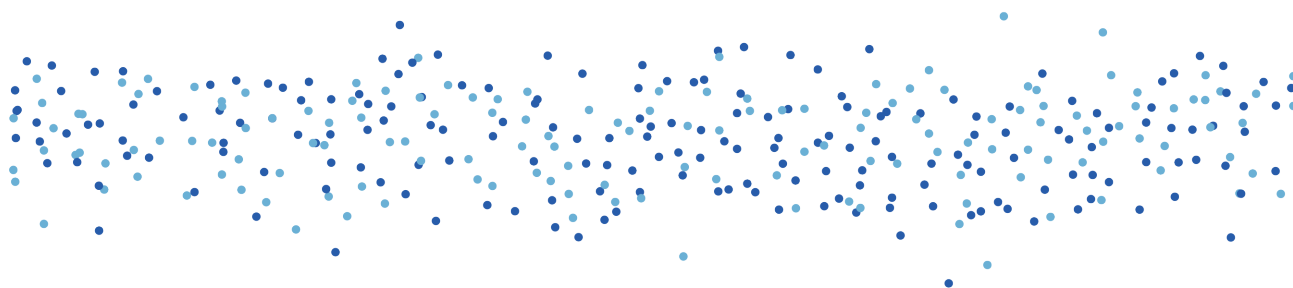
Re: Detection of submicroscopic chromosomal aberrations by array-based comparative genomic hybridization in fetuses with congenital heart disease

Y. Yan, Q. Wu, L. Zhang, X. Wang, S. Dan, D. Deng, L. Sun, L. Yao, Y. Ma and L. Wang.
Ultrasound Obstet Gynecol 2014; 43: 404-412.



Addendum

PhD portfolio



PhD portfolio

Name PhD candidate	M.C. de Wit
Erasmus MC department	Obstetrics and Gynecology
PhD period	2012-2017
Promotors	Prof.dr. E.A.P. Steegers Prof.dr. R.W. Hofstra
Co-promotors	dr. A.T.J.I. Go dr. R.J.H. Galjaard

1. PhD Training

General Courses

Biomedical English writing and communication	2013	4.0
Systematic literature search in Pubmed and other databases	2013	0.3
Scientific integrity	2014	0.3
Basiscursus Regelgeving en Organisatie voor Klinisch onderzoekers (BROK)	2014	1.0
Principles of research in medicine and epidemiology	2015	1.0
Introduction to data analysis	2015	1.0
Methods of clinical research	2015	1.0
Cohort studies	2015	1.0
Crew Resource Management (CRM) training	2015	0.3
Course Patient Oriented (CPO) Research	2015	0.3

Specific Courses

FMF certificate 20 weeks anomaly scan	2012	0.9
FMF certificate 13 Weeks scan	2012	0.9
Endnote	2013	0.1
Open Clinica	2014	0.1
Advances in genomics research	2015	0.7
The practice of epidemiologic analysis	2015	1.0
Prenatal screening	2016	0.3

Seminars and Workshops

Jubileumcongres Sophia kindziekenhuis	2013	0.3
LOGLODLOC & WPDT/NVOG "Prenatale diagnostiek: Nieuw, nieuwer, nieuwst!"	2013	0.3
"Schisis en craniofaciale afwijkingen bij kinderen: Alles wat de verwijzend arts moet weten"	2013	0.3
WPDT "Meer toevalsbevindingen in de prenatale diagnostiek: Hoe nu verder?"	2013	0.3
NVOG/NVK Pijlerdag foeto-maternale geneeskunde	2014	0.3
SCEM "Ethiek in de praktijk"	2014	0.3
"Science in transition, the Erasmus MC responsibility"	2014	0.3
"Spina bifida: met het oog op de toekomst: Waar liggen de grenzen van de behandelars?"	2014	0.3
"Big Brains, small brains, genes and mechanisms"	2015	0.3
SPSZN "Juridische aspecten prenatale screening en update ontwikkelingen"	2016	0.3
Erasmus MC course "Prenatale geneeskunde voor de gynaecoloog: Aangeboren afwijkingen en het perspectief na de geboorte."	2017	1.0
Fetal pathology meeting, monthly	2012-2017	1.0
Prenatal ultrasound meeting, weekly	2012-2017	2.0
Multidisciplinary obstetrics and pediatrics meeting (chairman), weekly	2012-2017	2.0

Presentations at Seminars

Refereeravond obstetrie en gynaecologie (oral presentation)	2013	1.0
WFE/WPDT symposium prenatale diagnostiek(oral presentation)	2013	1.0
Clinical genetics research meeting (oral presentation)	2015	1.0
Star-MDC/PCR verloskundig symposium (oral presentation)	2015	1.0
Sophia research day (oral presentation)	2016	1.0
Obstetrics and gynecology research meeting, weekly (2 oral presentations)	2012-2017	2.0

Presentations at (Inter)national Conferences

17th International conference on prenatal diagnosis and therapy, Lisboa, Portugal	2013	1.0
24 th World congress on ultrasound in obstetrics and gynecology, Barcelona, Spain (poster presentation)	2014	1.0
Erasmus MC course "Prenatale geneeskunde voor de gynaecoloog: Aangeboren afwijkingen en het perspectief na de geboorte", Rotterdam (oral presentation)	2016	1.0
20th International conference on prenatal diagnosis and therapy, Berlin, Germany (poster presentation)	2016	1.0
52 ^e Gynaecongres, Amersfoort (2 oral presentations)	2017	1.0

Other

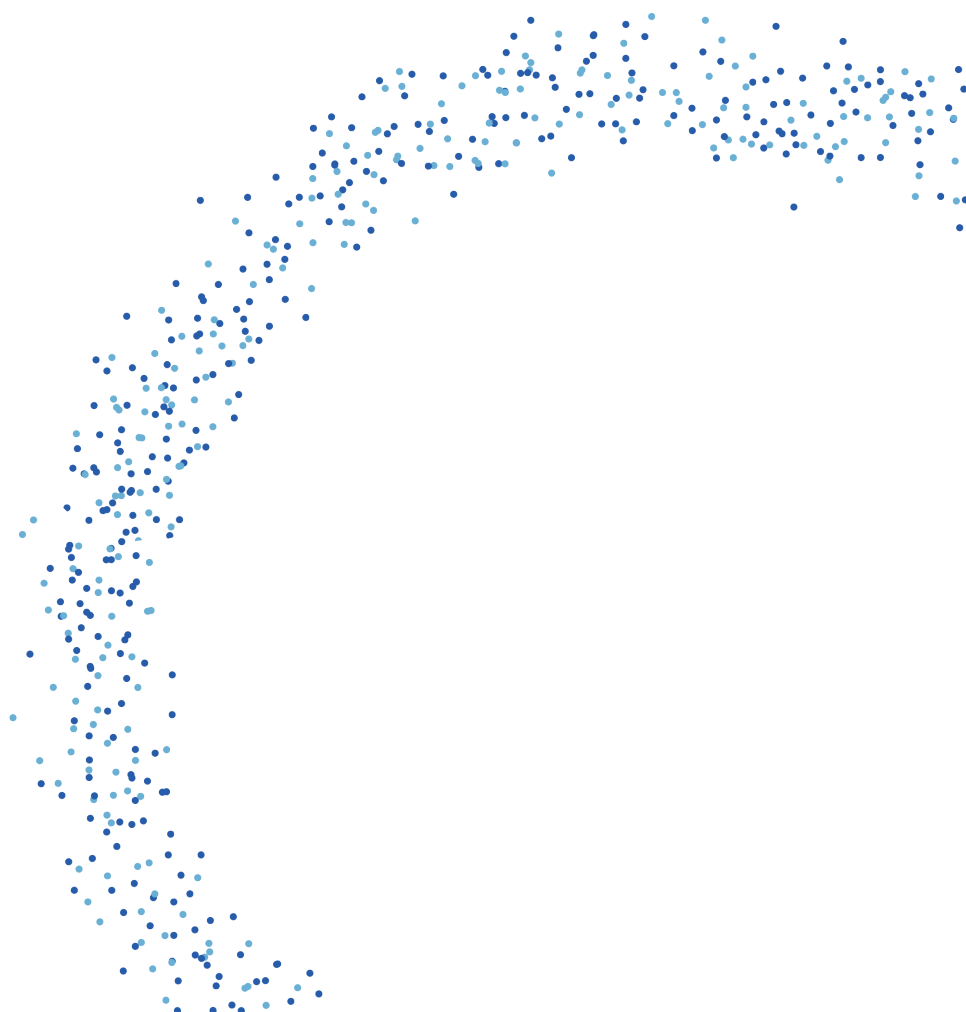
Ultrasound in Obstetrics and Gynecology Reviewer certificate	2016	1.0
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2. Teaching

Ultrasound in Obstetrics and Gynecology Journal club	2014	1.0
Prenatal screening on fetal structural abnormalities: Lecturing and practical classes	2013-2016	2.0
SEO Masterclass: Hands-on SEO sonography training	2014-2017	1.0
Mystery of creation: Lecturing, practical classes and training	2015-2017	2.0

Addendum

Curriculum Vitae



Curriculum Vitae

Naam	Merel Charlotte de Wit
Roepnaam	Charlotte
Adres	Rodenrijselaan 14B 3037 XE Rotterdam
E-mailadres	m.c.dewit@erasmusmc.nl / m.c.d.wit@gmail.com
Telefoon	+31 (0)6 43546029
Geboortedatum	24 februari 1986
Geboorteplaats	Apeldoorn
Nationaliteit	Nederlandse
BSN	101219180
BIG registratie	69915078701

Professionele ervaring

2017 – heden	ANIOS gynaecologie en verloskunde, Ikazia ziekenhuis Rotterdam
2011 – 2017	Arts prenatale geneeskunde, Erasmus MC Rotterdam

Opleidingen

2012 – heden	PhD onderzoek, Erasmus MC Rotterdam
2004 – 2011	Geneeskunde, Universiteit Utrecht
2005 – 2006	Culturele antropologie, Universiteit Utrecht
1998 – 2004	Atheneum, Baudartius College Zutphen

Onderzoeksprojecten

2012 – heden	Advanced Genomic Testing for Fetal Ultrasound Anomalies Co-promotoren: dr. A.T.J.I. Go en dr. R.J.H. Galjaard Promotoren: Prof. dr. E.A.P. Steegers en Prof. dr. R.M.W. Hofstra Erasmus MC Rotterdam
2009 – 2011	Cortical Brain Development in Fetuses affected by Intrauterine Growth Restriction, with or without Malaria Supervisors: Dr L.R. Pistorius and Dr M.R. Rijken UMC Utrecht and Shoklo Malaria Research Unit Mae Sot, Thailand

Onderwijservaring

2015 – 2017	Mystery of Creation: Hoorcolleges, vaardigheidsonderwijs en live-echoscopie Minor in curriculum geneeskunde, Erasmus Universiteit Rotterdam
2014 – 2017	SEO Masterclass: hands-on SEO echoscopie training Erasmus MC Rotterdam
2013 – 2016	Prenatale screening op foetale structurele afwijkingen en De Wervelkolom: Vaardigheidsonderwijs Curriculum geneeskunde, Erasmus Universiteit Rotterdam
2012 – heden	Echoscopie training voor AIOS, begeleiding van co-assistenten en studenten verloskunde Erasmus MC Rotterdam

Talen

Nederlands (moedertaal), Engels (vloeiend), Frans (niveau B1, *IS Institut International de Langue, Aix en Provence, Frankrijk*)

Passies

Surfen, ontwerpen en klussen, muziek



Addendum

About the author

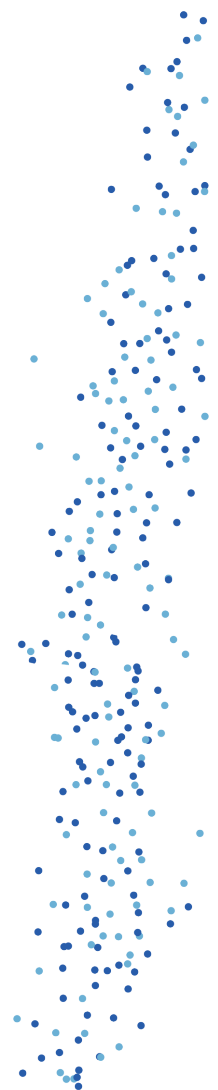
About the author

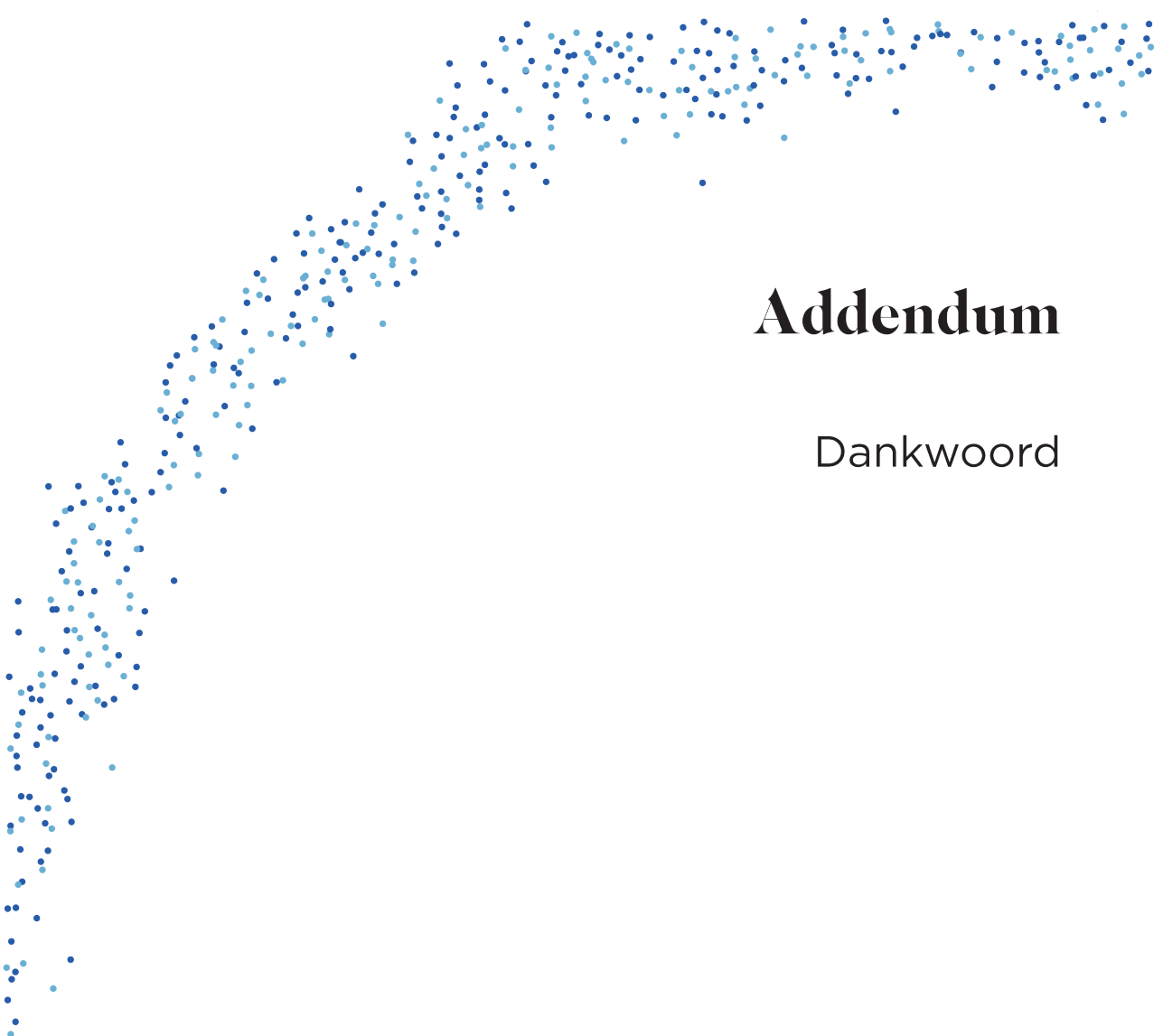
Merel Charlotte de Wit was born on the 24th of February 1986 in Apeldoorn. She attended the Baudartius college in Zutphen, where she completed athenaeum in 2004.

In the same year she started her medical training at the University of Utrecht. In her fourth year she did an extracurricular research project on fetal cortical brain development under the supervision of dr. L.R. Pistorius. At this point she got inspired to pursue a career in Obstetrics and Gynecology. In 2011 she assisted in a research program about the effect of maternal malaria on fetal cortical brain development in the Shoklo Malaria Research Unit in Mae Sot, on the border Thai border with Myanmar.

After graduating from medical school, Charlotte started working as a fetal ultrasound specialist in the department of Obstetrics in Gynecology of the Erasmus Medical Centre in Rotterdam. In 2012 she initiated her dissertation under guidance and supervision of dr. A.T.J.I. Go, dr. R.J.H. Galjaard, prof. dr. E.A.P. Steegers and prof. dr. R.W. Hofstra. This work resulted in her PhD thesis: "Advanced genomic testing for fetal ultrasound anomalies". She was given the opportunity to present her work at several (inter)national conferences. On the 1st of December 2017 she started as a junior resident in the department of Obstetrics and Gynecology at the Ikazia Hospital, Rotterdam.







Addendum

Dankwoord

Dankwoord

Gedurende de zes jaar van dit onderzoek heb ik van zo veel mensen steun en inspiratie gekregen. Dit is dan mijn kans om mijn woorden van dank op te schrijven en ik hoop dat ik jullie allen genoeg recht kan doen.

Allereerst alle (aanstaande) ouders en hun kinderen die hebben deelgenomen aan dit onderzoek. Een afwijkende echobevinding bij een ongeborn kind brengt enorme onzekerheid met zich mee. Ook wordt het vertrouwen in het krijgen van een gezond kind verstoord of geheel verloren. Ik heb er heel veel bewondering voor dat zij op dit moeilijke moment in hun leven meewerken aan wetenschappelijk onderzoek, zodat ouders en kinderen in de toekomst beter geholpen kunnen worden.

Dr. Attie Go. Omdat ik me geen leukere copromotor had kunnen wensen. Ik heb vele mooie herinneringen aan onze ad hoc brainstormsessies. Bedankt voor de vrijheid die je me hebt gegeven in het onderzoek. Voor je positieve support die me steeds hielp om te relativeren. En voor je inspirerende wijsheid.

Dr. Robert Jan Galjaard. Bedankt voor de fijne samenwerking. En voor je inzet en expertise om de koers van het onderzoek te bepalen en zo het onderzoek (terug) op de rails te zetten.

Prof. Dr. Eric Steegers en Prof. Dr. Robert Hofstra. Voor jullie vertrouwen in het onderzoek, jullie sturing en kritische beoordeling van dit proefschrift.

Paulien, Nina en Carsten. We got it together, baby. Wie heeft nou het geluk om drie en een halve paranimf te hebben. Ik heb altijd de grootste lol met jullie. En dat was precies wat ik nodig had. Bedankt voor het meedenken,

helpen en – Nina speciaal – voor alle voorkennis over promoveren. My kind of wonderful, that's what you are. – Barry

Alle collega's bij de prenatale geneeskunde, verloskunde en klinische genetica in het Erasmus MC. Voor de tijd en energie die jullie hebben besteed om mij op te leiden. Voor het last minute overnemen van poli's zodat ik naar meetings kon. En voor het extra poli's draaien zodat ik een week schrijfvrij kon hebben. Jullie zijn een geweldig team en patiënten zijn bij jullie in de beste handen.

Gosia. Omdat je zo enorm betrokken was bij de opzet van het onderzoek. Er zijn maar weinig mensen met zo veel kennis over en toewijding aan dit onderwerp als jij.

Gynaecologen, arts-assistenten en verloskundigen in het Ikazia. Het was kort maar krachtig. Het moment in het leven waarop je de grootste stumper bent, leer je het allermeeest. En van wie het beter te leren dan van jullie. Ik hoop jullie in de toekomst weer tegen te komen.

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Maria en Idemargreet. We zien elkaar zeker te weinig, maar jullie geven me altijd het gevoel dat jullie in de buurt zijn. Bedankt dat jullie mij onder jullie hoede hebben genomen. Ik kom gauw een weekend logeren.

Glastofamily. You know who you are. For letting our hair down at Glastonbury or any other place in the world. For providing hilarious title suggestions (I'm sorry that "Invasive prenatal testing isn't like tits on a fish" didn't make it). Hope to see you soon again.

Fleur. Voor onze gesprekken over het leven en de keuzes die we maken. Ik ben er dol op. Wie weet waar we ooit eindigen. Maar ik hoop dat we dan nog steeds samen zijn.

Scott. For having so much patience and trust in this process. For making me take the time to enjoy the good things in life. Thank you caterpillar. I promise I'll keep it up.

Rixt. Alles wat uit jouw handen komt is mooi. Ik ben vereerd dat je in je drukke schema de tijd vond om mijn proefschrift te ontwerpen. En wat was het leuk om samen te werken met een lieve vriendin als jij.

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Geerten, Jelmer, Carlijn en Sophie. Ik ben zo trots dat ik jullie zusje ben. Bedankt voor jullie niet aflatende interesse in mijn onderzoek. Voor het leuke stellingen diner. Voor alle gezellige borrels om mijlpalen te vieren. En voor dat jullie er altijd voor me zijn.

Papa. Van jou heb ik het denkvermogen gekregen voor het doen van dit onderzoek. Bedankt voor je waardevolle advies en steun. Ik ben blij dat we elkaar steeds vaker zien. Wanneer zullen we weer gaan zeilen?

Mama. Omdat je je me altijd zo inspireert, ik bewonder jou het allermeest. Niets is belangrijker voor mij dan jouw onvoorwaardelijke en eindeloze steun.

