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Implementation and effectiveness of new prenatal diagnostic strategies in Estonia

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Implementation and effectiveness of new prenatal diagnostic strategies in Estonia

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ABBREVIATIONS

AC	amniocentesis
ACMG	American College of Medical Genetics and Genomics
ACOG	American College of Obstetricians and Gynecologists
AFP	alfa-fetoprotein
ARSA	aberrant right subclavian artery
ART	assisted reproductive techniques
AVSD	atrio-ventricular septum defect
BMI	body mass index
BWS	Beckwith-Wiedemann syndrome
CGH	comparative genomic hybridization
cf-DNA	cell-free DNA
cff-DNA	cell-free fetal DNA
cFTS	combined first trimester screening
CHD	congenital heart disease
CI	confidence interval
CRL	crown-rump length
CMA	chromosomal microarray analysis
CNS	central nervous system
CNV	copy-number variant
CVS	chorionic villous sampling
DR	detection rate
DS	Down's syndrome
DV	ductus venosus
EHIF	Estonian Health Insurance Fund
ES	exome sequencing
ETCH	East-Tallinn Central Hospital
FBS	fetal blood sampling
FF	fetal fraction
FP	false-positive
FPR	false-positive rate
GS	genome sequencing
HCG	human chorionic gonadotropin
ISCN	International System for Human Cytogenetic Nomenclature
ISUOG	International Society of Ultrasound in Obstetrics and Gynecology
LCSH	long contiguous stretches of homozygosity
MKS	Meckel-Gruber syndrome
MoM	multiple of the median
MPGS	massively parallel genomic sequencing
MPSS	massively parallel shotgun sequencing
NB	nasal bone
NPV	negative predictive value

NT	nuchal translucency
PAPP-A	pregnancy associated plasma protein A
PD	prenatal diagnosis
PGD	preimplantation genetic diagnosis
PPV	positive predictive value
PS	prenatal screening
RAT	rare autosomal trisomies
SGB	Simpson-Golabi-Behmel syndrome
SL	susceptibility locus
SNP	single nucleotide polymorphism
TAT	turnaround time
TR	tricuspid regurgitation
T13	trisomy 13
T18	trisomy 18
T21	trisomy 21
TT	Triple test
TUH	Tartu University Hospital
UPD	uniparental disomy
US	ultrasound
VOUS	variant of uncertain clinical significance
β-hCG	beta human chorionic gonadotropin

LIST OF ORIGINAL PUBLICATIONS

1. Ridnõi, K.; Muru, K.; Keernik, M.; Pajusalu, S.; Ustav, E-L.; Tammur, P.; Mölter-Väär, T.; Kahre, T.; Šamarina, U.; Asser, K.; Szirko, F.; Reimand, T.; Õunap, K. A two-year prospective study assessing the performance of fetal chromosomal microarray analysis and next-generation sequencing in high-risk pregnancies. Mol Genet Genomic Med. 2021 Sep 6;e1787. Online ahead of print.
2. Ridnõi, K.; Kurvinen, E.; Pajusalu, S.; Reimand, T.; Õunap, K. Two Consecutive Pregnancies with Simpson-Golabi-Behmel Syndrome Type 1: Case Report and Review of Published Prenatal Cases. Mol Syndromol. 2018 Jul;9(4):205–213.
3. Ridnõi, K.; Šois, M.; Vaidla, E.; Pajusalu, S.; Kelder, L.; Reimand, T.; Õunap, K. (2019). A prenatally diagnosed case of Meckel-Gruber syndrome with novel compound heterozygous pathogenic variants in the TXNDC15 gene. A prenatally diagnosed case of Meckel-Gruber syndrome with novel compound heterozygous pathogenic variants in the TXNDC15 gene. Mol Genet Genomic Med. 2019 May;7(5): e614.
4. Ridnõi, K.; Muru, K.; Ustav, E-L.; Tammur, P.; Möler-Väär, T.; Asser, K.; Szirko, F.; Reimand, T.; Õunap, K. Loote kromosoomhaiguste esimese trimestri sõeluuringu tulemused Eestis kahe aasta jooksul. Eesti Arst (2021), accepted.

My contribution to the original publications:

Publication 1: participation in clinical evaluation of the patients, performing ultrasound examinations, performing invasive procedures. Gathering and analyzing the cFTS data. Participation in gathering and analyzing the data of CMA and NGS results. Writing the manuscript.

Publication 2: clinical and ultrasound examinations of all probands. Participation in pathoanatomical evaluation of the newborns and fetus. Writing the manuscript with the analysis of the published literature.

Publication 3: analysis of ultrasound, clinical and pathoanatomical examinations. Research of the published literature regarding MKS diagnosis. Writing the manuscript.

Publication 4: performing clinical assessment of fetuses in first trimester, assessment of indications for invasive diagnosis, performing invasive diagnostic procedures. Gathering and analyzing the data of all patients and performing statistical analysis. Writing the manuscript.

1. INTRODUCTION

Every pregnant woman has a compound risk of about 4% for carrying a structurally or genetically abnormal fetus [Harper 2010]. The main aim of developing prenatal screening (PS) programs is to identify pregnancies that are at high-risk of being affected by a disease and to offer diagnostic options to these women [ACOG 2020]. By contrast, the technologies used in prenatal diagnosis (PD) are developed with the intention of determining whether a specific chromosomal or genetic disorder is present in the fetus, aiming to allow a definitive diagnosis [ACOG 2016b]. These possibilities are essential for enabling pregnant women to exercise their reproductive rights: making decisions regarding pregnancy termination or expectant management in cases of diagnosed congenital malformation or genetic disease.

Different types of PS for chromosomal disease have been used during the past 40 years; nonetheless, a general shift has occurred towards testing during the first trimester of pregnancy [Nicolaides 2011a]. One of the main changes in the field of PS for aneuploidy was made in 1992, when fetal nuchal translucency (NT) was discovered and its potential diagnostic utility was described by Nicolaides *et al*. [Nicolaides *et al.* 1992]. A model for combined first-trimester risk assessment was introduced in 1999, in which maternal age, serum biochemical markers, free β-chorionic human gonadotropin (β-hCG), and pregnancy-associated plasma protein A (PAPP-A) were used in combination with the sonographic measurement of fetal NT [Spencer *et al.* 1999]. This program achieved a detection rate (DR) for trisomy 21 (T21) of almost 90%; however, the relatively high invasive-testing rate of 5% was a major concern. In the following years, several ultrasound (US) markers were introduced into combined first-trimester risk assessment including absence of the fetal nasal bone (NB) [Cicero *et al.* 2001], tricuspid valve regurgitation (TR) [Faiola *et al.* 2005], and the ductus venosus (DV) flow pattern [Matias *et al.* 1998]. A large multicenter study of 75,821 pregnancies showed that by using a combination of maternal age, serum biochemistry, fetal NT, and additional sonographic markers in a specifically defined group of women, it was possible to increase the T21 DR to over 90% and to reduce the false-positive rate (FPR) to below 3% [Nicolaides *et al.* 2005].

In Estonia, the use of PS was initially started in 1995, and the main known risk factor at that time was maternal age. In 1999, with the support of the Estonian Health Insurance Fund (EHIF), a national program of PS was implemented, based on second-trimester serum markers, which was known as the triple test (TT). However, further investigations were needed before fully converting to combined first-trimester screening (cFTS), and new PS and US diagnostics guideline was implemented in 2016 [Ustav *et al.* 2016].

Subsequent developments in PS required new technological solutions. The presence of fetal DNA in maternal plasma was described for the first time in 1997 [Lo *et al.* 1997]. This initiated a new era of non-invasive prenatal testing

(NIPT) in the field of PS. Large-scale validation studies based on the sequencing of cell-free fetal DNA (cff-DNA) were published almost ten years ago, in which the T21 DR was almost 100% with low false-positivity [Bianchi *et al.* 2012; Palomaki *et al.* 2011]. In Estonia, a local NIPT assay was recently developed and validated [Žilina *et al.* 2019].

The next step after a high-risk result from PS is definitive diagnosis: confirmation or exclusion of chromosomal disease. The gold standard for whole-chromosome aneuploidy has been karyotyping of fetal cells that are acquired via an invasive diagnostic procedure. Conventional karyotyping remained the first-tier diagnostic test for chromosomal disease for a long period. However, a major problem with this method was its low (5–10 Mb) resolution, which did not allow the diagnosis of submicroscopic rearrangements.

Chromosomal microarray analysis (CMA) or 'molecular karyotyping' is a DNA-based technology that detects genome-wide DNA losses or gains, copy-number variants (CNV), at a 100-fold higher resolution than karyotyping [Shearer *et al.* 2007]. Debates remain over the universal use of CMA in PD. The American College of Obstetricians and Gynecologists (ACOG) stated in 2013 that CMA should be used in PD as a first-tier diagnostic test in fetuses with US anomalies [ACOG 2013]. However, the incidence of pathogenic CNVs in the prenatal setting can be as high as 1 in 270 pregnancies and is not dependent on maternal age [Srebniak *et al.* 2018]. This is concerning in the present era of NIPT, due to possible underdiagnoses of clinically relevant submicroscopic chromosomal anomalies. In Estonia, CMA has been funded by the EHIF since 2011. Its clinical utility has been investigated mostly in the pediatric population, with a diagnostic yield of 25% [Žilina *et al.* 2014].

One of the greatest challenges in PD is to reach a definitive diagnosis in cases of fetal congenital anomalies that are discovered by US examination. CMA can identify disease-related alterations at the chromosomal level in about 27.4% of such cases [Fiorentino *et al.* 2013]. Thus, the majority of these fetuses do not receive a diagnosis [Monaghan *et al.* 2020]. The main difficulty in these cases is establishing phenotype–genotype correlation. Next-generation sequencing (NGS) methods in PD range from targeted sequencing with phenotype-specific gene panels, to large-scale gene panels, exome sequencing (ES), and even genome sequencing (GS) [Ferretti *et al.* 2019]. Several studies have shown that additional diagnostic information can be found in between 8.5% and 81% of these cases [Chandler *et al.* 2018; Lord *et al.* 2019]. In Estonia, NGS diagnostic performance has been evaluated in a pediatric and adult population, and a diagnostic yield of 26.3% was found in a cohort of 501 probands [Pajusalu *et al.* 2018].

The aim of this doctoral thesis was, firstly, to establish the diagnostic effectiveness of the new strategies for PS and PD of chromosomal diseases in a large cohort of pregnancies, representative of the whole population. For that purpose, a standardized cFTS protocol was applied and CMA performed in high-risk cases. Secondly, this thesis focused on using NGS in PD in a selected group of fetuses with congenital malformations.

2. LITERATURE REVIEW

2.1. General principles of prenatal screening and diagnosis

Screening is the systematic application of a test or inquiry to identify those individuals at sufficient risk of a specific disorder to benefit from further investigation or direct preventive action, among persons who have not sought medical attention on account of symptoms of that disorder [Wald 2006]. The foundation of prenatal screening (PS) for any disease process requires a fundamental understanding of the differences between diagnostic and screening tests. Diagnostic tests are often expensive, invasive and performed only in those individuals, who are believed to be "at-risk". Screening tests on the other hand should be cheap, easy to use and widely applicable for the population [Evans *et al.* 2005]. Criteria for worthwhile screening programs include a well-defined medically important disorder with known prevalence and tests that are cost-effective, safe, accessible, and have well-defined performance [Benn 2002]. Four key measures are used in the evaluation of screening tests: sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) [Evans *et al.* 2005].

Due to the above-mentioned reasons, PS was initially focused on the identification of pregnancies, which are at higher risk for being affected with T21 due to its relatively high prevalence in the population, severity of the disease and its importance in terms of public health costs. Recent decades have seen the development of PS approaches from using multiple biochemical and ultrasound markers in the first and second trimesters to analyzing cff-DNA in the maternal plasma [Kagan *et al.* 2017; Rink and Norton 2016]. These developments now give the opportunity to screen prenatally for broader variety of chromosomal disease.

However, as understanding of the molecular basis of many genetic conditions has increased, so have the opportunities for prenatal diagnosis (PD) of a wide range of disorders [Skirton *et al.* 2014]. Prenatal genetic testing has evolved considerably over the past decades, and new tests are being introduced into the prenatal setting at a very rapid pace [Dukhovny and Norton 2018]. In 2021 we can already discuss not only non-invasive aneuploidy screening, but even non-invasive PD for monogenic disorders [Scotchman *et al.* 2020]. These possibilities are very promising, but can be confusing not only for the patients, but for the medical professionals as well.

The goals of PD and PS from a public health perspective may be different from the goals of an individual patient. In public health model, the basic aim of prenatal testing is to reduce the frequency of select birth defects, improving population-level health along with reducing the burden of disease on society. The reproductive autonomy model is focused on providing women with crucial information that can help them make important reproductive decisions, such as whether to continue the pregnancy [Begovic 2019]. Women choose PD methods

and weigh the outcomes of prenatal genetic testing quite differently [Kuppermann *et al.* 2016]. For some patients, a PD of a genetic disease may mean choosing to terminate the pregnancy, while for others this may mean preparing for the birth of an infant with medical needs [Dukhovny and Norton 2018].

The latest recommendations to date on PS for chromosomal abnormalities are clearly stating that possibilities of PS and PD should be discussed with every woman, regardless of age and her ´a priori´ risk for chromosomal disease [ACOG 2020].

Further, in this literature review we will discuss different methods, used in PS for chromosomal anomalies as well as molecular analyses, used for PD of chromosomal or genetic disease.

2.2. Combined first-trimester screening

Antenatal care has developed dramatically over the past 30 years. The initial approach of simple observation and management of pregnancy complications as they appear has undergone a paradigm shift to the new pyramid of care, in which attention focuses on the first trimester to select cases at high-risk of aneuploidy and to predict complications [Nicolaides 2011b].

Down's syndrome (DS), or T21, is the most frequent chromosomal disease at birth, with a prevalence of 1.17 per 1,000 livebirths in Estonia reported in 2006 [Reimand *et al.* 2006]. Its frequency differs among countries, ranging from 5.03 per 10,000 livebirths in Korea to 12.6 per 10,000 livebirths in the USA [de Graaf *et al.* 2015; Park *et al.* 2019]. During the last 10 years, the incidence of DS livebirths has decreased to less than five cases per year in Estonia (Department of Clinical Genetics, Tartu University Hospital, unpublished records). The major known risk factor for T21 is advanced maternal age and its incidence increases markedly after the age of 35 [Hook 1981]. Initial estimates of the maternal age-specific live-birth prevalence of Down's syndrome were revised using predictive models based on a large dataset and corrected in later publications [Morris *et al.* 2005] (Table 1).

Table 1. Predicted odds of DS live birth by maternal age. Adapted from [Morris *et al.* 2005].

Maternal age at birth (years)	Predicted odds
20	1:1476
30	1:938
35	1:352
40	1:85
45	1:35

While the prevalence of T21 increases with maternal age, it decreases with gestational age due to spontaneous abortions of fetuses with DS [Savva *et al.* 2006]. At 12 weeks of pregnancy, the prevalence of T21 is 30% higher than at 40 weeks [Snijders *et al.* 1999]. Therefore, maternal and gestational age-specific risks should be used in the estimation of a-priori risk for chromosomal disease during PS [Snijders *et al.* 1994]. These estimates from 1994 are still valid and used in the risk calculations.

Prenatal aneuploidy screening tests have been developed to identify pregnancies that are at a high-risk of DS and to offer diagnostic procedures to this particular group of women [ACOG 2020]. Developments in the field of PS were necessary to increase the DR of T21 and to ensure that invasive diagnostics were offered to as few women as possible, due to the potential for fetal loss after the procedure, although such losses are relatively rare [Akolekar *et al.* 2015; Martins *et al.* 2020; Wulff *et al.* 2016]. Screening by maternal age and second-trimester US examination alone is not highly effective, detecting no more than 68% of DS cases. Moreover, in women younger than 35 years, the T21 DR is only 53% using this strategy [Howe *et al.* 2000]. PS on the basis of maternal age alone is therefore not recommended [Benn *et al.* 2015]. Different approaches have been used across European countries for PS for chromosomal diseases including combinations of maternal age, US examinations, and serum markers in the first and second trimesters [Boyd *et al.* 2008]. These national strategies have shown different performances. The Estonian national PS program, TT, using the second-trimester serum markers alfa-fetoprotein (AFP), total HCG, and unconjugated estriol, was first implemented in 1998 [Sitska *et al.* 2008]. Initial reports of the TT screening model in the early 1990s showed a good uptake of 74%, but a relatively low DR of T21 of 48% with an FPR of 4.1% [Wald *et al.* 1992]. This method was quickly adopted in our small population, and had reached a coverage of over 90% of all pregnant women by 2006. However, major problems remained with the strategy in terms of the high proportion of false-positive results (4.7%) and the relatively low DR of T21 (57.8%) [Sitska *et al.* 2008].

Clinical and laboratory research eventually started to focus on the first trimester of pregnancy, due to the relatively poor performance of second-trimester serum screening, the developments in research into new serum markers, and discoveries of the diagnostic value of first-trimester US scans.

2.2.1. NT and first-trimester serum markers

In 1992, the discovery of an association between the fetal NT thickness and trisomies by Nicolaides *et al*. marked the beginning of a new era in the field of PS for chromosomal diseases [Nicolaides *et al.* 1992]. NT is the subcutaneous accumulation of lymphatic fluid behind the fetal neck, which can be effectively measured during US examination between 11 and 14 weeks of gestation (Figure 1). Measurements of the NT thickness made by well-trained operators were

shown to be highly reproducible [Pandya *et al.* 1995]. Guidelines for the measurement of NT have developed over time due to increases in the quality of US probes and are freely available on the Fetal Medicine Foundation (FMF) website (Appendix 1).

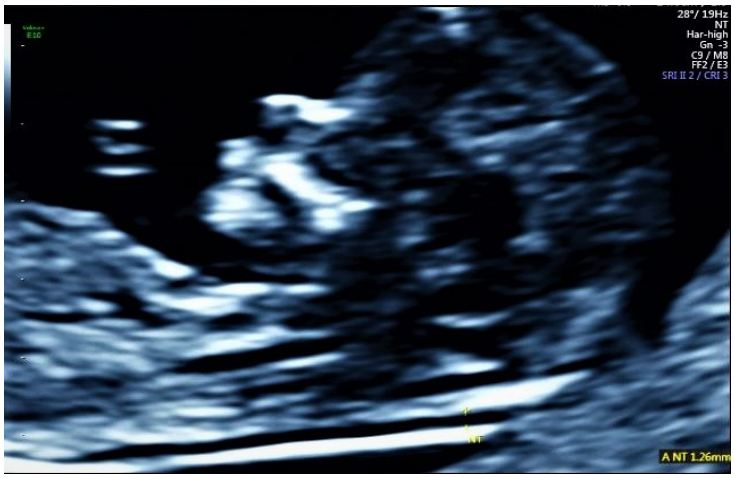

Figure 1. Sonographic measurement of NT thickness following the FMF rules in the mid-sagittal plane of the fetal head (from an unpublished personal archive).

Such a strict protocol and external quality assessment was necessary, because failure to perform a good quality scan results in a much poorer performance for screening, as was shown in a French population-based study [Fries *et al.* 2018]. For this reason, all US operators should have annual recertification by a FMF reviewer online. Some attempts have been made to measure NT-thickness automatically, but this method is not currently widely accepted [Sciortino *et al.* 2017].

Fetal NT thickness is the single US marker that is highly associated with T21 and all other major chromosomal diseases [Kagan *et al.* 2006]. When the risk of T21 was calculated from the maternal age and gestational-age-related prevalence, multiplied by the likelihood ratio depending on the deviation from normal NT thickness for the crown-rump length (CRL), almost 80% of cases could be detected prenatally [Snijders *et al.* 1998]. In this large study, the screen-positive group comprised 8.3% of all participants and the risk cut-off point for invasive testing was 1 in 300.

There were originally two approaches to quantifying NT deviation from the normal median: subtraction of the normal median from the NT measurement to produce a deviation in millimeters, referred as the delta NT; or division of the NT by the normal median to produce a multiple of the median (MoM) value [Spencer *et al.* 2003]. Furthermore, a study by Wright *et al*. revealed that NT

distribution followed two patterns, namely CRL-dependent and CRL-independent, which differed in normal and chromosomally abnormal fetuses [Wright *et al.* 2008]. They showed that 95% of fetuses affected by T21 followed a CRL-independent distribution of NT thickness. By contrast, 95% of chromosomally normal fetuses followed a CRL-dependent distribution of NT [Wright *et al.* 2008].

Running in parallel to the developments in US diagnostics was a search for first-trimester biochemical markers. Of the several serum markers that have been investigated, only two have shown clinical usefulness in terms of detection of fetal trisomy: free β-hCG [Spencer *et al.* 1992a; Spencer *et al.* 1992b] and PAPP-A [Wald *et al.* 1996]. Initial attempts to develop a combined screening program using fetal NT and first-trimester serum markers have shown that at a fixed FPR of 5%, the DR for T21 could be about 90% [Spencer *et al.* 1999]. This original methodology was developed further over the subsequent 10 years, due to ongoing research aiming to increase the performance of screening.

Research has shown that to accurately estimate the MoMs of both these biochemical markers, it is essential to adjust their values according to several maternal and pregnancy characteristics, including gestational age, ethnicity, smoking status, maternal weight, and type of conception [Kagan *et al.* 2008].

2.2.2. First-trimester additional US markers for chromosomal disease

In terms of the US element of cFTS, further developments have involved the inclusion of additional markers in the risk calculation with the aim of better delineating the risk of chromosomal disease and thereby lowering the FPR. These US markers are as follows: presence or absence of fetal nasal bone (NB) (Figure 2); tricuspid valve blood-flow pattern (Figure 3); and DV-flow assessment (Figure 4). The same strict standards for the performance techniques are applied to each of these markers with annual certification for the operators [Sonek and Nicolaides 2010].

The inclusion of additional US markers into cFTS is possible in different ways. The first large-scale study by Nicolaides *et al*. of almost 76,000 pregnancies used a contingent model with additional sonographic markers. After initial cFTS, using a standard combination of maternal age, NT thickness, and serum markers, the women were divided into three groups according to the T21 risk level. If the risk was high, which was defined as greater than 1 in 50, diagnostic invasive testing was offered. If the risk was low, defined as less than 1 in 1,000, no further testing was needed. If the risk was deemed intermediate, defined as a combined value ranging from 1 in 51 to 1 in 1,000, assessment of additional US markers was offered and the risk was then recalculated. As a result, only about 15% of women needed two-step US evaluation, the DR for T21 exceeded 90%, and the FPR was about 3% [Nicolaides *et al.* 2005].

Different strategies to increase the effectiveness of cFTS were evaluated in terms of the performance of each marker [Kagan *et al.* 2009a; Kagan *et al.* 2009c; Maiz *et al.* 2009]. All these approaches yielded a DR for T21 of over 90% with a low FPR of 2–3%. The low FPR was achieved by the better estimation of aneuploidy risks through the use of additional markers, due to clear differences in the incidence of their pathological findings between euploid and chromosomally abnormal fetuses (Table 2). The use of all three markers in the first trimester with NT measurement as a screening model can yield the same performance in terms of T21 DR, even without biochemical markers [Abele *et al.* 2015].

The use of additional US markers requires specific skills and the continuous performance of these examinations in everyday practice [Maiz *et al.* 2008; Sonek and Nicolaides 2010]. Nevertheless, the 'final' model of cFTS, which is used nowadays was prospectively validated in 2009 without the use of additional US markers and yielded 90% DR of T21 with FPR of 3% [Kagan *et al.* 2009b].

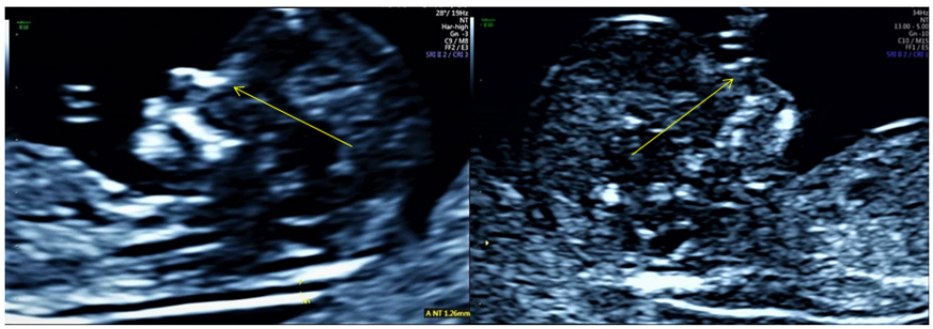

Figure 2. Appearance of the fetal NB. Left: a normal fetus showing the presence of the NB (arrow), which is more echogenic than the nasal skin overlying it. Right: a fetus with T21, in which there no echogenic NB (arrow) is observed in the same diagnostic plane. Images taken from an unpublished personal archive.

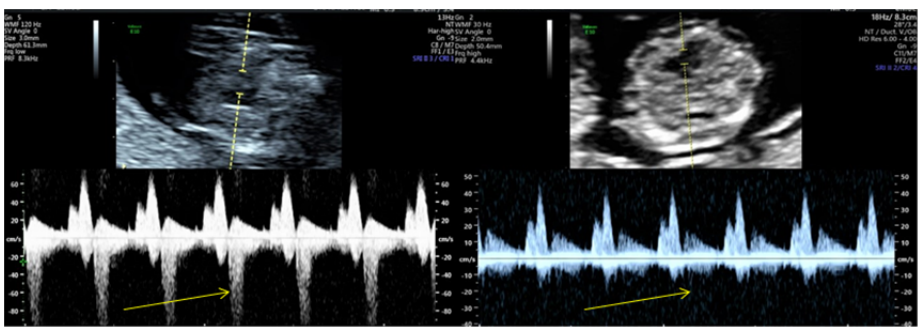

Figure 3. Tricuspid valve blood-flow. Left: a fetus with T21 and TR showing a regurgitation jet of more than 60 cm/s (arrow) during ventricular contraction. Right: a normal fetus showing no reversal in flow (arrow) at the same point in the cardiac cycle. Images taken from an unpublished personal archive.

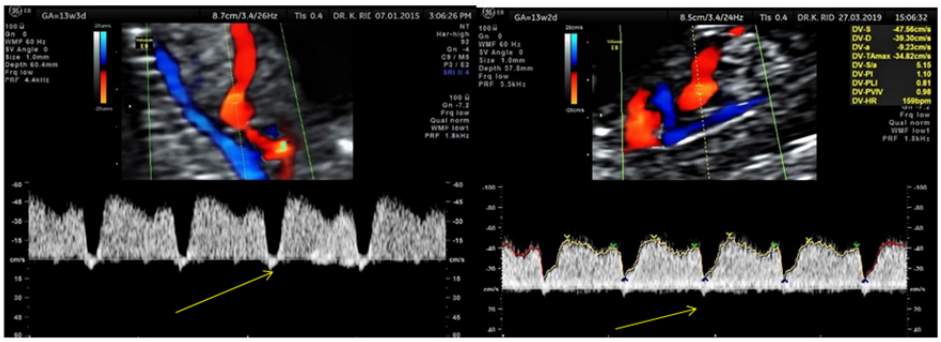

Figure 4. Doppler waveforms of the DV flow pattern. Left: a reversed α-wave (arrow). Right: a normal waveform with a positive α-wave during atrial contraction (arrow). Images taken from an unpublished personal archive.

The main cFTS US, biochemical characteristics and their differences between euploid fetuses and fetuses with common trisomies are summarized in Table 2.

Table 2. Characteristics of euploid and aneuploid fetuses in cFTS. Adapted from [Kagan *et al.* 2017]. Confidence intervals for MoM medians were not available in the review.

Condition	Standard cFTS			Extended cFTS		
	Median NT (mm)	Median free b-hCG (MoM)	Median PAPP-a (MoM)	Hypoplastic NB (%)	Tricuspid regurgitation (%)	Reversed DV flow (%)
Euploid	1.8	1.0	1.0	0.6	0.9	3.2
T21	3.5	2.0	0.5	63	56	66
T18	5.1	0.2	0.2	55	33	58
T13	3.9	0.5	0.3	35	30	55

β-hCG – beta-human chorionic gonadotropin; cFTS – combined first trimester screening; DV – ductus venosus; MoM – multiple of median; NB – nasal bone; NT – nuchal translucency; PAPP-a – pregnancy-associated plasma protein a; T13 – trisomy 13; T18 – trisomy 18; T21 – trisomy 21.

2.2.3. Impact of cFTS in clinical practice

Establishing a systematic protocol in PS programs is essential for providing women with the best antenatal care possible. The impact of introducing cFTS into routine antenatal care remains considerable. A large population-based study conducted in France showed, that the introduction of cFTS in comparison to second-trimester serum screening resulted in a 47% reduction in invasive testing at a national level, due to a two-fold lower screen-positive rate (9.5% versus 4.8%, respectively). The DR for T21 increased by 2.7% [Royere *et al.* 2016]. A very high (97.5%) DR for T21 was reported recently in a Taiwanese population study using cFTS with a risk cut-off point for invasive testing of 1 in 270 [Lan *et al.* 2018]. The first European country to adopt a national T21 screening program was Denmark [Lou *et al.* 2018]. Starting from 2006, cFTS was offered to all pregnant women in Denmark. A Danish population-based study reported that this had a huge impact. The introduction of combined risk assessment during the first trimester at a national level halved the number of infants born with DS. These changes in screening strategy resulted in a considerable decline in the number of invasive procedures that were carried out [Ekelund *et al.* 2008]. According to the Danish Fetal Medicine Database, during the period 2008–2012 the prenatal DR for T21 ranged from 82% to 90% with a maximum screen-positive rate of 4.7% [Ekelund *et al.* 2015].

In Estonia, initial attempts to perform cFTS were undertaken in 2005 in the public healthcare system. Between 2006 and 2016, several PS protocols were used, but there was no clear national policy. A model of contingent screening, which used cFTS as a first-tier test followed by TT, was introduced in the majority of hospitals from 2008. In this method, all pregnant women were offered cFTS if they had attended their first antenatal visit before 13 weeks of gestation. In cases where the combined risk for T21 was higher than 1 in 270 and the trisomy 18 (T18) risk was higher than 1 in 100, an invasive diagnostic procedure was suggested. Integrated TT, which combined second-trimester biochemical markers with NT measurement, was performed as a second-tier test in pregnancies where the combined risk for T21 was between 1 in 270 and 1 in 1,500 and the T18 risk ranged from 1 in 100 to 1 in 400 (Figure 5). Using a variation of this strategy, with a different definition of the intermediate-risk group, the T21 DR increased to 88.3% and the FPR decreased to 3.4% [Muru *et al.* 2010]. The new national prenatal diagnostics guidelines designated cFTS as the primary PS test for all pregnant women in Estonia [Ustav *et al.* 2016].

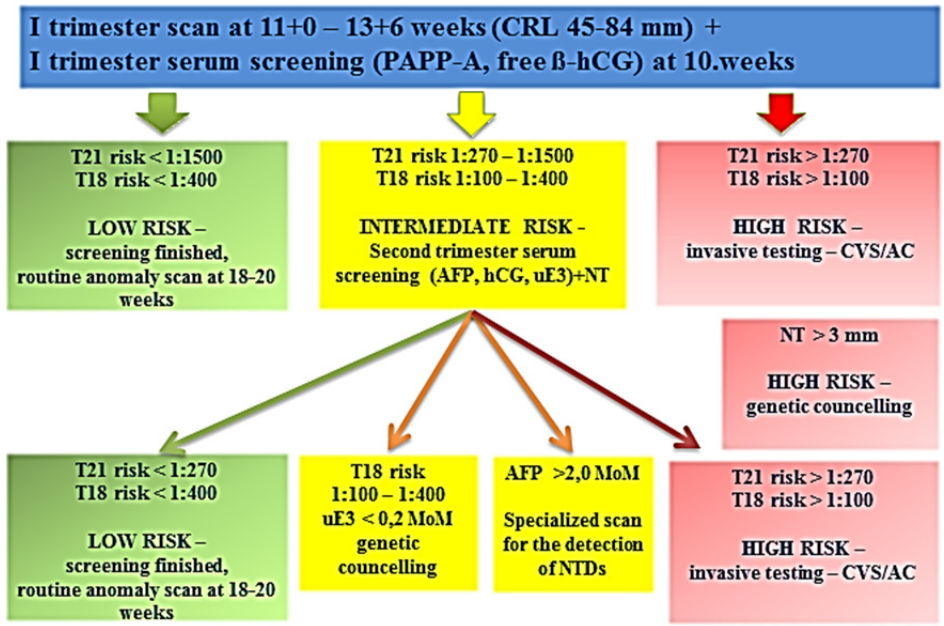

Figure 5. Protocol for PS in Estonia in the years 2008–2015. Adapted from [Muru *et al.* 2010].
AC – amniocentesis; AFP – alfa fetoprotein; β-hCG – beta human chorionic gonadotropin; CRL – crown-rump length; CVS – chorionic villous sampling; hCG – human chorionic gonadotropin; NT – nuchal translucency; NTD – neural tube defect; PAPP-A – pregnancy- associated plasma protein A; T18 – trisomy 18; T21 – trisomy 21; uE3 – unconjugated estriol.

Conventional cFTS had shown effectiveness in the detection of aneuploidy for almost two decades. Some countries have included this method of screening for chromosomal disease as a gold standard in their national health programs [Ekelund *et al.* 2015; Royere *et al.* 2016; Ustav *et al.* 2016]. A T21 DR of 90%, a T18 DR of 95%, and a trisomy 13 (T13) DR of 95% have been reported [Kagan *et al.* 2017]. Despite the relatively high FPR of 3–5% [Kagan *et al.* 2017], conventional cFTS is the most appropriate choice as a first-line screening method for most women in the general obstetric population [ACOG 2015]. This is particularly true in developing countries. A recent retrospective analysis of different contingent screening models in Southwestern China recommended cFTS as the first choice in cases when only traditional PS methods were available. The DR of T21 in this study was 78.8% [Luo *et al.* 2020]. The findings of this analysis were in accordance with the Cochrane review, which also highlighted the effectiveness of cFTS over second-trimester serum markers [Alldred *et al.* 2017].

2.3. Non-invasive prenatal testing

2.3.1. Cell-free fetal DNA

Despite effective cFTS, there is a need for further developments in PS in the direction of new technological solutions. In 1997, the presence of fetal DNA in maternal plasma was described for the first time [Lo *et al.* 1997]. The authors reported that plasma from pregnant women contained cell-free DNA (cf-DNA), including a fraction that is thought to be of placental origin, resulting from apoptosis of the trophoblasts. This fraction was called the 'fetal fraction' (FF) [Lo *et al.* 1997; Lo *et al.* 1998]. Later, it was understood, that 'fetal' cf-DNA was actually of cytotrophoblastic origin in the placenta [Bianchi *et al.* 2012; Faas *et al.* 2012]. The FF of cf-DNA in the maternal plasma is estimated to be about 10–20% of the whole circulating cf-DNA [Lun *et al.* 2008]. It can be detected as early as 4 weeks of gestation [Illanes *et al.* 2007] and is no longer detectable in the maternal plasma shortly after the delivery [Lo *et al.* 1999; Smid *et al.* 2003]. The whole fetal genome is represented in the maternal plasma in small fragments of cf-DNA, approximately 150 base pairs in size [Chan *et al.* 2004; Li *et al.* 2004]. The fact that fetal cf-DNA represents only a small fraction of the total DNA in the maternal plasma, where the main quantity of DNA is contributed by the pregnant woman, offered a great challenge for developing technology that could allow the use of cff-DNA in aneuploidy screening. In 2008, a proof-of-principle study was published, in which massively parallel genomic sequencing (MPGS) of DNA in maternal plasma was used to detect T21. It was suggested that this approach could potentially be applicable to all pregnancies for the purpose of NIPT of aneuploidies [Chiu *et al.* 2008].

2.3.2. NIPT methods

There are several NIPT methods, but the three that have been studied the most and are therefore implemented in clinical practice are massively parallel shotgun sequencing (MPSS), targeted massively parallel sequencing (t-MPS), and single nucleotide polymorphism (SNP) based sequencing [Benn *et al.* 2013]. Brief summary of these methods is given in Table 3.

Table 3. Summary of NIPT methods.

NIPT method	Validation studies	Special features/points to consider
MPSS	[Palomaki *et al.* 2011] [Bianchi *et al.* 2012] [Chiu *et al.* 2011]	- Possible incidental findings, confined to the placenta. - Possible detection of maternal mosaicism or occult malignancies
t-MPS	[Sparks *et al.* 2012] [Ashoor *et al.* 2012b] [Ashoor *et al.* 2013b]	- Possible reduction of cost - Possible quicker turnaround time - Possible lower detection of trisomy 13
SNP-based	[Zimmermann *et al.* 2012] [Nicolaides *et al.* 2013a]	- Distinguishing fetal genotype from maternal - Detection of triploidy - Detection of zigosity in twin pregnancies

The initial approach is based on the identification and counting of large numbers of DNA fragments in maternal plasma. This method utilizes MPSS of millions of fetal and maternal DNA fragments sequenced at the same time after each piece has been mapped to a locus on the chromosome from which it came. If fetal aneuploidy is present, there should be a relative excess or deficit of the quantity of DNA from the chromosome of interest [Chiu *et al.* 2008]. Validation studies using this method were the first in the field of NIPT, and all reported a high DR for T21 of about 97–99% [Bianchi *et al.* 2012; Chiu *et al.* 2011; Palomaki *et al.* 2011].

The targeted approach relies on selectively amplifying only those chromosomal regions that are of clinical interest (that is, chromosomes 21, 18, and 13). The advantage of this method is that it involves considerably less sequencing and therefore gives possible reductions in test costs [Sparks *et al.* 2012]. There have been several validation studies of this method, which have reported over 99% detection of T21 and 97.4–98% of T18 with a very low FPR [Ashoor *et al.* 2012b; Norton *et al.* 2012]. The sensitivity for T13, using this method, was much lower at 63.6%, but the FPR remained good at 0.05% [Ashoor *et al.* 2013b].

An approach for NIPT that uses DNA polymorphisms was demonstrated in 2007 [Dhallan *et al.* 2007]. The authors demonstrated that by using SNPs, it was possible to distinguish fetal DNA from maternal DNA. A validation study taking the different approach of using SNPs was published in 2012. In this work, cff-DNA from maternal blood was isolated, amplified using a multiplex polymerase chain reaction (PCR) assay targeting 11,000 SNPs on chromosomes 13, 18, 21, X, and Y in a single reaction, and sequenced. The authors reported the correct identification of all aneuploidy cases [Zimmermann *et al.* 2012]. The only NIPT platform using SNP-based selective sequencing was validated in 2013 by Nicolaides *et al*. [Nicolaides *et al.* 2013a].

In 2019, an Estonian-based NIPT platform was validated and is now in clinical use under the name of the NIPTIFY® test. This platform uses MPSS methodology with the new bioinformatics tool NIPTmer [Sauk *et al.* 2018], which counts pre-defined per-chromosome sets of unique k-mers from sequencing data. NIPTmer uses less computer resources and is faster than the other available NIPT-tools. NIPTIFY® was validated with 424 samples taken from pregnant women in the local population and proved its readiness to be used in prenatal settings in Estonia by correctly identifying all samples with T21, T18, and T13 [Žilina *et al.* 2019].

2.3.3. Fetal fraction

The FF is the percentage of the total maternal plasma cf-DNA that is of feto-placental origin [FF=fetal cfDNA/(fetal cf-DNA+maternal cf-DNA)][Hui and Bianchi 2020]. The median of the FF at the time of the first-trimester screening has been reported to be 11.4% [Ashoor *et al.* 2012a]. The FF increases with increasing levels of maternal β-hCG and PAPP-A and decreases with higher maternal weight. No correlation between the FF estimates was found in terms of smoking status, racial origin, or maternal age [Ashoor *et al.* 2012a]. A larger study by the same group subsequently showed an association between the FF and smoking status, Afro-Caribbean racial origin, fetal CRL, and T21 karyotype [Ashoor *et al.* 2013a]. Associations between the FF estimates and assisted reproduction [Galeva *et al.* 2019; Lee *et al.* 2018; Revello *et al.* 2016] as well as among twins were also reported [Galeva *et al.* 2019; Zhou *et al.* 2015]. Still, the strongest independent factor affecting the FF in a negative way remains as high maternal body-mass index (BMI) [Hou *et al.* 2019].

Measuring the FF in maternal plasma is an important factor affecting the performance of NIPT, as was highlighted by Canick *et al*. in 2013 [Canick *et al.* 2013]. Some laboratories do not measure the FF in NIPT samples, thereby possibly providing patients with false-negative results [Takoudes and Hamar 2015]. It is believed that measuring the FF should be one of the main quality control factors in future developments of NIPT platforms [Grati 2016]. Still, according to the consensus opinion on standards for reporting NIPT results by experts in the field, no clear guidance on the need to measure the FF was given [Deans *et al.* 2017]. Nevertheless, clinicians must understand the biological influences on the FF to be able to provide post-test counselling and clinical management [Hui and Bianchi 2020].

2.3.4. Screening performance of NIPT for common trisomies

The performance of cff-DNA screening for aneuploidy was extensively investigated in both high-risk and general populations. The largest meta-analysis of NIPT performance to date was based on 35 high-quality studies, in which

different methods of NIPT were used in various obstetric populations, both high-risk and low-risk, in either prospective or retrospective ways [Gil *et al.* 2017]. It was clear that in terms of screening performance, NIPT superseded conventional cFTS. A summary of the NIPT performance for T21, T18, and T13 in singleton pregnancies is shown in Table 4.

Table 4. Performance of cff-DNA testing with weighted pooled DR and FPR for common trisomies. Adapted from [Gil *et al.* 2017].

Condition	DR (%) (95% CI)	FPR (%) (95% CI)
T21	99.7 (99.1–99.9)	0.04 (0.02–0.07)
T18	97.9 (94.9–99.1)	0.04 (0.03–0.07)
T13	99.0 (65.8–100)	0.04 (0.02–0.07)

CI – confidence interval; DR – detection rate; FPR – false-positive rate.

A recent meta-analysis of the performance of NIPT using microarray-based quantification reported similar results: the sensitivity was 99.5% for T21 (95% CI, 96.3–99.9%), 97.7% (95% CI, 87.9–99.6%) for T18, and 100% (95% CI, 83.2–100%) for T13 [Geppert *et al.* 2020].

In terms of aneuploidy screening in twins, there is insufficient data to support the routine use of cff-DNA testing, thus it is not recommended [ACOG 2015]. The latest meta-analysis of NIPT performance in twins states that the performance of cff-DNA testing for T21 in twin pregnancies is similar to that reported in singleton pregnancies and is superior to cFTS performance; however, the number of cases of T18 and T13 are too small to accurately assess the predictive performance of the cff-DNA test [Gil *et al.* 2019]. By contrast, in the largest prospective study to date on the performance of NIPT in twin pregnancies, good results were demonstrated. Khalil *et al*. showed that T21 can be detected in 100% of cases using the IONA test: the pooled estimated DR for T21 in their systematic review was 95% (95% CI, 90–99%) [Khalil *et al.* 2021]. The NIPT performance for T18 and T13 may be less than that of T21 due to the small numbers of affected fetuses in the studies included in the systematic review. It is likely that with more studies to come in the future, a better understanding of NIPT performance in twins will be achieved.

2.3.5. NIPT implementation into clinical practice

The implementation of NIPT into clinical practice has been mainly laboratory driven and all NIPT assays have been commercially developed. Despite the obvious advantages of NIPT over cFTS, the latter remains the first-line screening test for aneuploidy in the general obstetric population [ACOG 2015]. One of the reasons for this is the fact that during first-trimester US examination it is possible to diagnose fetal malformations [Minnella *et al.* 2020; Syngelaki *et al.*

2019] and to predict certain pregnancy complications due to the changes in maternal biomarkers and hemodynamics [Wright *et al.* 2019]. The greatest advantage of NIPT over cFTS is its low FPR; thus, the main expectation from the implementation of NIPT in clinical practice is a reduction of the invasive-testing rate.

Nicolaides *et al*. in 2013 showed with the use of a mathematical model that applying cff-DNA testing in a contingent way after cFTS, the DR of T21 could reach 96.9% with an invasive testing rate of 0.66% [Nicolaides *et al.* 2013b]. Invasive testing carries a very low risk of pregnancy loss [Akolekar *et al.* 2015; Martins *et al.* 2020; Salomon *et al.* 2019; Wulff *et al.* 2016], but still the majority of women would prefer NIPT, at least in some settings [Oepkes *et al.* 2016; Seror *et al.* 2019]. The major question arising from this fact is how to implement NIPT into routine clinical practice.

There are essentially three possibilities for NIPT use in screening for chromosomal disease: routine use for all pregnancies; offering NIPT as a second-tier test only in high-risk groups after cFTS; or a contingent model of NIPT use in cFTS settings [Kagan *et al.* 2017]. The uptake of NIPT varies between different populations. In countries where NIPT is reimbursed by health insurance, the main factors influencing the uptake of NIPT are maternal education, racial origin, pregnancy termination acceptance, level of risk for aneuploidy after cFTS, and possibilities of molecular diagnostics after invasive testing [Gil *et al.* 2015; Lou *et al.* 2018; Miltoft *et al.* 2018].

Offering NIPT only to the group at high-risk after cFTS would lower the invasive-testing rate, while the DR of trisomies would stay at the same level as was shown in the TRIDENT-1 trial [Oepkes *et al.* 2016]. In the TRIDENT-2 study, where NIPT was implemented, as a first-tier screening test for chromosomal disease, the uptake of NIPT was only 42%, and 54% of women still did not participate in the screening program. This study showed a high DR of aneuploidies, but some questions regarding the reporting and managing of incidental findings of NIPT using the MPSS method, have arisen [van der Meij *et al.* 2019]. Latest nationwide NIPT implementation study from Belgium showed a much higher uptake (78.7%) of NIPT as a first-tier PS test. The Belgian approach of managing RATs after MPSS-based NIPT was based on national guidelines [Van Den Bogaert *et al.* 2021]. Some studies conducted in the public healthcare system have shown that the implementation of NIPT is feasible and straightforward in countries with established cFTS programs [Gil *et al.* 2016; Guy *et al.* 2021; Miltoft *et al.* 2018] and can be cost-effective, if applied in a contingent model with cFTS [Colosi *et al.* 2017; Xie *et al.* 2020]. Examples of current use of NIPT in the world are shown in Table 5.

Table 5. Examples of current NIPT use in different regions of the world, adapted from [Gadsboll *et al.* 2020].

Country	NIPT implementation, reimbursement
Denmark	High-risk after cFTS, reimbursed
Iceland	High-risk after cFTS, reimbursed
USA	No national policy, partly-reimbursed
Australia	No national policy, NIPT self-funded
Netherlands	NIPT for all women, partly reimbursed
Belgium	NIPT for all women, reimbursed
France	High-risk after cFTS, reimbursed
UK	High-risk after cFTS, reimbursed
Russian Federation	No national policy, self-funded

A local NIPT assay was recently developed and validated in Estonia, using the MPSS method with a novel bioinformatics algorithm [Žilina *et al.* 2019]. This gives us an opportunity to start using NIPT in clinical settings in our population, but the basis for this initiative is still cFTS. In 2020, the EHIF decided that NIPT will be offered as a second-tier screening test to women with certain indications after cFTS. During the COVID-19 pandemic in 2020, the EHIF in collaboration with the Estonian Gynecologist's Society decided to reimburse NIPT to all pregnant women in Estonia as a primary test for PS of chromosomal disease.

2.3.6. Challenges in clinical use of NIPT

Several factors can influence the performance of NIPT in terms of false-positive or false-negative results, including an insufficient or absent fetal fraction [Ashoor *et al.* 2013a; Takoudes and Hamar 2015], fetoplacental mosaicism [Grati *et al.* 2014], and the presence of a vanishing twin [Gromminger *et al.* 2014]. Furthermore, false-positive NIPT results can rarely be explained by maternal chromosomal mosaicism or maternal malignancies [Bianchi *et al.* 2015; Wang *et al.* 2014]. The origin of cff-DNA is in the external layer of the placenta, the trophoblast, or more accurately in the cytotrophoblast [Faas *et al.* 2012]. The fact that cff-DNA testing targets a mixture of DNA fragments of maternal and fetoplacental origin leads to certain biological limitations; consequently, cff-DNA testing can present both false-positive and false-negative results, and although it is a highly sensitive screening method it is not a diagnostic tool [Grati 2016; Van Opstal *et al.* 2016].

Based on the known data regarding the presence and types of fetoplacental mosaicism, this biological phenomenon could cause both false-positive and false-negative NIPT results [Grati *et al.* 2014; Suzumori *et al.* 2021]. Fetal US examination in the first trimester is still necessary and important in cases of high-risk NIPT results [Salomon *et al.* 2014] and can influence the timing of confirmatory diagnostic procedures depending on the type of aneuploidy

present [Grati *et al.* 2015; Van Opstal and Srebniak 2016]. This is of particular concern for high-risk NIPT results for T18 and T13, in which US evaluation of the fetus is of great importance [Zhen *et al.* 2019a; Zhen *et al.* 2019b]. Recent systematic reviews of discordant NIPT results have summarized the available data highlighting the need for transparent reporting of discordant or failed NIPT results [Hartwig *et al.* 2017; Samura and Okamoto 2020].

Another issue, which concerns only MPSS-based NIPT platforms, is incidental findings of rare autosomal trisomies (RATs). The incidence of such findings can be as frequent as 1 in 835 cases and can be related to poor pregnancy outcome [Scott *et al.* 2018]. However, the majority of such findings are limited to the placenta, but can trigger parental anxiety or unnecessary invasive testing [van der Meij *et al.* 2019]. Reporting such findings is still under debate in terms of pregnancy follow-up and parental counselling. The only RAT that showed a strong association with poor pregnancy outcome was trisomy 16; therefore, this was the only one that should be reported through cff-DNA testing [Grati *et al.* 2020]. Recently, according to leading experts in the field of PS, major concerns regarding MPSS-based NIPT platforms in the universal screening of the population have been raised. They stressed the following points of concern: clinical significance of RAT findings; lack of ability to predict the pregnancy outcome in those cases; and ethical and legal challenges in terms of how to counsel parents before the test, since accurate information is lacking [Jani *et al.* 2020].

Reviewing the available evidence, the recommendations for cff-DNA screening for fetal aneuploidy were detailed in the professional societies' guidelines [ACOG 2015; Benn *et al.* 2015]. Some points to consider in clinical practice are as follows:

- definitive diagnosis of T21 and other chromosomal anomalies can be achieved only by the analysis of fetal cells obtained by CVS or AC;
- cff-DNA testing can be offered in different ways: as a primary test for all women, secondary to a high-risk assessment after cFTS, or in contingent way to a broader group of women ascertained to have high or intermediate risk after conventional screening;
- pregnancy management decisions should not be based on the cff-DNA screening results alone;
- in cases of fetal structural anomaly, diagnostic testing, and not cff-DNA screening, should be offered;
- cff-DNA testing does not screen for fetal structural abnormalities; therefore, US examination of the fetus should be offered to all women;
- routine cff-DNA screening for microdeletion syndromes should not be performed;
- A discussion of the risks, benefits, limitations, and alternatives to the different methods of PS and diagnosis should take place with all patients.

The benefits and harms of screening programs must be carefully considered before implementation. There is clear evidence of excellent performance of NIPT in PS of common trisomies, but expanding the scope of NIPT to the microdeletions and genome-wide assessment of chromosomal imbalances could be premature [Di Renzo *et al.* 2019]. A good example of how NIPT could be implemented as a nationwide first-tier PS test comes from Belgium, which was the first country to implement fully reimbursed NIPT as a first-tier PS test for all pregnant women from 2018. The uptake of NIPT in the study reported by Van Den Bogaert *et el*. was 78.7% [Van Den Bogaert *et al.* 2021], which is almost two-fold higher than in a Dutch population-based study [van der Meij *et al.* 2019]. The authors are discussing in detail the performance of NIPT, the decrease in the invasive testing rate, and the management of RATs with the outcomes of pregnancies in most cases.

2.4. Invasive procedures in prenatal diagnosis

2.4.1. Amniocentesis

During this procedure, a small sample of amniotic fluid is obtained. The procedure is performed transabdominally after completing 15 weeks of pregnancy. An earlier procedure is related to a higher risk of miscarriage [CEMAT 1998]. The first report of AC with chromosomal analysis of cultivated amniocytes was published in 1966 [Steele and Breg 1966]. An evidence-based technique for performing AC has been described in detail in the recent guidelines issued by the International Society of Ultrasound in Obstetrics and Gynecology (ISUOG) [Ghi *et al.* 2016]. The rate of fetal losses is lower if 100 or more procedures are performed annually. Laboratory failure of culturing amniocytes is a very rare event, reported in less than 0.1% of the cases: main reasons for this are advanced gestational age and blood-stained amniotic fluid [Ghi *et al.* 2016].

2.4.2. Chorionic villous sampling

CVS is a procedure through which a sample of trophoblastic cells from the placenta is obtained. There are two ways of performing CVS: the transabdominal or the transcervical route. The only randomized-controlled trial (RCT) regarding the safety of these two methods did not find any difference [Jackson *et al.* 1992]. The choice of the method of CVS should be made by the operator, according to their experience or preference [Young *et al.* 2013]. The rate of fetal losses is lower if 100 or more procedures are performed annually [Ghi *et al.* 2016]. The main issue of CVS in PD is the biological phenomenon of 'chromosomal mosaicism' [Grati *et al.* 2017]. Different types of fetoplacental mosaicism with incidence rates are described. In the largest study to date, 60,347 chorionic samples were investigated and mosaic cases were detected in 2.18%: 87% of these were confined to the placenta and 13% were confined to

the fetus [Malvestiti *et al.* 2015]. Confirmatory AC is therefore a suggested procedure after mosaic findings in CVS samples along with proper counselling regarding the probability of the true fetal mosaicism [Malvestiti *et al.* 2015].

Invasive prenatal testing focuses on each woman's reproductive goals and preferences. Appropriate, nondirective pre-test counseling is essential to explain the benefits, limitations, and risks of these procedures. Indications for prenatal diagnostic testing are usually high-risk results from aneuploidy screening, fetal anomaly, detected on US examination, familiar history or maternal request [Norton and Rink 2016]. Prenatal genetic testing requires direct analysis of fetal tissue. There are three possibilities to obtain fetal cells: chorionic villous sampling (CVS); amniocentesis (AC); and fetal blood sampling (FBS). CVS and AC are the most frequent procedures in PD [Ghi *et al.* 2016].

The main issue with invasive diagnostics during pregnancy is safety. It has been stated for two decades that performing AC carries an additional risk for miscarriage of 1% [Tabor *et al.* 1986]. More recent data from two large systematic reviews show that procedure-related risk is actually much lower, being 0.20–0.22% for CVS and 0.11–0.30% for AC [Akolekar *et al.* 2015; Martins *et al.* 2020; Salomon *et al.* 2019; Wulff *et al.* 2016]. In the latest systematic review on the safety of invasive procedures it is clear that CVS might actually be a safer procedure than AC [Salomon *et al.* 2019].The decision to undergo invasive diagnostics should be made only by the patient. Women weigh-up the risk of miscarriage against the possible diagnostic information widely, but tend to choose non-invasive options [Kuppermann *et al.* 2014]. However, in a recent randomized controlled trial on the influence of prenatal counselling (the INVASIVE trial), it was shown that after extensive prenatal counselling women were 32% more likely to choose invasive diagnostics over other PS possibilities [Paz *et al.* 2020]. The decline in the invasive-testing rate raises some concerns in term of its effect on education, training, and the maintenance of clinical competence in PD [Rose and Eller 2014]. In the era of NIPT, the decline in the invasive-procedure rate is universally appreciated. It has been shown with the use of mathematical models that the invasive-testing rate can be as low as 0.66% after cFTS [Nicolaides *et al.* 2013b]. In terms of traditional indications for PD, such as advanced maternal age or high-risk after cFTS, such a decline is justified given the high DR and low FPR of NIPT for common trisomies [Gil *et al.* 2017; Gil *et al.* 2014]. However, with the developments in molecular PD, indications for invasive testing as well as methods used in genetic diagnosis have also evolved [Norton and Rink 2016]. Typical changes in indications for invasive procedures and in the amount of procedures performed are most clearly seen in a recent study from Belgium, where NIPT testing was implemented as a nationwide first-tier PS test [Van Den Bogaert *et al.* 2021]. An increase in the indications for PD such as fetal anomalies or single-gene disorder testing have been reported [Awomolo *et al.* 2018]. Given the outcomes of the INVASIVE trial, we can expect that more women will choose invasive PD over traditional PS options [Paz *et al.* 2020]. Women should be reassured that invasive pro-

cedures carried out by an experienced operator are not associated with a significant increase in miscarriage rate [Salomon *et al.* 2019].

There are several possibilities for PD after invasive procedure. Prenatal samples, acquired after CVS or AC, can be used for rapid aneuploidy detection or can be cultured for karyotyping. In cases, where CMA and NGS analysis are required fetal DNA can be extracted either directly for the sample or from the cell culture. General pathway of PD after invasive procedure is shown in Figure 6.

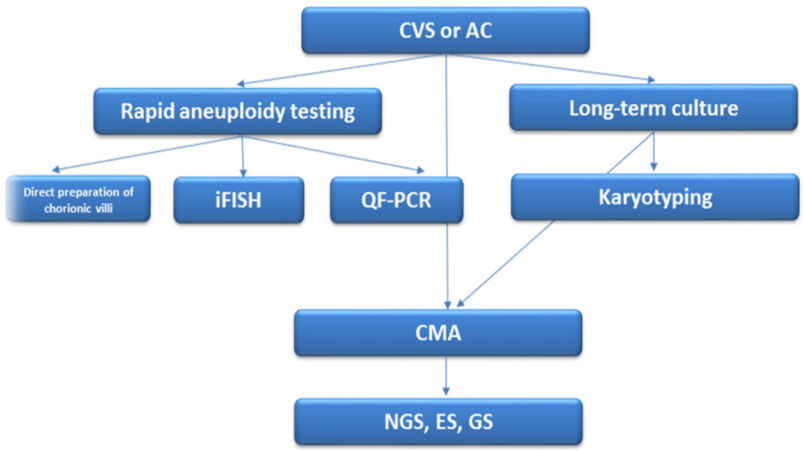

Figure 6. General possibilities for analysis of prenatal sample.
AC – amniocentesis; CMA – chromosomal microarray analysis; CVS – chorionic villous sampling; ES – exome sequencing; GS – genome sequencing; iFISH – interphase fluorescence in situ hybridization; NGS – next-generation sequencing; QF-PCR – quantitative fluorescent polymerase chain reaction

2.5. Chromosomal microarray analysis in prenatal diagnostics

2.5.1. Implementation of CMA in prenatal diagnosis. Indications for CMA

The aim of prenatal diagnostic tests is to reach a definitive diagnosis in each case that is referred for evaluation [ACOG 2016b]. Accurate genetic PD is essential in providing parents with better counseling about the prognosis of the child after birth or specifying the recurrence risk in cases of lethal anomaly and pregnancy termination.

Indications for PD may differ between countries, but generally can be divided into the following groups (adapted from [Silva *et al.* 2019]):

- High-risk for aneuploidy after cFTS or NIPT;
- Fetal anomaly detected on US examination;
- Previous pregnancy or birth with a chromosome abnormality;
- Parental chromosomal rearrangements;
- Possible fetal mosaicism on prior prenatal study;
- Familial history of monogenic disorders.

Despite clear PD indications, and considering all possible relevant information on fetal health, invasive diagnostic testing for aneuploidy should be available to all women, regardless of maternal age [ACOG 2007].

PD of chromosome abnormalities was introduced in 1966, when karyotyping of fetal amniocytes was performed [Steele and Breg 1966]. Conventional G-band karyotyping has been used for over 40 years as a gold standard in cytogenetics [Wou *et al.* 2016]. The diagnostic resolution of karyotyping is about 5–10 million base pairs, which does not allow the detection of smaller chromosomal lesions: microdeletions and microduplications [Silva *et al.* 2019]. CMA or 'molecular karyotyping' is a DNA-based technology, which detects genome-wide DNA losses or gains, CNV, at a 100-fold higher resolution than karyotyping [Kin Chau and Choy 2021; Shearer *et al.* 2007]. CMA was initially introduced into clinical practice as a first-tier test for the postnatal diagnostic evaluation of individuals with unexplained developmental delay/intellectual disability, autism spectrum disorders, or multiple congenital anomalies. Compared to conventional karyotyping, the additional diagnostic yield of CMA with these indications was 10–15% [Miller *et al.* 2010]. Nevertheless, at that point in time the routine use of CMA in PD was under debate and the use of CMA as a substitute for conventional karyotyping was not recommended [Novelli *et al.* 2012]. There were several reasons for that: interpretation difficulties with CMA results in cases of normal fetal US; lack of experienced cytogeneticists to interpret results in the prenatal setting; and the high cost of CMA analysis due to the frequent necessity to examine both the fetus and the parents [Vetro *et al.* 2012].

In 2012, Wapner *et al*. published a large study comparing karyotype with CMA diagnostic performance in prenatal settings. This prospective blinded

study of 4,406 pregnancies demonstrated an incremental diagnostic yield of 6.0% in cases with fetal anomalies and 1.7% for all other indications [Wapner *et al.* 2012]. These findings triggered further research, and meta-analysis of this period states that it was likely that CMA would replace karyotyping in high-risk pregnancies (cases with US anomalies). CMA DR of chromosomal disease over karyotype was found to be 7% (95% CI, 5–10%) [Hillman *et al.* 2013]. Considering these data, the ACOG issued a statement in which CMA was recommended as a first-tier test for PD in cases with fetal US anomalies, fetal demise, or stillbirth [ACOG 2016a]. It was also suggested that either karyotype or CMA was appropriate for prenatal testing in women with structurally normal fetuses, but no clear statement for choosing one method over the other was given. One of the main arguments against the routine prenatal use of CMA was the possibility of finding CNV of uncertain clinical significance (VOUS), which may complicate further counselling and decision-making processes regarding pregnancy follow-up [Lou *et al.* 2020; Richardson and Ormond 2018; Vetro *et al.* 2012]. Nevertheless, an approach for the implementation of CMA as a first-tier cytogenetic test in PD for cases without US anomalies had already been proposed in 2013 [Srebniak *et al.* 2013]. In Estonia, CMA has been funded by the EHIF since 2011. It is used as a first-tier diagnostic test if one of the following indications is present [Ustav *et al.* 2016]:

- NT greater than 3.5 mm;
- Fetal US abnormality;
- Family history or genetic referral;
- Findings in karyotype requiring submicroscopic evaluation.

2.5.2. CMA technology in prenatal settings

There are two main types of CMA performed currently in prenatal and postnatal settings: comparative genomic hybridization (CGH) and SNP microarrays [Kin Chau and Choy 2021; Stosic *et al.* 2018; Wou *et al.* 2016].

In the CGH approach, a patient's DNA is compared to a reference genome DNA sample to identify regions of under- or over-representation. Two DNA samples are labelled in different colors, using fluorescent dyes; the information about losses and gains of DNA is therefore derived by calculation of the difference in intensity of the fluorescent color signals for each probe. The normal copy number represents an equal intensity between the sample and reference DNA. A higher intensity of sample DNA over the reference would represent a gain; a lower intensity would mean a loss in the sample DNA [Karampetsou *et al.* 2014].

SNP array platforms are another approach. They do not use a comparative reference sample, but rather determine the patient's genotype in specific areas of the genome that are highly polymorphic among individuals. These differences are referred to as SNPs. In this way, absent or duplicated stretches of DNA can be identified [Stosic *et al.* 2018]. An additional value of SNP-array techno-

logy is the ability to detect uniparental disomy (UPD), mosaicism, zygosity, maternal cell contamination, and possible consanguinity. Triploidy can also be detected by SNP arrays [Levy and Wapner 2018]. This CMA technology has been used in Estonia since 2009.

2.5.3. Reporting of CMA findings

Chromosomal abnormalities observed on karyotype are generally associated with major clinical findings, due to the fact, that aberrations visible by a microscope involve hundreds to thousands of genes. The CMA ability to detect small submicroscopic lesions may be appealing for parents in terms of getting more diagnostic information; however, it can constitute a great challenge in PD, especially in cases where CNV has been found and can have a wide-ranging phenotype [Richardson and Ormond 2018].

The American College of Medical Genetics (ACMG), in joint consensus with the Association for Molecular Pathology, published guidelines for the interpretation of sequence variants in 2015. It was proposed that CVNs should be classified into the following five groups based on the degree of likelihood of pathogenicity [Richards *et al.* 2015]:

- benign, Class 1;
- likely benign, Class 2;
- uncertain clinical significance, Class 3;
- likely pathogenic, Class 4;
- pathogenic, Class 5.

This classification was based on CNV-frequency differences between affected individuals and the general population [de Leeuw *et al.* 2012]. A CMA report should consist of the following parts [Kearney *et al.* 2011]:

- cytogenetic location (chromosome and bands);
- CNV category (loss or gain of DNA);
- size and coordinates within genome;
- classification of significance (five-class system);
- genes involved in CNV, with specifications of disease-related genes, if applicable;
- recommendations for clinical follow-up.

Recently, the ACMG updated their technical standards for the interpretation of CNVs, introducing a quantitative scoring framework, encouraging the implementation of the five-tier system into the interpretation of CMA results, as described above [Riggs *et al.* 2020].

Different approach to the classification of array findings was proposed by Srebniak *et al*. in 2014, with a more detailed subcategorization of clinically relevant CNVs into causative array findings, variants in susceptibility loci (SL), and unexpected diagnoses [Srebniak *et al.* 2014]. Another, more simplified

approach was suggested in 2017 for clinical reporting in regards to classification of CNVs [Nowakowska 2017].

A major challenge in the use of CMA in prenatal cases is the interpretation of VOUS findings, especially in cases with a structurally normal fetus [Daum *et al.* 2021; Mardy *et al.* 2020; Vetro *et al.* 2012]. Several public databases provide helpful tools to facilitate the accuracy of CMA reporting, including the Database of Genomic Variants (DGV), the Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources (DECIPHER), the European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations (ECARUCA), and the Database of genomic variation and its relationship to human health (ClinVar). These collect data from healthy individuals as well as from subjects with congenital anomalies or developmental delay [de Leeuw *et al.* 2012].

The incidence of VOUS varies among published studies. Initially an incidence of 1.5% was reported [Wapner *et al.* 2012], which was confirmed by later meta-analysis, stating that the VOUS rate, with all indications considered, was about 1.4% [Hillman *et al.* 2013]. In a recent population-based study, with CMA being the primary test for all indications, a higher incidence of VOUS of 5.6% was reported [Muys *et al.* 2018]. Different incidences of VOUS can be explained by several factors, including differences in reporting guidelines, the CMA platform used, whole-genome versus targeted CMA, choices of CMA resolution, and ongoing research in terms of describing associations of CNVs with different phenotypes [Karampetsou *et al.* 2014; Levy and Wapner 2018; Srebniak *et al.* 2013; Vanakker *et al.* 2014]. In a recent study, focusing on VOUS findings, after performing CMA for various indications, the overall rate was 5.8%, which showed a decrease over time. The authors have suggested a classification of VOUS findings depending on the likelihood of an affected phenotype [Mardy *et al.* 2020].

The key-role of successful reporting of CMA findings is in the pre- and post-test counseling of patients. Pre-test counseling should be focused on the diagnostic options available, the patient's perception of risks and benefits, and their attitudes towards parenting a child with disabilities [Fonda Allen *et al.* 2016]. It should also include a discussion of the CMA potential to discover consanguinity or non-paternity [Dugoff *et al.* 2016]. The patient should understand what types of finding in terms of disease association that CMA can detect: early-onset severe conditions, autism-related findings, schizophrenia, and other late-onset conditions. These findings may not be related to the indication for the initial testing [Stosic *et al.* 2018]. Finally, patients should understand that CMA cannot exclude all genetic conditions or congenital anomalies. When counselled in terms of PD possibilities, most women choose to get maximal information about their fetuses, but many wish to avoid uncertain results [Hochner *et al.* 2020; Millo *et al.* 2021]. The post-test counseling should be done by an expert who has access to the databases that provide updated information concerning genotype–phenotype correlations [Dugoff *et al.* 2016]. Healthcare professionals should also bear in mind that parents' perception and interpretation of CMA

findings and their ability to manage their uncertainty may vary [Lou *et al.* 2020]. Understanding the genetic bases of disease, which could be identified with CMA, is also important for the providers of counselling, especially in cases where healthcare professionals do not have special training in genetics, because attitudes towards the discussion of CMA results can be different [Hui *et al.* 2020].

2.5.4. Diagnostic performance of CMA in different groups of prenatal indications

CMA has now become the first-tier prenatal diagnostic test in following-up the pregnancies, where fetal structural anomalies were identified on US examination [Oneda and Rauch 2017]. Furthermore, recent data support the use of CMA in cases with fetal growth restriction (FGR), where additional diagnostic information can be found in 4% of structurally normal fetuses and in 10% of FGR fetuses with US malformations [Borrell *et al.* 2018].

The diagnostic yield of CMA in the group of fetuses with US anomalies varies between studies from 3.0% to 7.3% [Chong *et al.* 2019; Hillman *et al.* 2013; Hui *et al.* 2021; Srebniak *et al.* 2016; Wapner *et al.* 2012]. These differences can be explained by types of fetal anomaly, choice of CMA platform, and resolution [de Wit *et al.* 2014; Shaffer *et al.* 2012b]. The most common fetal malformations associated with CNVs are cardiac, renal, skeletal, and central nervous system (CNS) related [Chong *et al.* 2019; de Wit *et al.* 2014; Hillman *et al.* 2013; Hui *et al.* 2021; Muys *et al.* 2018; Shaffer *et al.* 2012b].

Congenital heart disease (CHD) is the most common birth defect in the world, with 1.35 million newborn cases every year. The reported total CHD birth prevalence in Europe is higher than that in North America at 8.2 per 1,000 live births vs. 6.9 per 1,000 live births [van der Linde *et al.* 2011]. The overall incidence of chromosomal disease detectable with CMA in this group of fetuses is as high as 22.1%. Numerical chromosomal abnormalities, mostly T21, T18 and T13, are found in 10.8% of cases [Wang *et al.* 2018]. An incremental yield of CMA over karyotype of 12% in fetuses with CHD was reported in a large systematic review [Jansen *et al.* 2015]. Similar results were found in a recent French retrospective nation-wide study with a CMA diagnostic yield of 10.4% [Hureaux *et al.* 2019]. The most frequent pathogenic CNV, found in fetuses with CHD, is a 22q11.2 microdeletion, which is accountable for up to 40% of the cases [Chong *et al.* 2019; Hureaux *et al.* 2019]. The incidence of 22q11.2 microdeletion is even higher in those with non-isolated CHD, especially in fetuses with oral clefts, where this particular CNV is found in 100% of cases [Wang *et al.* 2018]. This evidence clearly supports the fact that conventional karyotyping alone is not enough in the PD of CHD. The only exception is probably isolated ventricular septum defects, where the prevalence of abnormal chromosomal findings is only 0.7% [Vedel *et al.* 2021].

CNS anomalies are diverse in terms of etiology, difficult to diagnose prenatally, and therefore complex to handle [Van den Veyver 2019]. It was reported that the incidence of clinically relevant findings in a group of fetuses with CNS defects after CMA was 8.6%, being highest in the subgroup with posterior fossa and cerebellar anomalies (13.8–19.6%) [Shaffer *et al.* 2012b]. Similar results were found in a study of 46 fetuses with CNS anomalies, identifying pathogenic CNVs in 10.9% of the cases, and also highlighting Dandy–Walker syndrome (33.3%) and holoprosencephaly (28.6%) [Sun *et al.* 2015]. In a study of 77 pregnancies affected with posterior fossa anomalies, the incidence of relevant CNVs was 18.5% [Zou *et al.* 2018]. The authors highlighted the fact that cerebellar hypoplasia cases seemed the most likely to have pathogenic CNVs on CMA (54.6%). In an 8-year observational study of CMA results in fetuses with CNS anomalies, the incidence of pathogenic CNVs was 6.7% among all the cases. The association of pathogenic findings in CMA with posterior fossa anomalies was also highlighted in this work [Santirocco *et al.* 2020]. It is clear that in cases of complex CNS anomalies, CMA has a great additional value; moreover, even in cases with relatively common findings, such as mild ventriculomegaly, CMA can be considered. In a retrospective study of 101 fetuses with isolated mild ventriculomegaly, the application of CMA after an invasive procedure revealed pathogenic CNVs in 3.0% of the cases [Duan *et al.* 2019]. In a recent retrospective study of 312 fetuses with mild ventriculomegaly, the incidence of pathogenic CNVs was 5.0% [Chang *et al.* 2020].

Increased fetal NT is a well-known marker of chromosomal disease and fetal structural defects, especially heart anomalies and genetic disorders [Baer *et al.* 2014; Jelliffe-Pawlowski *et al.* 2015; Kagan *et al.* 2006; Kagan *et al.* 2017; Sotiriadis *et al.* 2013; Souka *et al.* 2005]. It has also been shown that increased NT is a marker for the risk of neurodevelopmental disorders in infants [Hellmuth *et al.* 2017]. The results of a large systematic review published in 2015 supported the use of CMA in fetuses with increased NT and normal karyotypes. The incremental yield of CMA in those fetuses was estimated at 5.0% (95% CI, 2.0–8.0%) with a low incidence of VOUS of 0.8% [Grande *et al.* 2015]. Subsequent studies have confirmed these data, reporting an incidence of pathogenic CNVs of 2.7–3.7% [Egloff *et al.* 2018; Leung *et al.* 2019]. Importantly, in the present era of NIPT, a considerable amount of pathogenic chromosomal aberrations in fetuses with increased NT would not be detected using cff-DNA testing [Sotiriadis *et al.* 2017]. Moreover, the additional value of NT measurement over NIPT or CMA use in the group of fetuses with increased NT remains under debate [Huang *et al.* 2014; Lichtenbelt *et al.* 2015]. It is widely accepted that the cut-off point for invasive testing in the present era of NIPT should be a NT measurement of 3.5 mm or higher. The latest evidence raises questions as the cut-off for the invasive testing with CMA could be an NT measurement of 3.0 mm or higher, because the residual risk for chromosomal aberrations other than common trisomies in such fetuses is 1 in 21 (all aberrations included, 4.8% [95% CI, 3.2–7.3%]) [Petersen *et al.* 2020]. Data from a large retrospective

study by Sagi-Dain *et al.* confirmed that the rate of abnormal CMA findings was significantly higher in fetuses with NT measurements between 3.1 and 3.4 mm compared to fetuses with normal US findings. Notably, genome-wide NIPT as well as karyotyping would miss these findings in 1.5% or 1 in 69 cases [Sagi-Dain *et al.* 2021].

2.5.5. Challenges in prenatal use of CMA

CMA is now recommended by several professional societies for diagnostic testing in high-risk pregnancies, focusing on fetuses with multiple or isolated US abnormalities, pregnancy loss or stillbirth, intrauterine growth restriction, and in fetuses with NT equal or greater than 3.5 mm [ACOG 2016a; Armour *et al.* 2018; Dugoff *et al.* 2016]. By contrast, it is also stated that most genomic changes, which can be identified by CMA, but not by conventional karyotyping, are not associated with maternal age. CMA can be considered for all women who undergo invasive testing [ACOG 2007; ACOG 2016a]. In some centers, CMA has been used as a primary prenatal cytogenetic test for several years [Fiorentino *et al.* 2013; Muys *et al.* 2018; Shaffer *et al.* 2012a; Srebniak *et al.* 2013; Stern *et al.* 2020; Vogel *et al.* 2018]. In a recent systematic review and meta-analysis, it was estimated that the frequency of pathogenic CNVs was 0.84% (95% CI, 0.55–1.30%) in pregnancies that were referred for invasive testing because of advanced maternal age or anxiety. What is more important is that pooled estimates from meta-analysis of studies including 10,314 fetuses indicate that early-onset syndromic disorder can be detected in 0.37% of the cases that are referred for invasive testing without US anomalies (95% CI, 0.27–0.52%). This means that the risk of carrying a fetus with such an aberration is 1 in 270, which is higher than the risk for carrying a fetus with T21 in young women [Srebniak *et al.* 2018]. In a Danish retrospective study of CMA as a primary diagnostic tool in high-risk pregnancies after cFTS, pathogenic CNVs were detected in 2.3% of the cases [Vogel *et al.* 2018]. In a nation-wide Belgian study of CMA performed in 13,266 fetuses, the incidence of pathogenic CNVs was 1.9%, all indications included [Muys *et al.* 2018]. The authors were particularly concerned about the fact that with the implementation of NIPT, invasive prenatal testing will increasingly become restricted to pregnancies with US anomalies and those with a known genetic defect in the family. If NIPT becomes the first-tier screening test for all, submicroscopic chromosomal anomalies can be missed. The amount of such cases can be as high as 31.5% [Muys *et al.* 2018]. In the latest review, which included 29,612 structurally normal fetuses, the incidence of clinically relevant CNVs was between 1 in 250 and 1 in 40 pregnancies. The probability of a pathogenic CNV, associated with severe early onset diseases, without any US malformation throughout the pregnancy, was 0.5% or less [Daum *et al.* 2021].These recent data support the routine use of CMA in PD, as was proposed in 2012 by Wapner *et al*. [Wapner *et al.* 2012]. Considering that the incidence of miscarriage after CVS or AC is as low as

0.2% and 0.3% respectively [Salomon *et al.* 2019], it is reasonable to inform women about the diagnostic yield of CMA, which should be a gold-standard test for all indications in invasive PD [Daum *et al.* 2021].

The use of CMA in all pregnancies that undergo invasive testing raises several ethical questions concerning the diagnosis of uncertain prognosis, an unexpected diagnosis, or getting a result of uncertain or unknown clinical significance [Richardson and Ormond 2018]. Expecting parents should understand, after pre-test counselling, that CMA cannot detect everything in the genome. This can reduce unrealistic expectations of the 'perfect' prenatal test. Medical professionals who are providing such counselling should help their patients to appreciate the fact that total predictability is not always a possible or preferred outcome [Werner-Lin *et al.* 2016]. Still, from available studies of parental choices about getting information from genetic testing, it is clear that most women are making fully informed decisions regarding PD at this time [Hochner *et al.* 2020; Millo *et al.* 2021].

2.6. Next-generation sequencing and exome sequencing in prenatal diagnostics

The introduction of US into routine obstetrical care gave the possibility of the detection fetal structural abnormalities. Unexpected fetal anomalies occur in approximately 2–3% of all pregnancies and many have an underlying genetic condition [Ferretti *et al.* 2019]. Depending on the abnormality type, the number of organ systems involved, and the severity of the findings, up to 30% of such cases will have clinically relevant abnormal karyotypes [Zhang *et al.* 2017]. The application of CMA in this specific group of fetuses will reveal pathogenic CNVs in about 5–6% of cases [Chong *et al.* 2019; Hillman *et al.* 2013; Hui *et al.* 2021; Lin *et al.* 2020; Wapner *et al.* 2012]. Thus, using conventional karyotyping and molecular cytogenetics, more than 50% of fetuses with structural anomalies will be left without a diagnosis [Monaghan *et al.* 2020]. Due to the limitations of antenatal US imaging, there is an inability to identify subtle dysmorphic features, which hinders the ability to narrow the differential diagnosis [Kilby 2021]

Traditional prenatal evaluation of malformed fetuses in terms of possible associations with monogenic disorders in the absence of a family history is challenging, expensive, and time consuming; it is therefore not practical in a pregnancy setting [Ferretti *et al.* 2019]. NGS is a term used to describe many methods of high-throughput nucleotide-sequencing technologies [Kilby 2021]; these differ from Sanger sequencing [Sanger *et al.* 1977], which was the only method to evaluate the molecular causes of genetic diseases that was available for almost 30 years [Schuster 2008]. NGS is now widely used in the field of PS. All major NIPT platforms are based on NGS technology [Benn *et al.* 2013]. The evolution of NGS technologies in PD has enabled the screening of multiple genes in a single analysis, which is important and time saving in cases of *de*

novo monogenic disease in the fetus [Ferretti *et al.* 2019]. Traditionally such cases would require the sequential sequencing of many genes, exploring a potential molecular diagnosis of monogenic disorder [Normand *et al.* 2018].

Two technologies are interrogating the genome at a nucleotide level: GS and ES: GS assesses both the coding and noncoding regions of the genome; while ES is limited to the protein-coding regions, representing 1–2% of the whole genome, and involving more than 20,000 genes [Kilby 2021; Monaghan *et al.* 2020]. In clinical settings, ES is the preferred diagnostic option because most of the disease-related genes involve exons; thus, ES is more cost-effective than GS. Additionally, our current ability to interpret intronic regions of the genome is limited [Jelin and Vora 2018].

In the pediatric population, ES yields a diagnosis of underlying molecular cause in 25% of cases of presumed genetic syndrome after negative findings in karyotyping and CMA [Yang *et al.* 2014]. These findings in children suggested that ES could be an important diagnostic tool in the evaluation of genetic causes of fetal structural anomalies [Petrovski *et al.* 2019]. Accurate and precise diagnosis in cases of fetal congenital anomalies would increase the ability to provide parents with better counselling regarding the prognosis and possible treatment of neonates or fetuses [Jelin and Vora 2018]. In Estonia, the NGS diagnostic performance was evaluated in the pediatric and adult population, and in a cohort of 501 probands, the diagnostic yield was 26.3% [Pajusalu *et al.* 2018]. This study did not focus on PD.

The diagnostic yield of ES in prenatal cases ranges widely between published studies. Being a relatively new and expensive method in PD, which focuses on the evaluation of rare cases, the initial reported findings of diagnostic effectiveness have been based on small, highly selected cohorts. The diagnostic yield in these small case series was reported to be between 10 and 50% [Alamillo *et al.* 2015; Carss *et al.* 2014; Drury *et al.* 2015]. The largest prospective cohort study of the diagnostic performance of ES in PD in unselected cases of fetal anomalies to date reported a DR of 8.5% [Lord *et al.* 2019]. In another prospective cohort of 234 fetuses analyzed in trios, an additional diagnostic yield of 10.3% was reported [Petrovski *et al.* 2019]. Differences among studies are explainable by several factors including selection criteria of the fetal cases in terms of anomalies and methods of NGS used in the study. The highest DR of 81% of pathogenic variants was reported in a case series of 16 fetuses that were referred for NGS analysis with suspicion of skeletal dysplasia after multidisciplinary counselling [Chandler *et al.* 2018]. Thus, one of the factors affecting the diagnostic performance of ES in PS is actually a type of referral for the ES, unselected fetal anomalies, or highly selected cases, as referred by a geneticist [Mone *et al.* 2018]. The PAGE study identified specific phenotypes, which are associated with the highest yield of pathological variants. These are multiple anomalies (15.4%), skeletal anomalies (15.4%), and cardiac malformations (11.1%) [Lord *et al.* 2019]. The diagnostic yield appears to be considerably lower in large cohorts and higher in small cohorts with tight inclusion criteria. In a recent systematic review of performance of ES in PD, a

weighted diagnostic rate among all the studies included in the review was 19% [Guadagnolo *et al.* 2021].

In a recent study by Mone *et al*. (the CODE study), a prospective cohort of 197 trio ES was evaluated in fetuses with CHD identified prenatally. The additional diagnostic yield over karyotyping and CMA in those fetuses was 12.7% [Mone *et al.* 2021]. Similar results came from another large prospective cohort of 260 fetuses with CHD analyzed in trios. The diagnostic yield of ES was 10% [Li *et al.* 2020]. In a systematic review focusing on fetuses with CHD done by the CODE study group, data on 636 fetuses with CHD were analyzed. The pooled incremental yield of ES was 21% [Mone *et al.* 2021]. There are only a few studies published with fetal cohorts of more than 100 cases. A summary of their findings is given in Table 6.

There have been no reported studies of prenatal ES in the absence of fetal abnormalities [Harris *et al.* 2018]. According to a position statement issued by several professional societies, the routine use of ES in prenatal settings is not recommended [ISPD *et al.* 2018]. There is ongoing debate regarding the appropriate use of ES in PD: some researchers advocate unrestricted testing and others suggest that ES is justified only in severe cases [Harris *et al.* 2018]. Still, it is accepted now that ES in PD should be a phenotype-driven test. ES can result in thousands of detected variants and their interpretation is dependent on the precise phenotypical description, which is limited in prenatal settings [Kin Chau and Choy 2021]. There is evidence to show an increase in the detection of pathogenic variants after ES in cases where prenatal and postnatal phenotypes were combined, highlighting a need for deep phenotyping [Aarabi *et al.* 2018; Aggarwal *et al.* 2020].

Table 6. Results of ES diagnostic performance studies with cohorts of more than 100 fetuses.

Number of probands	Inclusion criteria	DR (%) of pathogenic variants	Reference
196	Live fetuses with US abnormalities	**24**	[Fu *et al.* 2018]
146	Live and terminated/miscarried fetuses with US abnormalities	**32**	[Normand *et al.* 2018]
610	Fetuses with structural anomalies, increased NT	**8.5**	[Lord *et al.* 2019]
234	Fetuses with structural anomalies	**10.3**	[Petrovski *et al.* 2019]
197	Fetuses with CHD	**12.7**	[Mone *et al.* 2021]
260	Fetuses with CHD	**10**	[Li *et al.* 2020]

CHD – congenital heart disease; DR – detection rate; NT – nuchal translucency; US – ultrasound.

All patients diagnosed with fetal anomaly should be offered the option of genetic testing, in order to make fully informed decisions. This is essential in respect to patients' reproductive autonomy [Howe 2014]. Clear communication, with detailed pre-test and post-test counselling, is required in prenatal ES referrals [Horn and Parker 2018]. It is crucial to consider, that NGS and ES have the potential to result in uncertain findings. Couples' experiences of receiving uncertain results after genetic testing have been described in a recent systematic review, addressing both the clinical and the emotional aspects of the impact of uncertainty [Harding *et al.* 2020].

In the recent ACMG statement on the use of fetal ES, points to consider are discussed in detail, highlighting all aspects of referring for ES, the required genetic counselling, the need for detailed evaluation of the fetus, the use of available diagnostic modalities, and issues with reporting the ES results in prenatal settings. As the new diagnostic tool in fetal medicine, ES may be considered in cases where diagnosis cannot be obtained using other diagnostic methods to evaluate fetuses with congenital anomalies [Monaghan *et al.* 2020].

Unresolved ethical dilemmas in the use of prenatal ES include the reporting of VOUS, the possibility of finding nonpaternity or consanguinity, and incidental findings that are not related to the reason for the initial testing. These can include variants associated with low-penetrant disorders and adult-onset disorders, both treatable and non-treatable [Abou Tayoun and Mason-Suares 2020].

2.7. Prenatal diagnosis of rare monogenic disorders highlighted in the thesis

2.7.1. Simpson-Golabi-Behmel syndome

SGB type I (OMIM 312870) is a rare X-linked disorder with characteristic pre- and post-natal overgrowth and multiple congenital anomalies. This well-known genetic condition has been rarely diagnosed prenatally. The prevalence of SGB syndrome is unknown. There were about 250 cases published by the year 2014 [Tenorio *et al.* 2014]. Due to its specific presentation, the diagnosis is usually postnatal [Manor and Lalani 2020]. Typical findings of SGB syndrome are macroglossia, hepatosplenomegaly, cardiac malformations, skeletal anomalies, facial clefts, and high birthweight [Neri *et al.* 2013; Tenorio *et al.* 2014; Xiang *et al.* 2020]. These features can also be seen on prenatal US examination [Cottereau *et al.* 2013; Li and McDonald 2009; Reischer *et al.* 2021; Ridnoi *et al.* 2018].

Differential diagnosis of SGB syndrome is complex due to its overlap with other overgrowth syndromes [Manor and Lalani 2020]. Certain pathways of the diagnosis have been proposed, in which many factors should be considered, starting from pregnancy dating and exclusion of gestational diabetes [Vora and Bianchi 2009]. In terms of genetic disorders, the following four other syndro-

mes should be considered when dealing with fetal overgrowth evaluation: Beckwith–Wiedemann syndrome (BWS), Pallister-Killian, Sotos, and Perlman.

2.7.2. Meckel–Gruber syndrome

MKS is a rare, lethal autosomal recessive ciliopathy caused by pathogenic variants in one of at least 17 genes [Hartill *et al.* 2017]. The overall worldwide incidence has been estimated at 1 in 135,000 livebirths, but it is relatively frequent in some populations, such as Finland, where the MKS incidence is 1 in 9,000 livebirths [Salonen and Norio 1984]. The first disease-causing variant in the *MKS1* gene was identified in the Finnish population [Kyttala *et al.* 2006]. The incidence of MKS can be even higher in populations with a high consanguinity rate, reaching 1 in 2,000 livebirths in Qatar [Al-Belushi *et al.* 2016]. The highest incidence of MKS was reported in Gujarati Indians with 1 case per 1,304 livebirths, and a carrier rate of 1 in 18 [Young *et al.* 1985].

MKS is a lethal disorder. In Europe 88% of prenatally detected cases are terminated [Barisic *et al.* 2015]. Profound prenatal features of MKS such as encephalocele, postaxial polydactyly and polycystic kidneys can be detected during US examination already in the first trimester [Sepulveda *et al.* 1997]. The incidences of these findings in MKS are 83.8%, 87.3%, and 97.7%, respectively [Barisic *et al.* 2015]. Other CNS findings or heart defects can also be present, including holoprosencephaly, cerebellar anomalies, or anencephaly [Khurana *et al.* 2017; Radhakrishnan *et al.* 2019; Yaqoubi and Fatema 2018]. An elevation of maternal serum AFP levels was described by Sepulveda *et al.* and is explained by the neural tube defect in MKS [Sepulveda *et al.* 1997].

Targeted diagnosis of MKS is usually triggered by distinctive US findings and can be started with the use of targeted NGS gene panels [Ridnoi *et al.* 2019; Watson *et al.* 2020]. ES may be necessary to exclude rare gene associations [Ridnoi *et al.* 2019; Zhang *et al.* 2020]

2.8. Prenatal screening and diagnosis in Estonia, past and present

The development of antenatal care in Estonia was very rapid after regaining independency in 1991. Antenatal care is fully supported by EHIF for every pregnant woman with a residence permit in our country, regardless of citizenship. All screening and diagnostic procedures during pregnancy, as well as labor are free of charge. This gives a great opportunity to apply PS and PD to every pregnant woman, who has the indication for the latter.

The use of national PS program was initially started in 1995 with maternal age as the main risk factor. In 1999, with the support of the EHIF, a national program of PS was implemented, based on second-trimester serum markers. Between 2006 and 2016, several PS protocols were used, but there was no clear

national policy [Sitska 2018]. The new national prenatal diagnostics guidelines designated cFTS as the primary PS test for all pregnant women in Estonia [Ustav *et al.* 2016]. From 2020, with the support from EHIF, a local NIPT platform was implemented into existing PS program, based on the universal cFTS (see the Results and Discussion part of the thesis).

The prenatal diagnostics officially started with the performance of the first amniocentesis on October 29, 1990 by dr. Aivar Ehrenberg in Tartu University Hospital (TUH). Initially a conventional karyotyping was used in the diagnosis of chromosomal disease. From 2000 a rapid aneuploidy testing iFISH became available. The use of CMA in clinical practice started in 2011 and NGS analysis in 2015. Both analyses now are reimbursed by EHIF, so every patient with the indication for these tests gets them free of charge. In cases, where invasive diagnostics is required patients are referred to one of the three major hospitals. In Estonia there is only one Department of Clinical Genetics in the TUH and all prenatal as well as postnatal diagnostics of chromosomal and genetic disease is performed there.

2.9. Summary of the literature

The field of PS and PD is constantly evolving. Different strategies are in use to provide pregnant women with the best antenatal care. Despite the fact that cFTS was introduced into clinical practice almost 20 years ago, it is still the first-line screening choice for the majority of women in the obstetric population. More than 90% of Down, Edwards, and Patau syndrome cases can be identified prenatally using conventional cFTS. Nevertheless, advances in the technology cannot be ignored and with the development of NIPT it is likely that at some point in time the latter will replace current screening protocols. At the same time, NIPT is unlikely to replace the US assessment of both the fetus and the expectant mother for the early detection of congenital anomalies and pregnancy complications. In terms of adapting new technologies into clinical practice, it is important to acknowledge the need for prospective quality assurance and continuing education of healthcare professionals as well as patients. Understanding the scientific basis of the new molecular methods used in PS and PD is necessary for evidence-based implementation in routine care. The benefits of NIPT, CMA, and NGS in prenatal setting can easily be overestimated by biases in counselling. The possibility of unclear or incidental findings in fetal DNA, using new molecular methods, needs to be addressed before testing. With the emerging evidence of the diagnostic effectiveness of CMA and NGS in PD, one can expect further studies in the field of PS and PD. Knowing this, it is wise to remind ourselves that all of these technologies have been developed to ensure that women are afforded their essential reproductive rights.

3. AIMS OF THE PRESENT STUDY

The aims of the present study were:

1. To evaluate the effectiveness of combined first trimester screening in Estonia;
2. To assess the use and effectiveness of chromosomal microarray analysis in high-risk pregnancies for the detection of chromosomal anomalies;
3. To establish the next-generation sequencing methods and to evaluate their effectiveness for fetuses with high risk and/or combined anomalies;
4. To characterize prenatal phenotype of Simpson-Golabi-Behmel syndrome;
5. To characterize a pathogenic variant in the *TXNDC15* gene in prenatally diagnosed case of Meckel-Gruber syndrome.

4. MATERIALS AND METHODS

4.1. Study subjects

4.1.1. The effectiveness of cFTS (Publication 4)

The study period was two years from Jan 01, 2017, until December 31, 2018. A total of 14,859 pregnant women were followed in two major centers for antenatal care in Estonia: the East-Tallinn Central Hospital's Women's Clinic (ETCH) and the Women's Clinic of Tartu University Hospital (TUH). Of these, 293 cases were excluded due to multiple pregnancy and/or an incomplete screening protocol. The final study group consisted of 14,566 singleton pregnancy cases (Figure 7). Participation in the study was voluntarily and written informed consent was given as a part of routine antenatal care [Ridnõi *et al.* 2021b].

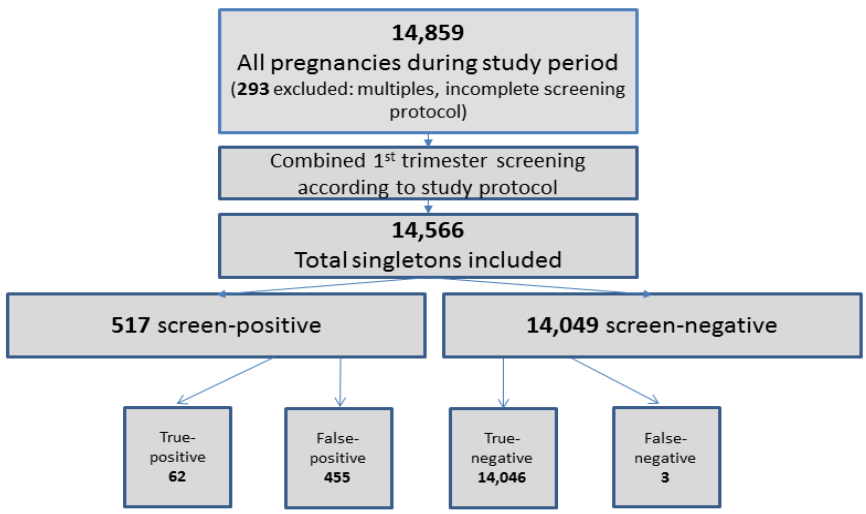

Figure 7. Study group of cFTS, from [Ridnõi *et al.* 2021b]

Among the cFTS study group, 517 women were screen-positive with risk for chromosomal disease higher than 1 in 100 (Figure 7). According to Estonian antenatal diagnostic guideline invasive diagnostics should be offered to every patient with high-risk for chromosomal disease [Ustav *et al.* 2016]. To all of them diagnostic invasive procedure was offered.

4.1.2. The effectiveness of CMA in high-risk pregnancies (Publication 1)

For the CMA diagnostic effectiveness evaluation, a total of 334 pregnant women were recruited out of 14,566 who underwent cFTS. Two groups were formed. Group A comprised 184 women all of whom were identified as at high-risk for trisomies after cFTS, but had normal NT measurement (below 3.5 mm) and no US malformations (Figure 8). All women in Group A were counselled before the procedure and additional written informed consent was obtained to perform CMA on fetal DNA. Group B comprised 150 women all of whom met the criteria for CMA as a first-tier diagnostic test (Figure 8). CMA in Estonia is performed as a first-tier diagnostic test after an invasive procedure if one of the following clinical indications is met: NT greater than 3.5 mm, fetal malformations, family history or known balanced translocation in one parent. All women in Group B gave written consent for a performance of invasive procedure, no additional consent was given for the performance of CMA [Ridnõi *et al.* 2021a].

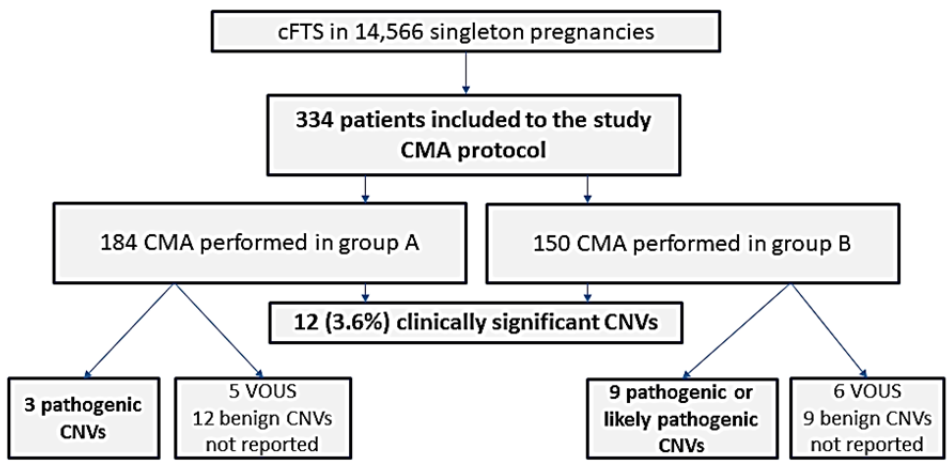

Figure 8. Study group of CMA: group A (high-risk after cFTS, normal ultrasound), group B (CMA indicated as first-tier diagnostic test), from [Ridnõi *et al.* 2021a].
cFTS – combined first-trimester screening; CMA – chromosomal microarray analysis; CNV –copy-number variant; VOUS – variant of uncertain clinical significance.

4.1.3. The effectiveness of NGS for fetuses with high risk and/or combined anomalies (Publication 1)

During the study period, a total of 28 cases (0.2%) were selected for the NGS panel analysis group. All cases were recruited from the cFTS study group. The inclusion criteria were as follows: fetal brain anomalies, non-immune fetal hydrops, combined heart defects, and multiple fetal anomalies, suggestive of an underlying genetic syndrome. The decision to perform NGS panel analysis was made by a clinical geneticist who also counselled the patient, and additional written informed consent was obtained for performing NGS analysis on fetal DNA. A detailed description of the indications for NGS analysis is given in Table 7. Only two pregnancies resulted in livebirths, all others were terminated according to medical indications before the 22nd gestational week [Ridnõi *et al.* 2021a].

Table 7. Description of the findings in cases selected for NGS analysis, adapted from [Ridnõi *et al.* 2021a].

Case no	Fetal US findings/types of anomaly on autopsy	
1	Corpus callosum dysgenesis. Bilateral ventriculomegaly	
2	Corpus callosum dysgenesis. Dysmorphic facial features	
3	Absence of corpus callosum. Additional spleen. Hydrops. Sandal gap	
4	Agenesis of corpus callosum	
5	Agenesis of corpus callosum. Dysmorphic facial features	
6	Agenesis of corpus callosum. Lissencephaly	
7	Cerebellar hypoplasia with ventriculomegaly	
8	Holoprosencephaly	
9	Brain atrophy with hemorrhage	
	Brain anomalies	**9**
10	Combined heart defect, asplenia. Malrotation of the gut	
11	Combined heart defect. Polysplenia	
12	Truncus arteriosus communis	
13	Stenosis of pulmonary artery	
14	Truncus arteriosus communis. Maternal 2q13 2.1Mb microdeletion	
15	Cardiomegaly, critical aortic stenosis	
	Cardiac anomalies	**6**
16	Cystic hygroma and generalized hydrops	
17	Enlarged NT and hydrops. Livebirth.	
18	Cystic hygroma and generalized hydrops	
19	Generalized hydrops	
	Non-immune hydrops	**4**
20	Facial cleft. Syndactyly of II–III toe. Absence of right kidney and ureter, aplasia of spleen	
21	Polycystic kidneys diagnosed at 29th week of pregnancy. Presence of ascites. Livebirth.	

Case no	Fetal US findings/types of anomaly on autopsy	
22	Unexplained anhydramnion at week 17	
23	Multiple anomalies: facial cleft, anencephaly, gastroschisis	
24	Large midline defect	
25	Enlarged NT, ectopia cordis, gastroschisis	
26	Holoprosencephaly, dysplastic cystic kidneys	
27	Large diaphragmatic hernia, dysmorphic facial features	
28	Large spina bifida in the thoracic region, dysmorphic facial features, deformation of the ribs on the right side	
	Multiple anomalies or syndromic suspicion	**9**

4.1.4. Family with SGB syndrome cases (Publication 2)

A 28-year-old primiparous Estonian woman was referred for cFTS at 13+3 weeks with a spontaneously conceived twin pregnancy (Probands 1 and 2). She had a history of spontaneous miscarriage in the first trimester but was otherwise healthy. The patient became pregnant four months after the first delivery. She was referred for a first-trimester scan at 12+6 weeks (Proband 3). The family history was negative for severe genetic disorders. Her partner was a healthy Estonian male. The pedigree is shown in Figure 9.

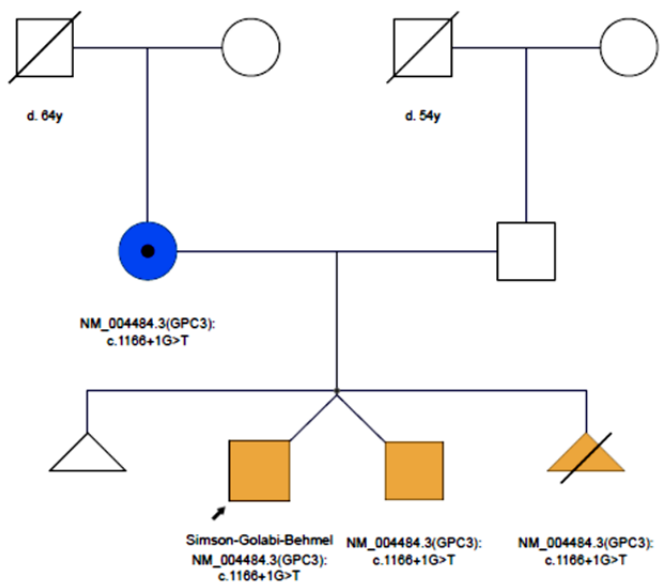

Figure 9. The pedigree of the family with SGB syndrome.

4.1.5 Family with Meckel–Gruber syndrome case (Publication 3)

A 33-year-old Estonian woman was referred for a clinical geneticist consultation at 13+0 weeks after abnormal US investigation results from a first-trimester scan. She was otherwise healthy but had experienced one ectopic pregnancy in the past. Her partner was a healthy Estonian male. The parents were not known to be related. The family history was negative for severe genetic disorders. The pedigree of the family is shown in Figure 10.

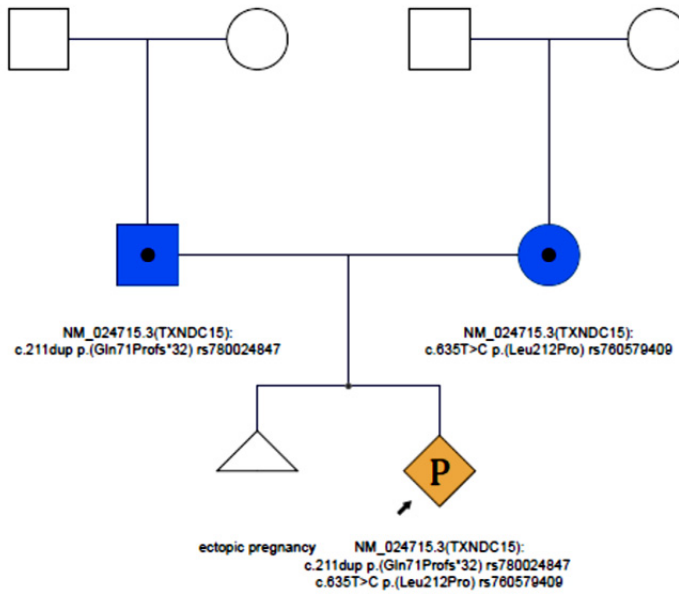

Figure 10. The pedigree of the family with MKS.

4.2. Methods

4.2.1. The effectiveness of cFTS

cFTS was performed in all pregnant women according to the study protocol (Figure 11), which was adapted from the Estonian prenatal diagnostics guidelines [Ustav *et al.* 2016]. The serum markers f-βhCG and PAPP-A were collected from patients between the 9th and 13th weeks of pregnancy. Two different systems for biochemical analysis were utilized: Roche Cobas (Roche Diagnostics, Basel, Switzerland) in TUH and KRYPTOR compact PLUS (Thermo Fisher Scientific, MA, USA) in ETCH.

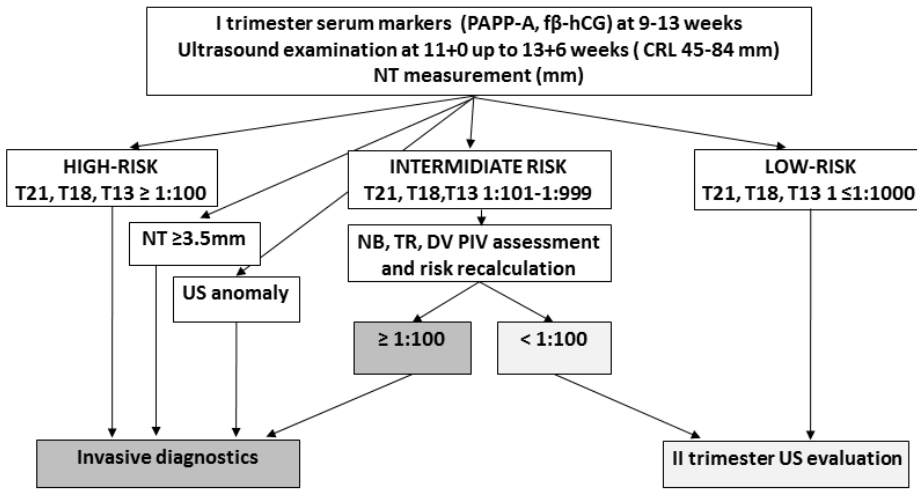

Figure 11. cFTS study protocol [Ridnõi *et al.* 2021b].
β-hCG – beta human chorionic gonadotropin; CRL – crown-rump length; DV PIV – ductus venosus pulsatility index; FHR – fetal heart rate; NB – nasal bone; NT – nuchal translucency; PAPP-A – pregnancy-associated plasma protein A; T13 – trisomy 13; T18 – trisomy 18; T21 – trisomy 21; TR – tricuspid regurgitation; US – ultrasound.

Ultrasound examinations were performed between 11+0 and 13+6 weeks of pregnancy, according to the FMF standards, for NT measurement (Appendix 1), pregnancy dating, and excluding major fetal defects. All US examinations were performed by FMF license holders. Patients were divided into three groups after the risk calculation. A risk for T21, T18, or T13 higher than 1 in 100 was defined as high-risk. A risk lower than 1 in 1,000 was defined as low risk. In the intermediate risk group, which was defined as a risk ranging from 1 in 101 to 1 in 999, additional US markers like NB; tricuspid flow, and DV pulsatility index were included in the calculation. All markers were assessed in accordance with

the FMF guidelines and a recalculation of aneuploidy risks was performed. After the recalculation, the high-risk group was defined as having a risk greater than 1 in 100. Risk calculations were performed with either Viewpoint 6 for OB/GYN (GE Healthcare, IL, USA) in ETCH or Astraia (Astraia software gmbh, Munich, Germany) in TUH. High-risk cases were offered an invasive diagnostic procedure. Pregnancy outcomes and information regarding livebirths with diagnosed trisomy were collected from the labor wards of the hospitals and the database of the Department of Clinical Genetics of TUH [Ridnõi *et al.* 2021b].

The performance of cFTS was measured as follows:

$$DR\% = \frac{\text{T21,T18 and T13 cases diagnosed after cFTS high-risk results prenatally}}{\text{all cases of T21,T18 and T13 diagnosed in cFTS study group pre- or postnatally}} *100.$$

The DR% was calculated for each trisomy separately.

$$FPR\% = \frac{(\text{all screen-positive cases after cFTS}) - (\text{true-positive cases of T21,T18 and T13})}{\text{all cases of cFTS}} * 100.$$

$$PPV\% = \frac{\text{true-positive cases of T21,T18 and T13}}{(\text{true-positive cases}) + (\text{false-positive cases})} *100.$$

$$NPV\% = \frac{\text{true-negative cases of T21,T18 and T13}}{(\text{true-negative cases}) + (\text{false-negative cases})} *100.$$

$$Sensitivity\% = \frac{\text{true-positive cases of T21,T18 and T13}}{(\text{true-positive cases}) + (\text{false-negative cases})} *100.$$

$$Specificity\% = \frac{\text{true-negative cases of T21,T18 and T13}}{(\text{true-negative cases}) + (\text{false-positive cases})} *100.$$

4.2.2. Karyotyping

Fetal samples for cytogenetic and molecular genetic testing were obtained by CVS or AC and were cultured according to the standard protocols in the Department of Clinical Genetics of TUH. Karyotyping was performed by G-banding and chromosomes were analyzed at the 400–500 level according to the International System for Human Cytogenetic Nomenclature (ISCN 2016) criteria [McGowan-Jordan *et al.* 2016]. Twelve metaphases were analyzed for each sample; in cases of suspected mosaicism, 30 metaphases were karyotyped in total [Ridnõi *et al.* 2021a; Ridnõi *et al.* 2021b].

4.2.3. NIPT

Of the 517 screen-positive women after cFTS, 92 chose to perform NIPT as a second-tier screening to possibly avoid invasive diagnostics. During the study period in our two centers, two NIPT platforms were available: the PANO-

RAMA™ test (Natera Inc., SanCarlos, CA, USA); and the NIPTIFY ® test (Competence Centre on Health Technologies, CCHT, Tartu, Estonia), which is a new genome-wide NIPT assay developed in Estonia [Zilina *et al.* 2019]. Invasive diagnostics were offered only in cases with high-risk NIPT results [Ridnõi *et al.* 2021b].

4.2.4. The effectiveness of CMA in high-risk pregnancies

Genomic DNA for CMA was extracted either directly from AC or CVS or from a cultured sample. CMA was performed using Illumina HumanCytoSNP-12 BeadChips (Illumina Inc., SanDiego, CA, USA). Genotype analysis was performed using GenomeStudio software v2011.1 (Illumina Inc.) with additional input from QuantiSNP v2.3 software [Colella *et al.* 2007]. CNVs were classified into four classes: pathogenic, likely pathogenic, VOUS and benign/likely benign variants [Nowakowska 2017]. All CMA analyses were performed in the United laboratory of TUH and interpretation carried out by the Department of Clinical Genetics of TUH.

In prenatal cases, benign and likely benign findings or long contiguous stretches of homozygosity (LCSH) of any size were not reported. Several online databases were used in the decision making, particularly Online Mendelian Inheritance in Man (OMIM), human genome browsers (UCSC and Ensembl), DECIPHER, and the Database of Genomic Variants (DGV). PubMed was used for peer-reviewed article searches. In cases of reported findings, the parents' genomic DNA, extracted from blood lymphocytes, was also analyzed to determine the heredity [Ridnõi *et al.* 2021a].

The diagnostic yield in the CMA study group was measured in DR% for the whole study group and in Group A and Group B separately:

$$DR\% = \frac{\text{clinically relevant CNV cases}}{\text{all CMA performed in the group}} * 100.$$

4.2.5. The effectiveness of NGS for fetuses with high risk and/or combined anomalies

Fetal DNA was extracted either from fetal material acquired from invasive procedures prenatally or from fetal tissues after the termination of pregnancy. NGS was performed using the TruSight One (4,813 genes) or TruSight One Expanded (6,699 genes) sequencing panels (Illumina Inc., San Diego, California). Sequencing was carried out on the NextSeq 500 platform (Illumina) in the Department of Clinical Genetics of TUH. NGS was performed only on probands [Ridnõi *et al.* 2021a].

Reads were aligned to the reference genome hg19 by the Burrows-Wheeler Aligner (BWA) [Li and Durbin 2009] and variants were identified by Genome

Analysis Toolkit (GATK) [McKenna *et al.* 2010] tools using BWA Enrichment v2.1 workflow on the BaseSpace Onsite system (Illumina). Variants from variant call format (VCF) files were annotated by an in-house variant annotation pipeline involving ANNOVAR [Wang *et al.* 2010], SnpSift [Cingolani *et al.* 2012], and GATK [McKenna *et al.* 2010]. CNV detection was carried out by CoNIFER software [Krumm *et al.* 2012]. All reported pathogenic, likely pathogenic, and VOUS variants were validated by Sanger sequencing in the fetus and in both parents to confirm the inheritance pattern. Variants were reported according to the ACMG standards [Richards *et al.* 2015].

The diagnostic yield in the NGS study group was calculated in the same way as in the CMA study group.

4.2.6. Methods used to evaluate SGB syndrome cases

According to the national PD guidelines, the first-tier test in cases identified as high-risk after cFTS is conventional karyotyping, using standard techniques, as described in chapter 4.2.2. Considering the fact that both fetuses (proband 1 and 2) had increased NT over 3.5 mm, CMA was also performed on DNA from one twin. CMA was performed in the same way, which is described in chapter 4.2.4.

For definitive molecular diagnosis, parent-offspring trio ES was performed on DNA extracted from proband 1. Library preparation and a sequencing run were performed by GenomeScan B.V. (Leiden, The Netherlands) using a SureSelect XT Human All Exon v5 enrichment kit (Agilent Technologies, Santa Clara, CA) and an Illumina HiSeq 4000 sequencer. Interpretation of the results was carried out in the Department of Clinical Genetics of TUH.

4.2.7. Methods used in evaluating fetus with MKS

US examination was performed during the first trimester of pregnancy. Pregnancy was terminated due to profound US findings and aborted fetuses were referred for pathoanatomical autopsy. DNA was extracted from fetal tissues after pregnancy termination. NGS analysis and ES of the fetal DNA was performed in the same way described in chapter 4.2.5 and 4.2.6.

4.2.8. Statistical analysis

Statistical analysis was performed with STATA 16.2 software using Wilson confidence intervals for binominal proportion.

4.3. Ethics

The present study was approved by the Research Ethics Committee of the University of Tartu (protocol 263/M-19 17.10.2016) and supported by Estonian Research Council grants PUT355 and PRG471.

All of the women from the cFTS study group gave their written informed consent as part of a regular antenatal care.

Additional written informed consent for performing CMA on fetal DNA was taken from each woman recruited to CMA study Group A. For all of the women recruited to CMA study Group B, consent was obtained for a performance of diagnostic invasive procedure, no additional consent was taken for performing CMA. The obstetrician performing the invasive diagnostic procedure or a medical geneticist provided comprehensive counselling in terms of the benefits, advantages, risks, and possible incidental findings in CMA.

In the NGS study group, written informed consent was taken in each case for performing panel testing on fetal DNA and counselling was provided by a clinical geneticist.

In cases of SGB syndrome and MKS, written consent for the publication of fetal, neonatal, and autopsy images was obtained from parents.

5. RESULTS AND DISCUSSION

5.1. The effectiveness of cFTS

Combined FTS was performed in 14,566 singleton pregnancies. The screen-positive rate was 3.54%, with 517 cases having a risk of greater than 1 in 100 for T21, T18, or T13 (Figure 7). Results of the cFTS study group are summarized in Table 8.

Table 8. Results of cFTS: detected trisomies and screening performance, adapted from [Ridnõi *et al.* 2021b].

Condition	Cases after cFTS/All cases	DR% (95%CI)
T21	48/51	94 (84.09–97.98)
T18	11/11	100 (74.12–100)
T13	3/3	100 (43.85–100)
Screening performance		
Screen-positive		517
True-positive		62
False-positive		455
Screen-negative		14049
True-negative		14046
False-negative		3
Total		14566
Sensitivity% (95%CI)		95,38 (86,24–98,80)
Specificity% (95%CI)		96,86 (96,56–97,13)
PPV% (95%CI)		11,99 (9,38–15,18)
NPV% (95%CI)		99,97 (99,93–99,99)

CI – confidence interval; cFTS – combined first trimester screening; DR – detection rate; NPV – negative predictive value; PPV – positive predictive value

Among the 51 pregnancies affected by T21, 48 cases were diagnosed after cFTS. Three cases of T21 were false-negative after cFTS, one case was diagnosed after the first-trimester scan because of a major heart defect, and two other cases were diagnosed after the second-trimester US scan. In addition, 11 cases of T18 and three cases of T13 were identified. Additionally we diagnosed Turner syndrome in 10 cases (including one with the mosaic condition), as well as four cases of polyploidy, four atypical mosaic cases, two cases of translocations, one case of triple X syndrome, and two cases of Klinefelter syndrome. All were considered to have been false-positive cFTS results (Appendix 2). Therefore, the FPR was 3.12%. During the study period, there were no live births with undiagnosed trisomy in the initial cFTS study group of 14,566

pregnancies [Ridnõi *et al.* 2021b]. The characteristics of cFTS in pregnancies affected with chromosomal disease are summarized in Appendix 2.

All cases except for two (one of T21 and one of Turner syndrome) were diagnosed using an invasive procedure. One case of T21 was identified as high risk in the first trimester using the combined test, had a positive NIPT result, and was strongly suspected of having an atrioventricular septal defect (AVSD). Invasive diagnostics were offered, but the patient declined this option and requested a pregnancy termination. A similar scenario arose in the case of Turner syndrome: a generalized hydrops of the fetus was diagnosed in a first-trimester scan, but the patient declined invasive testing and opted for NIPT. Cytogenetic confirmation was not performed after termination; however, considering the high-risk NIPT results and US findings it was highly likely that the fetuses had chromosomal abnormalities [Ridnõi *et al.* 2021b]. In screen-positive group after cFTS 92 women (17.8%) chose NIPT as second-tier screening to avoid invasive testing, 33 women (6.4%) refused any further testing.

It has been mandatory to offer cFTS to all pregnant women in Estonia since 2016, due to the new national prenatal diagnostics guideline. The overall coverage of PS has reached more than 90% [Sitska 2018]. High coverage has been documented since 1998, when primary screening for T21 was based on second-trimester serum markers [Sitska *et al.* 2008]. The DR for common trisomies in the present study was comparable with previous results in the published literature [Ekelund *et al.* 2015; Nicolaides *et al.* 2005; Santorum *et al.* 2017; Vogel *et al.* 2019]. Notably, the DR for T21 of 94% in the first trimester was higher compared to a previous Estonian study that used a contingent model of cFTS and second-trimester serum screening and achieved a DR for DS of 88.3% [Muru *et al.* 2010]. cFTS has been shown to be superior to second-trimester serum screening in terms of the detection of T21 in several population-based studies and the Cochrane review [Alldred *et al.* 2017; Lan *et al.* 2018; Luo *et al.* 2020; Royere *et al.* 2016]. The present study proved that the shift towards chromosomal disease screening solely in the first trimester was justified in Estonia. The main weakness of cFTS is its relatively high FPR and low PPV due to high proportion of false-positive results. Comparing to cFTS in terms of FPR and PPV, NIPT is more accurate and superior in performance for common trisomies [Bianchi *et al.* 2014; Gil *et al.* 2017]. This is one of the main reasons why NIPT is actively implemented into current PS models nowadays.

In the screen-positive group after cFTS, we performed 392 diagnostic invasive procedures. The invasive testing rate after cFTS in our study group was only 2.7%. In a large Danish study regarding the safety of procedures, the rate of invasive testing after cFTS was 4.7% [Wulff *et al.* 2016]. The main reason for this reduced invasive testing rate was the definition of the high-risk group for trisomies being over 1 in 100 in our study [Ridnõi *et al.* 2021b]. In Estonia, there was a huge decrease in the invasive testing rate from 12% in 2007 to about 5% in 2016 [Sitska 2018]. This can be explained by the changes in national policies in PS from TT in 2007 to a contingent approach with a combination of cFTS and TT in 2016. Our study showed a continuing decline in these terms

with an invasive testing rate after cFTS of only 2.7%, which was attributed to universal cFTS.

Further improvements in the detection of T21, T18, and T13, and a marked decrease in the invasive testing rate are possible with the implementation of NIPT into existing models of screening. The potentially invasive testing rate could be reduced to below 0.5% by offering NIPT to a larger group of women [Nicolaides *et al.* 2013b].

With a cFTS coverage of over 90% in Estonia, NIPT has been implemented since 2020 as a second-tier screening test in a group with so-called intermediate risk after cFTS. NIPT is now offered to women with the following indications: a risk for T21 of 1 in 11 to 1 in 1,000, and risks for T18 and T13 of 1 in 11 to 1 in 100 after cFTS. Additionally, NIPT can be provided to women with a history of diagnosed trisomies in previous pregnancies or in cases where invasive diagnostics would have a greater risk of miscarriage. NIPT, performed with above-mentioned indications is covered by EHIF and free of charge.

In the present study, three false-negative cases of T21 were reported after prospective cFTS. All of them had a combined risk for DS lower than 1 in 1,000. The first case had an Ebstein's anomaly, such that an indication for invasive testing would remain. The second case had a low combined risk due to misinterpretation of additional US markers. The third case had a normal NT measurement and normal serum markers, and so would have been missed in the first trimester when NIPT was offered to women with a risk for T21 higher than 1 in 1,000. Assuming that NIPT could correctly identify all T21, T18, and T13 cases, we could potentially avoid over 300 procedures, performing invasive testing only in cases with NIPT high-risk results, but two cases of T21 would still have been missed [Ridnõi *et al.* 2021b]. Another model of using NIPT in the existing cFTS protocol showed that if all women with an increased risk (≥1:200) had an invasive test and it was performed up to a risk of 1:1,000, 95% of common trisomies/sex chromosome aberrations and 55% of atypical aberrations would be detected [Iwarsson and Conner 2020].

The use and uptake of NIPT varies greatly between different populations [Gadsboll *et al.* 2020]. In those countries where NIPT is reimbursed by public-health insurance the main factors influencing its uptake are the level of trisomy risk after cFTS, maternal education, maternal racial origin, pregnancy termination acceptance, and the possibilities of molecular diagnostics after invasive testing [Gil *et al.* 2015; Lou *et al.* 2018]. On the other hand there is a good evidence from a randomized trial (INVASIVE) that counselling before PS can shift women's preferences towards invasive diagnostics [Paz *et al.* 2020].

The main issues of NIPT implementation in routine PS systems are universal or contingent model, type of NIPT method, what would be the best in terms of additional benefits. There are reasonable concerns regarding using MPSS-based NIPT platforms as a first-tier screening test, such as managing incidental findings (RATs or submicroscopic chromosomal rearrangements) and the need for optimal and transparent pre-test counselling [Di Renzo *et al.* 2019; Jani *et al.* 2020]. The advances in US diagnostics should also be considered. NIPT cannot

substitute for first-trimester US examination. There is good evidence that the measurement of NT is still of importance even in the era of NIPT [Petersen *et al.* 2020; Sagi-Dain *et al.* 2021]. NIPT cannot diagnose fetal structural defects and negative NIPT results can sometimes be misleading for the management of pregnancy in the presence of fetal structural anomalies [Suzumori *et al.* 2021]. Therefore, in cases of fetal structural anomalies, invasive diagnostics rather than NIPT should be performed [Zhu *et al.* 2021].

Further studies are needed in Estonia to accurately estimate the effectiveness of NIPT implementation in the existing cFTS model of PS and the effect on perinatal outcomes.

5.2. The effectiveness of CMA in high-risk pregnancies

During the study period, cFTS was performed in 14,566 women with singleton pregnancies. In total, 334 CMA analyses were performed in two main indication groups. Group A comprised all patients with high-risk for trisomies after cFTS, but with a NT measurement below 3.5 mm and no US malformation. Group B comprised all patients who met the criteria for CMA as a first-tier diagnostic test based on the Estonian national PD guidelines [Ustav *et al.* 2016].

CMA was performed in 184 cases in Group A and in 150 cases in Group B. In total, 12 clinically significant pathogenic or likely pathogenic CNVs were found in both study groups (Figure 8), which gave an additional diagnostic yield of 3.6% (95% CI, 2.07–6.17%). Nine of these findings were, as expected, in Group B, where the diagnostic yield was 6.0% (95% CI, 3.27–11.29%). In Group A, the diagnostic yield of CMA was 1.6% (95% CI, 0.56–4.71%). Additionally, we found 21 benign or likely benign CNVs and 11 VOUS, with most of them being LCSH regions, which were not reported according to our laboratory protocol (Appendix 3) [Ridnõi *et al.* 2021a].

The main reason for dividing the cases into two groups was to compare the CMA diagnostic yield in terms of different indications. It was shown that clinically relevant CNVs were found in about 1.7% of fetuses when CMA was done with indications such as advanced maternal age, anxiety, or positive serum screening without US malformations [Wapner *et al.* 2012]. There is emerging evidence that CMA should be a first-tier diagnostic test in PD with all indications [Daum *et al.* 2021; Rodriguez-Revenga *et al.* 2020; Stern *et al.* 2020].

In the CMA study Group A, in which 184 CMA analyses were performed with broader indications, we found three pathogenic CNVs with clearly described associated phenotypes (Table 9). All of these had a normal conventional karyotype.

Among the pathogenic findings from Group A, a first case of 15q13.3 microdeletion of 1.6 Mb was diagnosed. This region of deletion covers five genes, including *FAN1* and *TRPM1*. In this particular case, microdeletion was inherited from an apparently healthy mother, so the prognosis can vary. A phenotypically normal baby was born with a normal early neonatal period. This child

will need close neurobehavioral follow-up [Ridnõi *et al.* 2021a]. This is a highly variable syndrome associated with an increased risk of intellectual disability, epilepsy, and autistic spectrum disorders. Heterozygous deletions in this region are inherited in approximately 85.4% of individuals with this syndrome [Lowther *et al.* 2015]. Commonly reported associations are developmental delay or intellectual disability, epilepsy, speech problems, autism spectrum disorders, and attention deficit disorders. Serious congenital anomalies are rare [Simon *et al.* 2019].

In a second case, a low-level mosaicism (10–20%) for monosomy X was found. Ultrasound examination of the fetus was normal and a postnatal examination of a child at the age of 1 year showed no developmental delay. Still, chromosomal analysis from the peripheral lymphocytes confirmed a mosaic Turner's syndrome [Ridnõi *et al.* 2021a]. In this case, regular follow-up by a pediatric endocrinologist and later by a gynecologist was recommended, due to the increased risk of infertility and endocrine disorders [Levitsky *et al.* 2015]. In a large population-based UK study of 244,000 women using UK Biobank data, the prevalence of mosaic Turner syndrome was estimated to be 76 in 100,000, but there was reduced penetrance in the adult population. The was no increased risk for cardiac disease or hypertensive disorders in these women, and the authors suggested that the clinical management of 45,X/46,XX individuals should be minimal [Tuke *et al.* 2019]. Still, there remain some concerns regarding pregnancy management and infertility issues in mosaic Turner syndrome [Calanchini *et al.* 2020].

The third pathogenic finding in Group A was a 3.7-Mb deletion in the 9q22.32q22.33 region. This region covers 23 genes, including *FANCC, PTCH1, ERCC6L2, HSD17B3, TDRD7, XPA, FOXE1, NANS,* and *GPR51.* A baby weighing 3,990 g was born at term and had dysmorphic features including trigonocephaly, hypoplasia of the eyebrow arches, postaxial polydactyly (a rudimentary finger on the left hand), broad nasal bridge, and dysmorphic ears [Ridnõi *et al.* 2021a]. This is a known 9q22.3 microdeletion syndrome in which craniosynostosis, hydrocephaly, macrosomia, and intellectual disability have been described [Muller *et al.* 2012]. This baby will need close follow-up due to a high risk of early craniosynostosis as well as early intellectual disability.

Additionally, we found five VOUS cases, four of which were reported (Appendix 3) as follows: a 1.9-Mb deletion in the 15q13.3q14 region inherited from the father; a *de novo* 0.5-Mb duplication in the 3p25.2 region; a 2.9-Mb duplication in the 8q21.13q21.2 region; and a 0.36-Mb duplication in the 15q11.2 region. The last variant was in a so-called susceptibility locus for neurodevelopmental disorders [Butler 2017] and so this child was followed-up by a medical geneticist and a pediatric neurologist.

The diagnostic yield of 1.6% (95% CI, 0.56–4.71%) in Group A was similar to those reported in several previous studies and reviews [Hillman *et al.* 2013; Stern *et al.* 2020; Vogel *et al.* 2018; Wapner *et al.* 2012]; however, compared to a recently published Danish study in which the CNV detection rate in screen-positive cases after cFTS was 2.3%, our number was smaller [Vogel *et al.*

2018]. This can be explained by the fact that the indication for the invasive diagnostics and CMA in our study was a risk higher than 1 in 100 after cFTS. By contrast, in the Danish study, the risk cut-off point was higher than 1 in 300.

In the CMA study Group B, 150 analyses were performed. We found nine clinically significant pathogenic or likely pathogenic CNVs (Table 9). Six pregnancies were terminated due to fetal malformations and three babies were born: two of these were cases of 15q11.2-region microdeletions and one was a case of a 0.1-Mb deletion in the 11p15.4 region [Ridnõi *et al.* 2021a].

In the first two 15q11.2 cases, the microdeletion was inherited from apparently healthy mothers, but this is a known region for possible intellectual disability and autistic spectrum disorders [Butler 2017], therefore these children will require thorough follow-up by a neurologist.

The literature on the 15q11.2 deletions is extensive but confusing. It has been stated that this deletion is associated with neurodevelopmental disorders, and mild enrichment of the deletion is observed in individuals with schizophrenia, epilepsy, and learning disabilities [Butler 2017]. A recent meta-analysis of previously published data on the 15q11.2 deletion recommends that this CNV should be classified as "pathogenic with a mild sized effect" and should not be discussed in prenatal settings [Jonch *et al.* 2019]. The changes in the classification and interpretation of this CNV are a good example of how difficult their prenatal interpretation can be.

In the second 11p15.4-deletion case, invasive diagnostics were performed due to a high-risk result after cFTS and the familial history. The mother had epsilon-gamma-delta-beta thalassemia due to an 11p15.4 microdeletion (OMIM 141900). The same microdeletion was diagnosed in the fetus. Thus, close antenatal surveillance was conducted. By week 30, the fetus had developed signs of anemia and an intrauterine blood transfusion was performed. After the procedure the fetus developed bradycardia and an emergency cesarean section was required. The hemoglobin level was 90 g/l after birth and several blood transfusions were performed. In this case, CMA provided relevant clinical information not only for postnatal management but also for antenatal surveillance [Ridnõi *et al.* 2021a].

The diagnostic yield of CMA in Group B was 6.0% (95% CI, 3.27–11.29%), which was similar to those in other studies and reviews [Chong *et al.* 2019; Hillman *et al.* 2013; Hui *et al.* 2021; Kin Chau and Choy 2021; Lin *et al.* 2020; Vogel *et al.* 2018; Wapner *et al.* 2012].

The incidence of VOUS between both CMA study groups was 3.3%. The Department of Clinical Genetics of TUH uses an SNP-array CMA platform. This allows evaluation of the presence of LSCH regions, although these findings are usually not reported prenatally due to the lack of corresponding phenotypic description and the difficulties in interpreting the results. LSCH regions are occasionally reported prenatally in cases where the clinician suspects a specific recessively inherited disease on the bases of US or postmortem findings [Ridnõi *et al.* 2021a]. Decisions about whether to report specific findings are made on a case-by-case basis [Pajusalu *et al.* 2015]. The

incidence of VOUS findings differs between studies and is dependent on the CMA technology used, local protocols, and difficulties of interpretation during fetal life [Levy and Wapner 2018; Mardy *et al.* 2020]. Reporting VOUS in the prenatal setting is challenging but is worthwhile, as some cases can change in significance over time [Mardy *et al.* 2020; Stosic *et al.* 2018].

The present study results were in concordance with previously published data. Recently, a large study of 10,377 pregnancies with CMA application in every case showed an overall prevalence of CNVs of 2.1%, but this figure was twice as high in the subgroup of fetuses with US anomalies [Lin *et al.* 2020]. It is currently suggested that all women who undergo invasive diagnostics should be informed about the additional benefits of CMA in PD and should be offered this test rather than conventional karyotyping [Rodriguez-Revenga *et al.* 2020]. It is essential in the present era of possibilities for NIPT that women receive unbiased information regarding the diagnostic effectiveness and potential of CMA in PD as well as the incidence of pathogenic CNVs even in low-risk pregnancies [Daum *et al.* 2021]. In high-risk pregnancies, CMA has a superior diagnostic performance and should be offered to all women instead of expanded NIPT panels [Zhu *et al.* 2021].

Based on the results of the present study, as well as the published literature, we suggest that there is sufficient scientific data to support the routine use of CMA in PD for every indication in Estonia. Considering the implementation of NIPT in Estonia as a second-tier screening test after cFTS, we would predict a drastic decrease in the invasive testing rate. It is therefore important that women receiving invasive procedures for the purpose of PD have the most accurate information about the latest advances in molecular genetic diagnostics. In every case in which CMA is applied, comprehensive pretest advice should be provided by a specialist who has received appropriate training in PD and genetic counselling.

Table 9. Pathogenic or likely pathogenic CMA results in both groups: indications, karyotype, CMA results, interpretation, clinical significance and outcome, from [Ridnõi *et al.* 2021a].

Pathogenic findings in CMA Group A					
Combined risk for T21, ultrasound findings	**Karyotype**	**CMA result**	**Interpretation**	**Clinical significance**	**Pregnancy outcome (weight, length, Apgar)**
1:96	46,XY	arr[GRCh37] 15q13.2q13.3(30955149_32509892)x1 mat, 1.6Mb	15q13.3 microdeletion syndrome	Pathogenic	4344 g, 53 cm. Apgar 9/ 9
1:44	46,XX	arr(X)x1[0.2]	Mosaic Turner syndrome (10–20%)	Pathogenic	3692 g, 52 cm. Apgar 9/9
1:11	46,XY	arr[GRCh37] 9q22.32q22.33(97598966_101270230)x1 dn, 3.7 Mb	9q22.3 microdeletion syndrome	Pathogenic	3990 g, 54 cm. Apgar 8/9
Pathogenic findings in CMA Group B					
Complicated anamnesis	not done	arr[GRCh37] 11p15.4(5228708_5343533)x1 mat, 0.1 Mb	Epsilon gamma delta beta thalassemia	Pathogenic	1100 g, Apgar 1/4/5
Aortic arch pathology on ultrasound	47,XY,+idic(22)(q11.21)dn	arr[GRCh37] 22q11.1q11.21(16854770_18656495)x4, 1.8 Mb	Cat eye syndrome	Pathogenic	Termination
Megacystis on ultrasound	47,XY,+der(7)t(7;12)(q11.21;p13.33)mat	arr[GRCh37] 7p22.3q11.21(46239_63729722)x3, 12p13.33(191619_916310)x3	unbalanced translocation	Pathogenic	Termination
Truncus arteriosus communis with interrupted aortic arch on ultrasound	46,XY	arr[GRCh37] 16p11.2(29634212_30199805)x1, 0.6 Mb	16p11.2 microdeletion syndrome	Pathogenic	Termination
Left ventricle hypoplasia on ultrasound	mos 45,X[16]/ 46,X,r(X)(p11.3q21.31)[4]	arr[GRCh37] Xp22.33p11.3(93118–46179305)x1, Xp11.3q21.31(46203386–89413918)x1~2, Xq21.31q28(89536405–155235833)x1	Turner syndrome mosaic variant	Pathogenic	Termination

Combined risk for T21, ultrasound findings	**Karyotype**	**CMA result**	**Interpretation**	**Clinical significance**	**Pregnancy outcome (weight, length, Apgar)**
Increased NT 8.90 mm	not done	arr[GRCh37] 15q11.2(22754322_23140114)x1 mat, 17p13.3(1665862_1680318)x0 mat,pat, 17p13.3p13.1(783580_9127010)x2 hmz	Osteogenesis imperfecta, type VI	Pathogenic	Termination
Combined risk for T21 1:4; Increased NT 9.0 mm	46, XX	arr[GRCh37] 6q23.1q23.2(130397515_134259753)x1, 3.8 Mb	6q23.1q23.2 microdeletion	Likely pathogenic	Termination
Increased NT 3.6 mm	46,XY	arr[GRCh37] 15q11.2(22754322_23652850)x1 mat, 0.9 Mb	15q11.2 microdeletion syndrome	Likely pathogenic/ Susceptibility locus	3950 g, 50 cm. Apgar 8/9
Increased NT 3.5 mm	not done	arr[GRCh37] 15q11.2(22754322_23140114)x1 mat, 0.4 Mb	15q11.2 microdeletion syndrome	Likely pathogenic/ Susceptibility locus	3328 g, 48 cm. Apgar 9/9

CMA – chromosomal microarray analysis; NT – nuchal translucency; T21 – trisomy 21; VOUS – variant of uncertain significance

5.3. The effectiveness of NGS for fetuses with high risk and/or combined anomalies

Out of 28 selected cases of dysmorphic fetuses, we found five pathogenic variants through NGS analysis related to fetal phenotype. Two variants of unknown clinical significance, but with relevant phenotypes and two incidental findings were also reported (with informed consent). The additional diagnostic yield of the NGS analysis in our study group was therefore 17.9% (95% CI, 7.88–35.59%). Detailed descriptions of the cases with pathogenic results and those with uncertain clinical significance are given in Table 10 [Ridnõi *et al.* 2021a].

The incidence of pathogenic variants in NGS and ES prenatal cohorts varies widely between published studies. Reasons for such differences include the selection of cases according to fetal anomaly, the number of probands in the cohorts, and the selection of the NGS method used [Ferretti *et al.* 2019; Kilby 2021; Monaghan *et al.* 2020]. The additional diagnostic yield reported in NGS studies, most of which used ES, ranged from 8.5% to 10% in large unselected cohorts of 610 [Lord *et al.* 2019] and 234 [Petrovski *et al.* 2019] fetuses, respectively, to 81% [Chandler *et al.* 2018] in a small series of 16 fetuses, with a strong suspicion of skeletal dysplasia, using a targeted sequencing panel. A weighted diagnostic rate of 19% was reported in a recent systematic review on the performance of ES in PD [Guadagnolo *et al.* 2021].

Besides the general limitations associated with NGS several considerations are specific to prenatal settings. These include sample type and quality, variant calling and filtration, genotype–phenotype correlations, interpretation, turnaround time (TAT), and reporting [Abou Tayoun and Mason-Suares 2020]. The TAT of NGS analyses is an important issue in the prenatal setting, especially in countries like Estonia where pregnancy termination is not allowed after 22+0 weeks. The mean TAT of rapid ES using targeted gene panels was 10 days (with a range of 4–28 days) in an observational study from the Netherlands [Deden *et al.* 2020]. A rapid TAT is of great importance in terms of the clinical utility of ES. It has been shown that ES results can influence the management of pregnancy, delivery, and the postnatal period in 35% of cases [Tolusso *et al.* 2021].

ES increases the possibility of definitive diagnosis, but also increases the likelihood of identifying VOUS and incidental findings [Monaghan *et al.* 2020]. Similarly to CMA, challenges with reporting VOUS arise. It is important to follow standardized variant classifications [Richards *et al.* 2015] to separate pathogenic VOUS and benign variants. In fetal diagnostics, reporting VOUS is even more challenging than in post-natal cases due to the limited options for follow-up and phenotyping; however, in some cases reporting VOUS variants may still be considered, for example if the known phenotype associated with the gene is consistent with US findings [Monaghan *et al.* 2020]. In our study, there were two such examples: one case in which *PKD1* VOUS was reported for a

fetus with polycystic kidneys and another case in which a *NOTCH1* variant was reported after the US finding of a heart defect [Ridnõi *et al.* 2021a].

Managing the incidental or secondary findings raises several ethical and clinical dilemmas. According to the ACMG guidelines, secondary or unsolicited findings may be reported after adequate counselling [Monaghan *et al.* 2020]. Two pathogenic secondary findings in the cancer-predisposing genes *MLH1* and *BRCA1* were detected in this cohort and reported back to the ordering physician for cascade screening of family members. This illustrates how NGS-based tests may have additional benefits that are outside of the scope of fetal medicine [Ridnõi *et al.* 2021a]. The recent recommendations of the European Society of Human Genetics regarding opportunistic genomic screening include advice to be cautious when reporting secondary findings in GS [de Wert *et al.* 2020]. The overall incidence of inconclusive findings after prenatal ES is 12%. The incidence of secondary or incidental findings is difficult to estimate due to the small numbers of studies in which such findings have been reported [Guadagnolo *et al.* 2021].

In a previous study by our department, in which the NSG diagnostic performance was investigated in an adult and pediatric population, a diagnostic yield of 26.3% was reported [Pajusalu *et al.* 2018]. The present study is the first report from our department to use NGS in PD in a group of 28 probands showing good results for the detection of genetic disease. Our results are in accordance with previously published data on small cohorts of selected fetuses [Alamillo *et al.* 2015; Deden *et al.* 2020; Drury *et al.* 2015; Tolusso *et al.* 2021]. NGS has revolutionized clinical practice in medical genetics with its ability to rapidly analyze large sets of genes. Therefore, we can expect a significant impact on both genetic research and clinical diagnostics [Guadagnolo *et al.* 2021]. More research is needed for better delineation of the malformation groups in which NGS will give the best diagnostic results.

Table 10. Reported findings in the NGS study group [Ridnõi *et al.* 2021a].

Fetal findings, indication for NGS	Result	Zygosity	Pregnancy outcome	Classification	Inheritance	OMIM disease
Agenesis of corpus callosum. Dysmorphic features on autopsy.	NM_015443.3(KANSL1):c.1652+1G>A	Heterozygous	TOP	Pathogenic	de novo	Koolen-de Vries syndrome (OMIM#610443)
NT 8.0 mm. Fetal hydrops	NM_002295.5(RPSA):c.413_417del p.(Ser138Cysfs*2)	Heterozygous	TOP	Pathogenic	de novo	Isolated congenital asplenia (OMIM#271400)
Multiple anomalies. Malrotation of the bowl, cardiac anomaly on autopsy	NM_001492.5(GDF1):c.909dup p.(Val304Argfs*48)	Homozygous	TOP	Pathogenic	parents are hetero-zygous carriers	Right atrial isomerism, Ivemark syndrome (OMIM#208530)
Bilateral ventriculomegaly. Corpus callosum dysgenensis.	NM_017791.2(FLVCR2):c.927C>A p.(Asn309Lys); NM_017791.2(FLVCR2):c.952G>T p.(Gly318Cys)	Compound heterozygous	TOP	Pathogenic	parents are hetero-zygous carriers	Fowler syndrome (OMIM#225790)
Facial cleft, absence of right kidney. Agenesis of spleen.	NM_032458.2(PHF6):c.241–1G>A	Heterozygous	TOP	Pathogenic	de novo	Borjeson-Forssmann-Lehmann syndrome (OMIM#301900)
Bilateral polycystic dysplastic kidneys.	NM_000296.3(PKD1):c.8302G>A p.(Val2768Met)	Heterozygous	Livebirth	VOUS	carrier status not known	Polycystic kidney disease 1 (OMIM#173900)
VSD, truncus arteriosus communis.	NM_017617.4(NOTCH1):c.680G>A p.(Cys227Tyr)	Heterozygous	TOP	VOUS	the mother is a healthy carrier	Notch receptor 1 (OMIM#190198)
Agenesis of corpus callosum. Additional spleen. Fetal hydrops.	NM_000249.2(MLH1):c.1168del p.(Glu390Asnfs*11)	Heterozygous	TOP	Incidental finding	paternal	Lynch syndrome (OMIM#120435)

Fetal findings, indication for NGS	Result	Zygosity	Pregnancy outcome	Classification	Inheritance	OMIM disease
Spina bifida in thoracic region. Facial dysmorphism.	NM_007300.3(BRCA1):c.4035del p.(Glu1346Lysfs*20)	Heterozygous	TOP	Incidental finding	mother is not a carrier	Familiar breast – ovarian cancer (OMIM#113705)

NGS – next-generation sequencing; NT – nuchal translucency; TOP – termination of pregnancy; VOUS – variant of uncertain clinical significance; VSD – ventricular septum defect.

5.4. Prenatal phenotypes of SGB syndrome

At the time of the first-trimester US examination a dichorionic/diamniotic twin pregnancy was confirmed. The NT measurements were increased (5.25 mm and 4.18 mm, respectively). No anatomical defects were noted. CVS was performed, according to the PD guideline, due to the increased NT measurements. The karyotype of both twins was normal (46, XY) [Ridnoi *et al.* 2018].

A second US examination was performed at 20+2 weeks. The scan revealed numerous fetal anomalies. Dysmorphic features which were found in both twins including a flattened fetal profile with considerable prefrontal oedema, hypertelorism, dysgenesis of the corpus callosum, hepatomegaly, mildly enlarged hyperechogenic kidneys, and micropenis. Additionally, hypoplastic cerebellum was noted in one twin and one exhibited aberrant right subclavian artery (ARSA) with suspicion of double aortic arch (Figures 12–14). Both fetuses were near the 90th weight centile and had also marked polyhydramnios.

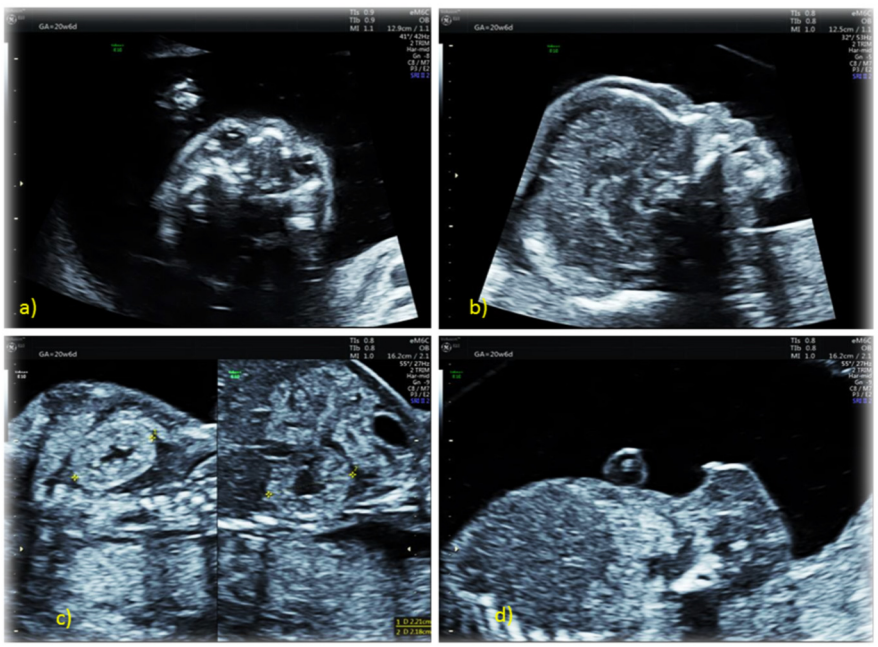

Figure 12. Dysmorphic features of fetuses at 20-week scan. a) Hypertelorism (proband 1). b) Prefrontal edema and flat facial profile (proband 2). c) Hyperechogenic kidneys. d) Micropenis (proband 1). Reproduced from [Ridnoi *et al.* 2018], with permission.

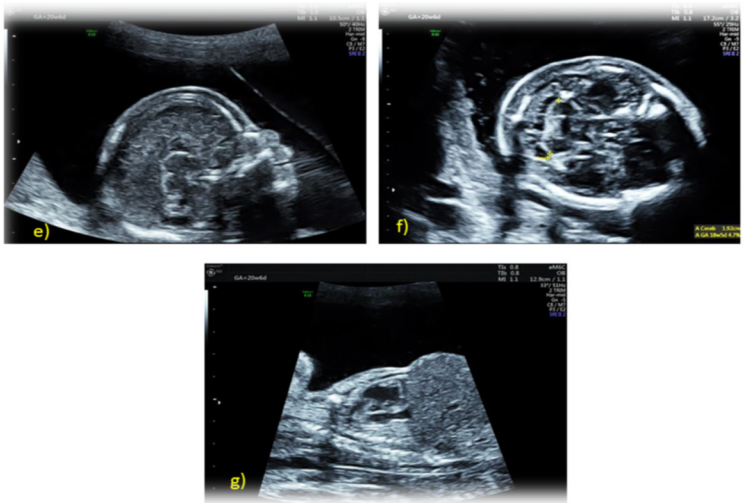

Figure 13. Dysmorphic features of fetuses at 20-week scan. e) Dysgenic corpus callosum and flat fetal profile (proband 1). f) Hypoplastic cerebellum (proband 1). g) Enlarged liver (proband 2). Reproduced from [Ridnoi *et al.* 2018], with permission.

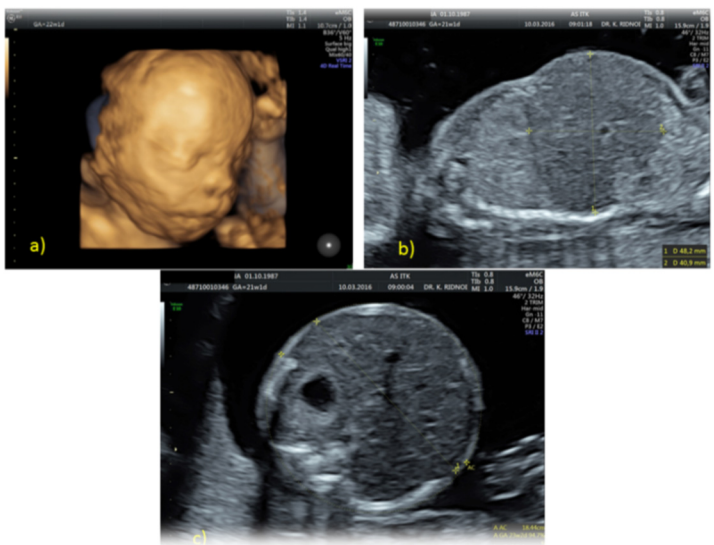

Figure 14. Dysmorphic features fetuses at week 22 scan. a) Round, edematous face (proband 2). b) Measurements of fetal liver (proband 1): antero-posterior (AP) length 48.2 mm, cranio-caudal (CC) length 40.2 mm. c) Abdominal circumference (proband 1) with a transverse width of the liver of 56.4 mm. Reproduced from [Ridnoi *et al.* 2018], with permission.

CMA was performed in one twin. A small (1 Mbp) microduplication was found in the 22q11.2–11.3 region, which was not consistent with the profound fetal findings. The couple were counselled by a team of clinical geneticists and fetal medicine specialists, according to the fetal findings. The conclusion was that there was a strong clinical suspicion of a genetic syndrome. Due to the US findings, the option of pregnancy termination, with post-mortem examination and ES, was offered. The patient refused the termination and continued the pregnancy. Severe polyhydramnios had developed at 24+6 weeks and despite tocolysis preterm labor occurred at 25+2 weeks. Two premature boys were born, weighing 1,044 g and 1,090 g and with Apgar scores of 2/4/6 and 5/4/7 respectively, both of which were on the 75th weight centile [Ridnoi *et al.* 2018].

Postnatal phenotypic findings included marked macroglossia, hypertelorism, low-set ears, contractures of the fingers, and dysmorphic genitalia. General body oedema in both neonates was also noted. Difficult intubation was performed for both twins due to macroglossia. All clinical findings were described by the neonatologist. After stabilization, both babies were transferred to the neonatal intensive care unit of Tallinn Children's Hospital. The first baby died on the 6th day of life due to acute necrotizing enterocolitis. The second twin died 2 days prior due to infant respiratory distress syndrome [Ridnoi *et al.* 2018].

A pathoanatomical autopsy was performed for both twins. The main findings included hypertelorism, cardiomegaly, and hepato-splenomegaly. Both twins had enlarged kidneys and adrenal glands. Both had cryptorchidism. No cardiac malformations were found.

To investigate the molecular etiology of any possible genetic syndrome, parent–offspring trio ES was performed on the fetal DNA. This identified a hemizygous splice site variant in the *GPC3* gene, NM_004484.3:c.1166+1G>T. The mother was a heterozygous carrier [Ridnoi *et al.* 2018]. This variant had not been previously described in the BIOBASE Human Gene Mutation Database (HGMD Professional) [Stenson *et al.* 2009] or the Exome Aggregation Consortium (ExAC) databases [Lek *et al.* 2016]. Splice-site variants usually cause a loss of protein function; therefore, the detected variant was classified as pathogenic according to the ACMG guidelines [Richards *et al.* 2015]. Pathogenic hemizygous variants in the *GPC3* gene cause SGB syndrome [Vuillaume *et al.* 2018].

In the following pregnancy, PD started in the first trimester. Right-sided facial cleft and NT enlargement were diagnosed at the US examination for cFTS. At this time, CVS was not performed, because ES results from the previous pregnancy were pending. The diagnosis of cleft palate and lip on the right side, dysmorphic male genitalia, and flattened facial profile with prefrontal edema was confirmed during a follow-up scan at week 17+0. Amniocentesis with targeted sequencing of fetal DNA was performed at 17+0 weeks. The same hemizygous c.1166+1G>T pathogenic variant in the *GPC3* gene was identified. Thus, SGB syndrome type 1 was molecularly confirmed in this fetus. Multi-

disciplinary counselling regarding prognosis of SGB syndrome was conducted and the patient opted for pregnancy termination [Ridnoi *et al.* 2018].

The pathoanatomical findings of the fetus were consistent with SGB syndrome: macrosomia (590 g), hypertelorism, macrostomia, and hepatosplenomegaly with three additional spleens. The fetus had a right-sided cleft of lip and hard palate (Figure 15). No cardiac malformations were found.

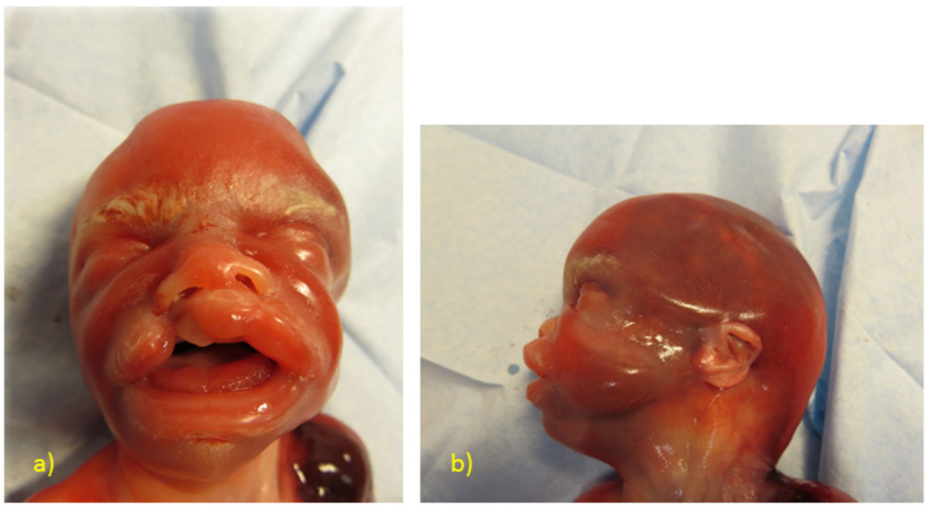

Figure 15. Pathoanatomical findings in proband 3. a) Right-sided cleft lip and palate and macrostomia. b) Facial edema. Reproduced from [Ridnoi *et al.* 2018], with permission.

SGB syndrome is usually diagnosed during the postnatal period [Manor and Lalani 2020]. Distinctive features of this syndrome are high birth weight, organomegaly (of the liver, spleen, and kidneys), facial clefts, cardiac malformations, abnormal genitalia, and CNS anomalies [Tenorio *et al.* 2014]. Some of the typical features of SGB syndrome can present during the antenatal period and can be seen as early as the first trimester of pregnancy [Li and McDonald 2009; Reischer *et al.* 2021; Ridnoi *et al.* 2018]. In suspected cases of SGB syndrome, CMA and ES can be used to aid diagnosis [Kehrer *et al.* 2016; Reischer *et al.* 2021; Xiang *et al.* 2020]. A recent review of published prenatal cases evaluated US features of SGB syndrome based on the 60 examples available at that time, including the three fetal cases described above [Ridnoi *et al.* 2018]. Our SGB syndrome case in twins was the first known report of this disease in multiple pregnancy to be diagnosed prenatally. Another case of SGB syndrome in a twin pregnancy was published recently, in which only one twin was affected [Reischer *et al.* 2021]. The prenatal findings were similar to our

case, but also included diaphragmatic hernia and malposition of the heart. Typical prenatal findings of SGB syndrome are summarized in Table 11.

Table 11. Estimated incidence of prenatal US findings in SGB syndrome. Incidence percentage adapted from [Ridnoi *et al.* 2018].

Prenatal findings	Proband 1	Proband 2	Proband 3	Incidence (%)
Macrosomia/overgrowth	+	+	+	86
Polyhydramnios	+	+		70
Organomegaly	+	+	+	60
Renal anomalies	+	+		32
CDH				30
Enlarged NT/NF	+	+	+	28
Craniofacial anomalies	+	+	+	13
Cardiac anomalies				13
Elevated MSAFP				12
Flat fetal profile	+	+	+	10
Genital anomalies	+	+	+	8
Ventriculomegaly				7
Cystic hygroma				5
Facial cleft			+	5
CNS anomalies	+	+		5
Polydactyly				5
Omphalocele				5
Skeletal anomalies				3
SUA				1.6

CDH – congenital diaphragmatic hernia; CNS – central nervous system; MSAFP – maternal serum alfa fetoprotein; NF – nuchal fold; NT – nuchal translucency; SUA – single umbilical artery.

The main prenatal feature of SGB syndrome is fetal macrosomia. This has been reported in 86% of cases. In the present case series, all three fetuses were macrosomic by week 20 of pregnancy [Ridnoi *et al.* 2018].

The next most frequent prenatal finding in SGB syndrome is polyhydramnios, which is reported in 70% of cases [Ridnoi *et al.* 2018]. Extreme polyhydramnios was a reason for the early preterm birth in the first pregnancy of our patient. The incidence of preterm birth in pregnancies with SGB syndrome is not clear. The largest review of a prenatal series with SGB syndrome in which pregnancy outcomes were followed reported a high proportion of preterm births (13/42, 31%), while most of the cases were moderately premature [Cottereau *et*

al. 2013]. Four pregnancies in that series were terminated late in the second trimester; therefore, the true incidence of preterm birth could be even higher. In a recent case report from China, polyhydramnios was also described in pregnancies affected with SGB syndrome [Xiang *et al.* 2020]. It is likely that the reason for most preterm deliveries in cases with SGB syndrome is a marked excess of amniotic fluid.

The internal organ anomalies that are seen in SGB prenatal cases are listed in Table 9. The most frequent of these are organomegaly (reported in 60% of prenatal cases), renal malformations (32%), and congenital diaphragmatic hernia (30%). Omphalocele is rare in SGB syndrome (reported in 5% of prenatal cases) [Ridnoi *et al.* 2018]. Cardiac malformations in SGB syndrome are common: in a reported series of 101 cases (mostly postnatal), structural cardiac anomalies were found in 26% [Lin *et al.* 1999]. In a review of the published prenatal cases, cardiac anomalies were present in only 13% [Ridnoi *et al.* 2018]. This difference was attributed to the fact that some minor cardiac anomalies are difficult to diagnose prenatally and are therefore identified only after birth.

The distinctive facial features of individuals with SGB syndrome, which are usually described as 'coarse', may already be present in the fetal stage. A 'flat' fetal profile has been described in 10% of prenatal cases [Ridnoi *et al.* 2018]. All three of the cases in the present series had prefrontal edema.

NT is a well-known marker for chromosomal anomalies. Enlarged NT may be present in cases of chromosomal pathologies [Kagan *et al.* 2006] and certain genetic disorders, especially Noonan syndrome [Pergament *et al.* 2011]. An increased NT measurement was described in 2009, during a first-trimester scan, in a fetus that was later diagnosed with SGB syndrome [Li and McDonald 2009]. In a recent report of SGB syndrome in a twin pregnancy, an enlarged NT measurement was also seen in the affected fetus [Reischer *et al.* 2021]. It is difficult to estimate the incidence of increased NT measurement as a marker of SGB syndrome because most published cases do not include data from the first-trimester scan. Among 47 cases that were presumed to have first-trimester scan information available, increased NT or nuchal fold measurements were found in 13 (28%) [Ridnoi *et al.* 2018]. In a French review of SGB syndrome in which prenatal symptoms were described, it remained unclear whether NT measurements were available for all cases [Cottereau *et al.* 2013]. Increased NT measurement could in reality be present in a larger proportion of SGB syndrome cases, but more observational data are needed to support this assumption. In all three of the fetuses presented in this case series, NT measurements were markedly increased, which was the reason for further chromosomal and genetic investigations [Ridnoi *et al.* 2018].

SGB syndrome is inherited in an X-linked recessive manner [Vuillaume *et al.* 2019]. If the mother of a proband has a pathogenic variant, the chance of its transmission in each pregnancy is 50%. Males who inherit the pathogenic variant will be affected. Females who inherit the pathogenic variant will be carriers, although due to X-chromosome inactivation they may also show various manifestations of SGB syndrome, including intellectual disability, heart

defects, and coarse facial features [Schirwani *et al.* 2019]. Males with SGB syndrome will pass the pathogenic variant to all of their daughters and none of their sons [Sajorda *et al.* 1993]. During genetic counselling, the possibilities of oocyte donation and preimplantation genetic diagnosis (PGD) may be discussed.

Differential diagnosis of SGB syndrome is challenging due to similarities in its clinical presentation with other overgrowth syndromes [Manor and Lalani 2020]. A diagnostic pathway in PD for these conditions was proposed in 2009 [Vora and Bianchi 2009], suggesting that many factors should be considered, including pregnancy dating and the possibility of gestational diabetes. However, in terms of genetic syndromes, the following five are the most likely: Pallister–Killian (OMIM 601803), Sotos (OMIM 117550), Perlman (OMIM 267000), Beckwith–Wiedemann (OMIM 130650), and SGB. From a pediatric perspective, additional possible overgrowth syndrome are Weaver syndrome, Malan syndrome, and DNMT3A-related overgrowth syndrome [Brioude *et al.* 2019; Manor and Lalani 2020]. These are rarely diagnosed prenatally. The main overlap with clinical presentation and US findings in the prenatal setting for SGB syndrome is with BWS, which is the most frequent overgrowth syndrome [Manor and Lalani 2020]. Despite each having specific traits, overgrowth syndromes often share clinical features. This is particularly true for patients with BWS or SGB who can have macroglossia, macrosomia, umbilical hernia and almost the same spectrum of internal organs' malformations. Careful and systematic evaluation of the fetus or newborn is required to reach to the precise diagnosis in cases with suspected overgrowth syndrome. The differential diagnosis of SGB syndrome with detailed description of similar overgrowth syndromes can be found in a review article from our department [Ridnoi *et al.* 2018].

5.5. Pathogenic variant in the *TXNDC15* gene in a prenatally diagnosed case of MKS

The diagnostic US examination was performed in the first trimester of pregnancy. Numerous fetal anomalies were revealed on US examination comprising enlarged NT (4.1 mm), bilateral polycystic kidneys, occipital encephalocele, and postaxial polydactyly of the hands and feet (Figure 16). Based on the US examination, there was a strong suspicion of MKS. After multidisciplinary counselling, the patient decided to terminate the pregnancy with post-mortem pathoanatomical evaluation of the fetus and genetic testing of the fetal DNA.

The initial targeted NGS analysis was performed using an Illumina TruSight One sequencing panel. No pathogenic variants were found in the *MKS1*, *TMEM216*, *TMEM67*, *CEP290*, *RPGRIP1L*, *CC2D2A*, *NPHP3*, *TCTN2*, *B9D1*, and *B9D2* genes, which are known to be associated with MKS. Subsequently, parent–offspring trio ES was performed.

After ES of fetal DNA two compound heterozygous variants in the *TXNDC15* gene we identified: NM_024715.3:c.211dup p.(Gln71Profs*32)

rs780024847 (inherited from the father) and NM_024715.3:c.635T>C p.(Leu212Pro) rs760579409 (inherited from the mother). Sanger sequencing confirmed these variants, which have never been described before in association with genetic disorders [Ridnoi *et al.* 2019]. According to the gnomAD database, such allele variants are very rare in the general population, with allele frequencies of 0.0029% and 0.00041%, respectively [Lek *et al.* 2016]. Based on these findings, we concluded that in our case, two pathogenic variants in the *TXNDC15* gene are likely to be the cause for the congenital malformations and MKS diagnosis [Ridnoi *et al.* 2019]. The family recurrence risk for affected offspring is therefore 25%.

The autopsy showed occipital encephalocele (0.9 × 1.0 cm) and bilateral enlarged polycystic kidneys (1.4 × 0.7 × 0.5 cm) with a total tissue mass of 0.503 g (normal values at 12 weeks = 0.16 ± 0.04 g [Enid Gilbert-Barness 2007]). Bilateral polydactyly of the hands (six fingers) and feet (seven toes) was also noted. No other anomalies were found. A histological study confirmed polycystic dysplastic kidneys (Figure 17).

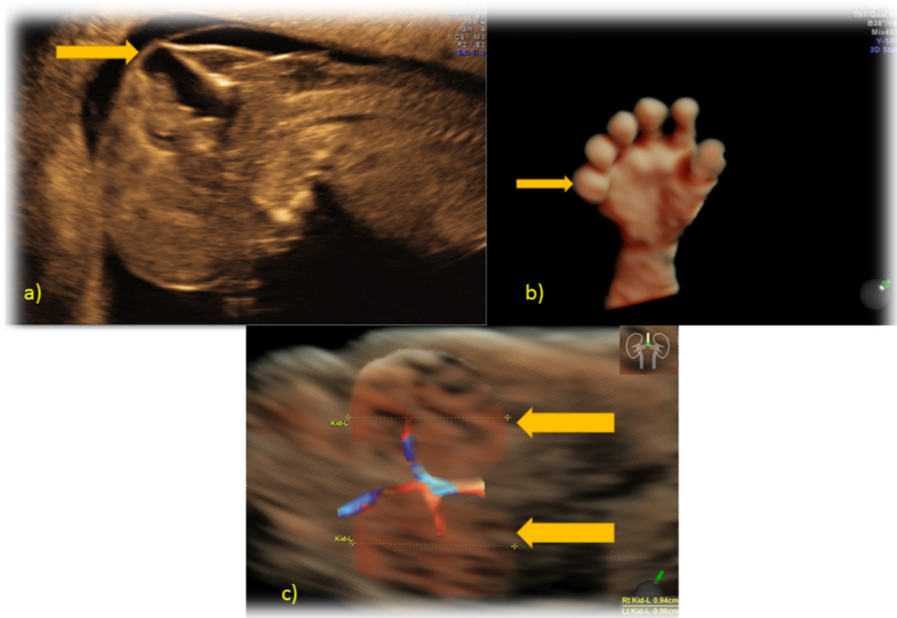

Figure 16. a) Occipital encephalocele in two-dimensional (2D) high-resolution transvaginal US: the midsagittal view shows the large occipital bone defect through which the meninges and cerebral parenchyma have migrated. b) Postaxial polydactyly of one hand in three-dimensional (3D) high-resolution transvaginal US in surface mode demonstrating a postaxial view of the extra (sixth) digit. c) Cystic renal dysplasia in 3D high-resolution transvaginal US in constructed glass-body mode: the coronal view shows enlarged hyperechoic kidneys leading to distention of the abdomen. Adapted from [Ridnoi *et al.* 2019].

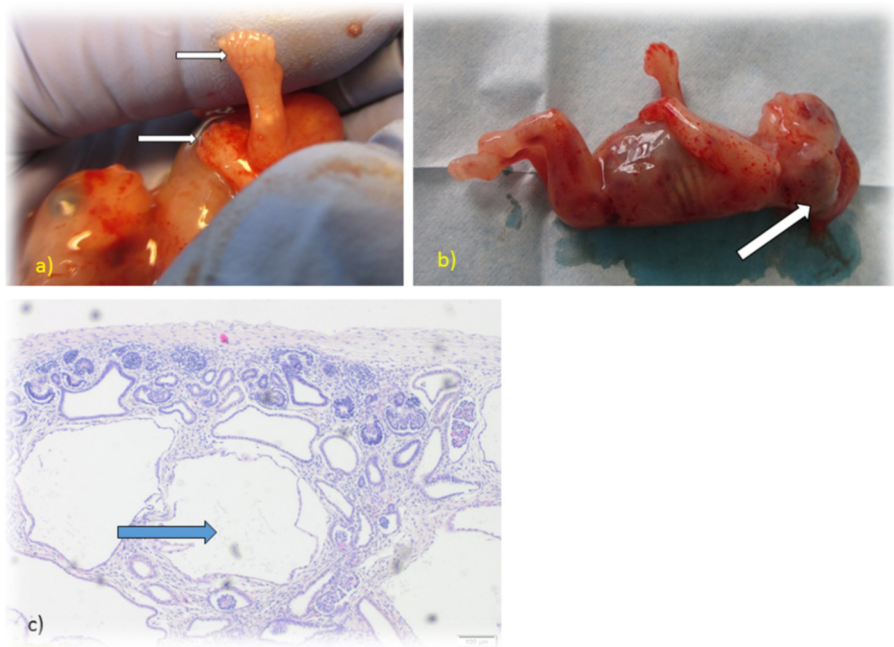

Figure 17. a) A fetus after termination of pregnancy. Postaxial polydactyly is seen in the six fingers and seven toes (arrows). b) Occipital encephalocele (arrow). c) Histological findings in the fetal kidneys. included a thin intermittent cortex with dysplastic cystic structures of varying sizes lined with a single layer of immature cubic nephrogenic epithelium. Adapted from [Ridnoi *et al.* 2019].

Homozygous pathogenic variants in the *TXNDC15* gene causing MKS have been reported in one publication before our case, where MKS was described in three consanguineous families: two Saudi and one Pakistani [Shaheen *et al.* 2016]. The prenatal findings in one stillbirth were typical for MKS: polydactyly, enlarged cystic kidneys, and occipital encephalocele. In the first Saudi family, a homozygous NM_024715.3:c.672_686del:p.(Ser225_His229del) pathogenic variant was identified. The second Saudi family had several affected pregnancies with typical MKS findings, and in their case, a homozygous NM_024715.3:c.103+1G>A variant was present. The third family in the report was of Pakistani origin and had two children with MKS; the pathogenic homozygous variant NM_024715.3:c.956dupT,(p.Ser321Lysfs*15) was identified in that case [Shaheen *et al.* 2016].

In the present case, we report same prenatal findings: bilateral postaxial polydactyly, occipital encephalocele, and bilateral polycystic kidneys caused by compound heterozygous variants in the *TXNDC15* gene. Recent experimental findings using a CRISPR-based screen for ciliary disorders suggest that the *TXDNC15* gene, which encodes a thioredoxin domain–containing transmembrane protein, is indeed a novel MKS gene [Breslow *et al.* 2018]. In our case we have identified frameshift variant NM_024715.3:c.211dup

p.(Gln71Profs*32), which causes reading frame change and results in formation of premature stop codon, which likely initiates nonsense-mediated decay eliminating mRNA and altering the protein synthesis in this frame. In missense variant NM_024715.3:c.635T>Cp.(Leu212Pro)rs760579409, which located in thioredoxin domain, we see two possibilities: damaging the protein function, with possible residual activity or generation of new splice-site exon in sequence which also can alter protein synthesis in this frame [Ridnoi *et al.* 2019].

After our case, a pathogenic homozygous variant c.844C>T, p.(Arg282Ter) in *TXNDC15* was reported in an Indian family also confirming that is a causative gene of MKS [Radhakrishnan *et al.* 2019]. In addition to classical fetal US findings of MKS holoprosencephaly, univentricular heart and bilateral bowing of tibia and fibula were described in this case. In other report an unusual finding of anencephaly was described with classical MKS 'triad" [Yaqoubi and Fatema 2018]. Although MKS is very specific in US presentation certain differential diagnostic difficulties can occur in cases of severe Smith–Lemli–Opitz (microcephaly, polydactyly), T13 or Patau syndrome (holoprosencephaly, polydactyly, heart defects) or Joubert syndrome (cerebellar anomalies, polydactyly, cystic renal lesions), which is also a ciliopathy [Khurana *et al.* 2017].

MKS is a heterogeneous syndrome in terms of the linked causative genes, but genotype–phenotype associations in particular genes are not fully understood. For example pathogenic variants in the *TCTN2* gene are associated with MKS, but experimental data show that this gene is not required for ciliogenesis in the kidney [Zhang *et al.* 2020]. In the first-line diagnosis, we used an NGS-based large gene panel, but only 10 MKS genes were available on the platform used. In the case of prenatal US findings that are highly suggestive of MKS and a negative NGS MKS gene panel, we recommend performing ES not to miss rare causative genes linked to MKS based on our case report and the available literature [Ridnoi *et al.* 2019].

5.6. Practical implication

The practical value of this work is to develop an evidence-based protocol for future directions in PS and PD in Estonia. Current study showed the effectiveness of existing national PS program, based on the universal cFTS and parallel to that we showed the positive implications of applying DNA based molecular analyses into routine PD model. In future, the results of our study can be used as a scientific base for further investigations. Possible directions of such investigations should include studies on routine use of CMA in the era of NIPT and larger studies on the application of NGS methods in PD in fetuses with congenital anomalies.

6. CONCLUSIONS

1. A two-year prospective cFTS-effectiveness study was conducted in a cohort of 14,566 pregnancies in two major hospitals in Estonia.
   1.1. cFTS was found to be an effective screening method for the prenatal detection of three major trisomies: the DR was 94% for T21, and 100% for T18 and T13, in the first trimester of pregnancy.
   1.2. Compared to the previous Estonian study, the DR of T21 increased from 88.3% to 94%.
   1.3. Our study results showed that the shift towards cFTS in Estonia led to an increase in the prenatal detection of chromosomal diseases and was justified.
   1.4. The invasive-testing rate of 2.7% after cFTS was lower in comparison with other published studies and local Estonian data from previous years.
2. The diagnostic effectiveness of CMA was investigated in a cohort of 334 fetuses with normal karyotype and high-risk results after cFTS, US anomalies, or specific genetic indications.
   2.1. CMA had a diagnostic effectiveness of 3.6% in high-risk pregnancies after cFTS or fetal US examination.
   2.2. In a subgroup of fetuses with high-risk results for trisomies after cFTS but normal US findings, CMA discovered clinically relevant CNVs in 1.6% of cases. This finding was in the accordance with previously published larger studies. It supported the use of CMA as a primary diagnostic test in cases with high-risk results after cFTS.
   2.3. The probability of pathogenic CNV in high-risk pregnancies after cFTS was 1 in 62 according to our results. Women should be aware of and appropriately counselled regarding the possibilities of CMA diagnostics.
   2.4. The use of CMA as a first-tier prenatal diagnostic test in pregnancies with fetal US anomalies or genetic indications had an additional diagnostic value of 6.0% over conventional karyotyping. This finding was in accordance with published studies.
   2.5. The VOUS incidence in the whole cohort of 334 CMA was 3.3%, which was lower than reported in most large CMA studies.
   2.6. Most of the benign findings were LSCH regions and were not reported in PD.
3. The diagnostic effectiveness of NGS analysis using a large-gene panel was investigated in a cohort of 28 fetuses with combined US anomalies and normal CMA.
   3.1. The diagnostic effectiveness of NGS analysis in pregnancies with combined US anomalies was 17.9%. This result was similar to other studies published in the literature, where the DR of pathogenic variants

after prenatal NGS analyses or ES ranged from 8.5% to 81%, depending on the inclusion criteria for fetal malformations.

3.2. We reported two VOUS findings after NGS analysis in *NOTCH1* and *PKD1* genes that were related to fetal phenotype. In the *NOTCH1* variant, we diagnosed a combined heart defect in the fetus, but the carrier mother was phenotypically normal. In the *PKD1* variant, we diagnosed polycystic kidneys in the fetus, but the parents were lost to follow-up.

3.3. We reported two clinically relevant incidental findings in the cancer-predisposing genes *MLH1* and *BRCA1*, which were unrelated to fetal phenotype, and these findings lead to cascade screening of the family members.

4. We reported three cases of SGB syndrome in two consecutive pregnancies. The prenatal findings of this syndrome were analyzed in detail with a review of the previously published prenatal cases.

4.1. We reported a novel hemizygous splice-site variant in the *GPC3* gene, NM_004484.3:c.1166+1G>T, which was diagnosed in three fetuses from two consecutive pregnancies.

4.2. The most frequent prenatal findings in SGB syndrome were macrosomia, polyhydramnios, organomegaly, and renal anomalies, which were detectable by detailed US examination.

4.3. Other overgrowth syndromes should be considered in differential diagnosis, particularly BWS.

5. A case of MKS was diagnosed prenatally and described in detail in terms of the antenatal US findings, the molecular findings of ES, and the pathoanatomical findings of the fetus.

5.1. We classified two compound heterozygous *TXNDC15* variants with no previous clinical annotations as disease causing: NM_024715.3: c.211dup p.(Gln71Profs*32) rs780024847 and NM_024715.3:c.635T> C p.(Leu212Pro) rs760579409. Association of this gene with MKS was previously reported only once in the literature.

5.2. Ultrasound anomalies of the fetus, molecular findings, and pathoanatomical features in our cases supported previously published experimental findings, suggesting that the *TXNDC15* gene is a novel MKS-related gene.

7. APPENDIX

Appendix 1. Protocol for the measurement of fetal NT thickness. From FMF webpage.

- The gestational period must be 11 to 13 weeks and six days.
- The fetal crown-rump length (CRL) should be between 45 and 84 mm.
- The magnification of the image should be such that the fetal head and thorax occupy the whole screen.
- A mid-sagittal view of the face should be obtained. This is defined by the presence of the echogenic tip of the nose and rectangular shape of the palate anteriorly, the translucent diencephalon in the center, and the nuchal membrane posteriorly. Minor deviations from the exact midline plane would cause non-visualization of the tip of the nose and visibility of the maxilla.
- The fetus should be in a neutral position, with the head in line with the spine. When the fetal neck is hyperextended, the measurement can be falsely increased, and when the neck is flexed, the measurement can be falsely decreased.
- Care must be taken to distinguish between fetal skin and amnion.
- The widest part of translucency must always be measured.
- Measurements should be taken with the inner border of the horizontal line of the calipers placed on the line that defines the NT thickness – the crossbar of the caliper should be such that it is hardly visible as it merges with the white line of the border, and not in the nuchal fluid.
- In magnifying the image (pre or post freeze zoom) it is important to turn the gain down. This avoids the mistake of placing the caliper on the fuzzy edge of the line, which causes an underestimate of the nuchal measurement.
- During the scan, more than one measurement must be taken and the maximum value that meets all of the abovementioned criteria should be recorded in the database.
- The umbilical cord may be round the fetal neck in about 5% of cases and this finding may produce a falsely increased NT. In such cases, the measurements of NT above and below the cord are different and, in the calculation of risk, it is more appropriate to use the average of the two measurements.

Appendix 2. cFTS characteristics of pregnancies affected with chromosomal disease, including three T21 false-negative cases.

Case	Age	CRL	NT	ßhCG MoM	PAPP-A MoM	Adjusted risk T21 1:	Adjusted risk T18 1:	Adjusted risk T13 1:	Karyotype	Outcome
Trisomy 21										
1	33	58.90	8.40	2.59	0.77	2	16	98	47,XX,+21	Termination
2	38	73.90	2.50	2.79	0.36	5	6996	2029	47,XX,+21	Termination
3	45	63.40	3.90	1.58	0.18	2	27	35	47,XX,+21	Termination
4	44	75.20	3.40	2.63	0.58	2	479	584	47,XX,+21	Termination
5	33	67.80	4.10	5.27	0.22	2	87	110	47,XX,+21	Termination
6	38	72.10	4.40	5.47	0.23	2	101	116	47,XX,+21	Termination
7	37	54.70	1.60	4.36	0.39	14	7090	16952	47,XX,+21	Termination
8	38	57.10	2.90	1.54	0.40	2	977	390	47,XY,+21	Termination
9	45	51.70	1.30	1.08	0.41	41	532	1324	47,XY,+21	Stillbirth
10	36	81.20	3.20	0.92	0.63	60	1654	1349	47,XY,+21	Termination
11	39	60.50	4.60	1.39	0.36	2	74	33	47,XY,+21	Termination
12	43	60.50	1.70	5.15	0.58	7	1601	5028	47,XY,+21	Termination
13	43	69.90	1.80	2.25	0.19	3	61	98	47,XY,+21	Termination
14***	40	56.10	3.00	1.73	0.25	2	127	68	NIPT/High risk for T21	Termination
15	43	65.50	3.60	1.89	0.72	2	87	534	47,XX,+21	Termination
16	38	71.60	1.90	1.98	0.33	17	3373	144	47,XX,+21	Termination
17	35	66.00	1.90	1.09	0.40	23	707	162	47,XX,+21	Termination
18	31	56.60	1.80	1.38	1.33	11	105	806	47,XX,+21	Termination
19	45	71.10	2.80	3.41	0.50	2	1201	1826	47,XX,+21	Termination
20	41	71.00	5.30	1.17	0.87	4	12	10	47,XX,+21	Termination
21	31	66.40	3.40	2.38	0.33	2	1521	834	47,XX,+21	Termination
22	35	57.80	1.80	3.01	0.29	18	4036	414	47,XY,+21	Termination
23	42	65.20	5.50	1.24	0.74	3	18	3	47,XY,+21	Termination
24	32	64.90	1.80	2.33	0.55	27	3848	7170	47,XY,+21	Termination
25	35	66.10	4.00	1.55	0.26	2	145	147	47,XY,+21	Termination

Case	Age	CRL	NT	ßhCG MoM	PAPP-A MoM	Adjusted risk T21 1:	Adjusted risk T18 1:	Adjusted risk T13 1:	Karyotype	Outcome
26	40	63.70	3.70	1.35	0.69	4	114	505	47,XY,+21	Termination
27	35	56.50	4.00	2.67	0.15	4	129	2	47,XY,+21	Termination
28	40	65.60	2.40	2.36	0.65	19	3834	12729	47,XY,+21	Termination
29	46	62.90	2.90	2.97	1.53	6	142	230	47,XY,+21	Termination
30	31	69.40	3.10	1.26	0.67	98	3970	9305	mos 47,XY,+21[2]/ 46,XY[50]	Live Birth
31	38	58.70	1.80	1.42	0.37	46	3 474	5 011	47,XX,+21	Termination
32	37	66.50	3.10	2.06	0.22	3	60	8 290	47,XX,+21	Termination
33	35	63.70	5.30	1.58	0.84	2	64	524	47,XY,+21	Termination
34	29	72.80	2.40	1.62	0.63	5	2 393	2 217	47,XY,+21	Termination
35	36	56.20	2.70	2.65	0.40	2	6 211	53	47,XY,+21	Termination
36	40	63.80	3.20	0.85	0.39	2	335	30	47,XY,+21	Termination
37	29	74.00	2.50	2.83	0.86	43	4289	12430	47,XY,+21	Termination
38	29	53.00	1.90	1.55	0.26	21	3740	3619	47,XY,+21	Termination
39	44	69.00	2.50	1.07	0.23	2	74	72	47,XY,+21	Termination
40	42	73.50	2.48	1.51	0.16	2	66	59	47,XX,+21	Termination
41	41	50.10	2.20	5.08	0.59	2	2846	8980	47,XX,+21	Termination
42	35	68.30	1.98	1.78	2.08	19	14013	43780	47,XX,+21	Termination
43	41	68.00	2.59	1.45	0.22	2	198	132	47,XX,+21	Termination
44	37	69.00	1.68	1.29	0.23	13	406	816	47,XX,+21	Termination
45	41	62.10	2.90	1.74	0.35	2	834	340	47,XY,+21	Termination
46	32	57.40	3.70	2.29	0.74	4	501	400	47,XY,+21	Termination
47	31	71.40	5.36	0.56	0.30	3	4	17	47,XY,+21	Termination
48	28	81.40	1.57	1.99	0.17	56	993	825	47,XY,+21	Termination
False-negative trisomy 21 cases										
49*	35	54.00	1.70	1.89	0.93	2617	12238	38556	47,XY,+21	Termination
50**	31	56.60	1.80	1.38	1.33	2547	20000	20000	47,XX,+21	Termination
51*	34	68.00	2.40	1.02	0.64	1996	15545	20000	47,XX,+21	Termination

Case	Age	CRL	NT	ßhCG MoM	PAPP-A MoM	Adjusted risk T21 1:	Adjusted risk T18 1:	Adjusted risk T13 1:	Karyotype	Outcome
Trisomy 18										
1	40	52.50	4.50	0.03	0.19	33	2	14	47,XX,+18	Termination
2	41	62.90	4.60	0.15	0.25	19	2	14	47,XY,+18	Termination
3	42	63.40	1.60	0.22	0.46	516	31	827	48,XXY,+18	Termination
4	46	51.50	7.00	0.16	0.95	29	2	99	47,XY,+18	Termination
5	39	45.40	5.00	0.10	0.41	73	2	30	47,XY,+18	Termination
6	36	48.60	7.20	0.17	0.47	120	2	77	47,XY,+18	Termination
7	37	57.50	1.90	0.17	0.15	73	23	264	47,XX,+18	Termination
8	33	60.30	7.20	0.31	0.80	5	3	3	48,XY,+18	Termination
9	41	69.00	9.10	0.29	0.37	15	2	11	48,XXX,+18	Termination
10	34	60.00	5.56	0.30	0.10	25	8	2	47,XY,+18	Termination
11	28	54.00	9.82	0.12	0.41	295	2	23	47,XY,+18	Termination
Trisomy 13										
1	40	60.30	7.20	0.31	0.80	88	21	2	47,XY,+13	Termination
2	37	58.00	2.10	0.27	0.69	3741	1500	99	47,XX,+13	Termination
3	26	74.00	1.63	0.24	0.46	18657	971	79	47,XY,+13	Termination
Turner syndrome										
1	40	62.30	2.80	2.56	0.30	2	1643	76	45,X	Termination
2	37	66.30	5.40	1.24	2.42	22	37	2	45,X	Termination
3	39	75.50	2.50	4.45	1.52	42	4833	16205	45,X	Termination
4	37	63,40	8.10	1.51	0.22	2	16	12	45,X	Termination
5	39	65,20	1.70	3.18	0.65	50	5134	16096	mos 45,X[29]/46, XX[71]	Live Birth
6	40	66,50	10.30	1.75	0.99	2	4	28	45,X	Termination
7	28	67,60	11.90	2.70	0.73	2	18	11	45,X	Termination
8	26	59,50	11.40	n/a	n/a	17	12	7	45,X	Termination
9	27	70,40	9.84	1.00	1.08	3	14	7	45,X	Termination
10****	20	60,00	6.60	0.36	0.86	52	32	48	NIPT/High risk for monosomy X	Termination

Case	Age	CRL	NT	ßhCG MoM	PAPP-A MoM	Adjusted risk T21 1:	Adjusted risk T18 1:	Adjusted risk T13 1:	Karyotype	Outcome
Polyploidy										
1	33	54,30	2.50	0.16	0.12	27	18	52	69,XXX	Termination
2	32	57,80	1.70	0.11	0.32	4527	66	1217	69,XXX	Termination
3	35	46,00	1.85	0.1	0.1	12	37	132	69,XXX	Termination
4	40	70,00	3.51	1.1	0.8	15	127	206	92,XXXX	Termination
Other anomalies										
1	39	64,6	1.80	1.83	0.60	17	825	68	mos 46,XX,fra(10)(q11.1)[7]/46,XX,del(10)(q11.1)[5]/47,XX,+del(10)(q11.1)[1]/46,XX[37]	Termination
2	29	66,60	2.60	2.71	0.67	82	23123	100320	mos 47,XY,+2[25]/46,XX[12]	Live Birth
3	39	49,50	1.00	2.35	0.69	73	846	6019	45,XX,rob(15;22)(q10;q10)	Live Birth
4	37	62,00	3.30	1.93	1.01	10	10	10	47,XY,+der(7)t(7;12)(q11.21;p13.33)mat	Termination
5	39	78,60	2.20	3.19	0.62	20	5026	15674	47,XXX	Live Birth
6	41	56,60	1.80	0.06	0.35	340	7	159	mos 47,XX,+9[22]/46,XX[28]	Live Birth
7	30	67,00	1.40	0.75	0.29	26	106	4	47,XX,+12[13]/ 46,XX[7]	Live Birth
8	39	76.00	3.60	2.21	2.46	25	126	583	47,XXY	Live Birth
9	42	67.30	1.80	0.62	0.33	58	146	529	47,XXY	Termination

* Diagnosed after second trimester scan. ** Diagnosed after first trimester scan. Ebstein's anomaly. *** Absent nasal bone. Invasive procedure declined. NIPT with high-risk for trisomy 21. Terminated on patient request. **** Generalized hydrops. Univentricular heart. Invasive procedure declined. NIPT with high-risk for monosomy X. Terminated on patient request.

β-hCG – beta human chorionic gonadotropin; CRL – crown-rump length; MoM – multiple of median; NT – nuchal translucency; PAPP-A – pregnancy-associated plasma protein A; T13 – trisomy 13; T18 – trisomy 18; T21 – trisomy 21.

Appendix 3. Benign and VOUS CMA results in both groups: indications, karyotype, CMA results, interpretation, clinical significance and outcome, from [Ridnõi *et al.* 2021a].

Indication: Combined risk for T21, ultrasound findings	Karyotype	CMA result	Reported/ not reported	Clinical significance	Outcome (weight, length, Apgar)
Group A					
1:5	46,XX	arr[GRCh37] 15q13.3q14(32922947_34807851)x1 pat	reported	VOUS	2984 g., 49 cm. Apgar 9/10
1:45	46,XY	arr[GRCh37] 3p25.2(12443657_12898941)x3 dn	reported	VOUS	2640 g., 46 cm. Apgar 8/9
1:100	46,XY	arr[GRCh37] 8q21.13q21.2(83691079_86547351)x3 pat	reported	VOUS	4930 g., 55 cm. Apgar 9/9
1:46	46,XX	arr[GRCh37] 15q11.2(22754322_23109890)x3	reported	VOUS/ Susceptibility locus	3510 g., 52 cm. Apgar 9/10
1:77	46,XX	arr[GRCh37] 11p15.5(630124_733639)x1	not reported	VOUS	3005 g., 50 cm. Apgar 9/9
1:79	46,XX	arr[GRCh37] 22q11.21(18877787_19008108)x1	not reported	Likely benign	3722 g., 51 cm. Apgar 9/9.
1:65	46,XX	arr[GRCh37] 3q25.2q25.33(154756700_159888920)x2 hmz	not reported	Benign	3410 g., 50 cm. Apgar 9/9
1:39	46,XX	arr[GRCh37] 2q13(110859672_110982530)x1, 2q22q23.3(148452586_154507406)x2 hmz	not reported	Benign	2720 g., 50cm. Apgar 7/8
1:31	46,XX	arr[GRCh37] Xp22.31(8439382_8539504)x3	not reported	Likely benign	2644 g., 47 cm., Apgar 9/9
1:7	46,XX	arr[GRCh37] 2q13(110859672_110982530)x1	not reported	Benign	4252 g., 53 cm. Apgar 9/10
1:79	46,XY	arr[GRCh37] 21q21.3q22.12(30676316_36232671)x2 hmz	not reported	Benign	4170 g., 55 cm. Apgar 9/9

Indication: Combined risk for T21, ultrasound findings	**Karyotype**	**CMA result**	**Reported/ not reported**	**Clinical significance**	**Outcome (weight, length, Apgar)**
1:68	46,XX	arr[GRCh37] 13q21.31q21.33(64081203_71311007)x2 hmz	not reported	Benign	3170 g., 50 cm. Apgar 8/8
1:56	46,XX	arr[GRCh37] 2q13(110859672_110982530)x1	not reported	Benign	662 g., Apgar 6/8
1:81	46,XX	arr[GRCh37] 12q21.31(83419976_88854647)x2 hmz	not reported	Benign	3332 g. Apgar 9/9
1:43	46,XX	arr[GRCh37] 8p12p11.23(32472617_37651477)x2 hmz	not reported	Benign	2700 g., 49 cm. Apgar 9/9
1:36	46,XY	arr[GRCh37] 5q14.3(83269151_88999206)x2 hmz	not reported	Benign	3188 g., 52 cm. Apgar 8/8
1:49	46,XX	arr[GRCh37] 11p13p12(35553538_41277361)x2 hmz	not reported	Benign	3420 g., 50 cm. Apgar 9/9
Group B					
Increased NT 3.6 mm.	46,XY	arr[GRCh37] 13q12.12(23359525_24860240)x3 mat	reported	VOUS	4174 g., 54 cm. Apgar 8/9
Pulmonary valve stenosis on ultrasound	46,XY,t(1;9)(q12;q12)mat	UPD(9)	reported	VOUS	2300 g., 46 cm. Apgar 8/9
Holoprosencephaly on ultrasound	46,XX	arr[GRCh37] 3q29(195959924_196616249)x1	reported	VOUS	Termination
Increased NT 3.56 mm.	46,XY	arr[GRCh37] 20q13.2(52571861_54907329)x1 pat	reported	VOUS	3088 g., 49 cm. Apgar 8/9
Familiar history. Siblings karyotype 5p15.33 del pat	not done	arr[GRCh37] 5p15.33(1_346610)x1 pat	reported	VOUS	Termination
Increased NT 3.8 mm.	47,XX,+12[13]/46,XX[7]	arr(12)x3[0.2]	reported	Likely benign	3775 g., 51 cm. Apgar 9/10
Increased NT 3.30–3.50 mm.	46,XY	arr[GRCh37] Xp22.31(8439382_9104639)x3	not reported	Likely benign	3880 g., 53 cm. Apgar 5/8

Indication: Combined risk for T21, ultrasound findings	**Karyotype**	**CMA result**	**Reported/ not reported**	**Clinical significance**	**Outcome (weight, length, Apgar)**
Increased NT 4.30 mm.	46,XY	arr[GRCh37] 16p13.11(15052746_16303388)x3,1p33p32.3 (49067236_55438925)x2 hmz	not reported	Likely benign	2520 g., 46 cm., Apgar 8/9
Diaphragmatic hernia on ultrasound	46,XX	arr[GRCh37] 2q13(110859672_110982530)x1	not reported	Benign	3690 g., 51 cm. Apgar 9/10
Critical aortic stenosis	46,XY	arr[GRCh37] 6q26(162006836_162183907)x1	not reported	Benign	Termination
Tetralogy of Fallot, suspicion of esophageal atresia on ultrasound	not done	arr[GRCh37] Xp13q21.1(71595785_81855868)x2 hmz	not reported	Benign	Termination
Increased NT 8.10 mm.	46,XY	arr[GRCh37] 15q15.1q21.3(41602866_53014003)x2 hmz	not reported	Benign	Termination
Increased NT 3.80 mm.	46,XX	arr[GRCh37] 16q11.1q12.2(25844990_31652151)x2 hmz	not reported	Benign	3988 g., 55 cm., Apgar 9/10
Fetal tricuspid regurgitation	46,XY	arr[GRCh37] 8q23.1q23.3(109965998_116102920)x2 hmz	not reported	Benign	3428 g., 50 cm., Apgar 9/10
Midline defect with large omphalocele on first trimester ultrasound	46,XY	arr[GRCh37] 5p15.2p14.3(13650272_22375786)x2 hmz	not reported	VOUS	Termination

CMA – chromosomal microarray analysis; NT– nuchal translucency; T21 – trisomya 21; VOUS – variant of uncertain significance

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9. WEB RESOURCES

- ClinVar. Database of genomic variation and its relationship to human health: https://www.ncbi.nlm.nih.gov/clinvar/
- DECIPHER. A DatabasE of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources: https://decipher.sanger.ac.uk
- DGV. A Database of Genomic Variants. http://dgv.tcag.ca
- ExAC. The Exome Aggregation Consortium (ExAC) is a coalition of investigators seeking to aggregate and harmonize exome sequencing data from a variety of largescale sequencing projects, and to make summary data available for the wider scientific community: http://exac.broadinstitute.org/
- ECARUCA. European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations: https://omictools.com/ecaruca-tool
- FMF. The Fetal Medicine Foundation: http://www.fetalmedicine.org
- GATK. Variant discovery toolkit for high-throughput sequencing data: https:// software.broadinstitute.org/gatk/
- GenBank. National Institute of Health genetic sequence database, an annotated collection of all publicly available DNA sequences. https://www.ncbi.nlm.nih.gov/ genbank/
- GeneReviews. An international point-of-care resource for clinicians, provides clinically relevant and medically actionable information for inherited condition: https://www.ncbi.nlm.nih.gov/books/NBK1116
- gnomAD. The Genome Aggregation Database, is a coalition of investigators seeking to aggregate and harmonize exome and genome sequencing data from a variety of large-scale sequencing projects, and to make summary data available for the wider scientific community: http://gnomad.broadinstitute.org
- OMIM. Online Mendelian Inheritance in Man. An Online Catalog of Human Genes and Genetic Disorders: http://omim.org
- Picard. A set of command line tools for manipulating high-throughput sequencing data: http://broadinstitute.github.io/picard/

SUMMARY IN ESTONIAN

Uute sünnieelse diagnostika strateegiate rakendamine ja nende efektiivsuse hindamine Eestis

Iga raseduse puhul on umbes 4%-line tõenäosus, et sünnib arengulise kõrvalekaldega laps [Harper 2010]. Sünnieelsete sõeluuringute eesmärk on selgitada välja rasedad, kellel on suurenenud risk sünnitada kromosoomhaiguse või kaasasündinud arenguhäirega laps ning pakkuda riskigrupile täpsustavaid diagnostilisi analüüse [ACOG 2020]. Nende diagnostiliste analüüsidega püütakse leida arengurikke etioloogilist põhjust – kromosoomhaigust või geenimuutust. Sünnieelse diagnostika võimaluste loomisega me tagame naistele reproduktiivse autonoomia õigused: valiku võimalused raseduse katkestamise või jätkamise suhtes. Läbi aegade on kromosoomhaiguste sõeltestimise alusena kasutatud erinevaid näitajaid: ema vanust, ema vereseerumi erinevaid biokeemilisi markereid, loote ultraheli markereid ja erinevaid kombinatsioone eeltoodud faktoritest [Sitska 2018]. Kõik sünnieelsed kromosoomhaiguste sõeltestid on käesolevaks hetkeks nihkunud raseduse esimesse trimestrisse.

Nicolaides jt näitasid 1992. aastal oma uuringus, et loodetel, kelle kukla piirkonna läbikumavus, e NT (*nuchal translucency*) rasedusesuuruses 10.–13. nädalat oli üle 3 mm, esines suurema tõenäosusega kromosoomhaigus [Nicolaides *et al.* 1992]. See avastus märgib uue ajastu algust sünnieelses sõeltestimises ehk esimese trimestri kombineeritud sõeluuringu (KS) kasutusele võttu. Kombineeritud kromosoomhaiguste sõeltestimise mudelit, kus ema vanuse juurde arvestati loote NT-väärtus koos ema vereseerumi vaba beeta-kooriongonadotropiini (β-hCG) ja rasedusega seotud plasmavalgu A (*pregnancy associated plasma proteiin-A* e PAPP-A) kohandatud väärtustega pakuti välja 1999. aastal. Sellega saavutati trisoomia 21 (T21) avastamismäär 89% ja fikseeritud vale-positiivsuse määr 5% [Spencer *et al.* 1999]. Esimese trimestri kombineeritud sõeluuringu avastamise määra (*detection rate,* DR) on võimalik tõsta, lisades riskikalkulatsiooni ultraheli lisamarkerid nagu loote ninaluu olemasolu/puudumine, kolmikhõlmalise klapi regurgitatsiooni esinemine ja venoosjuha α-laine iseloomustus või pulsatiilsuse indeks. Suures mitme keskuse vahelises uuringus, mis hõlmas 75 821 rasedat, näidati, et kasutades kombinatsiooni ema vanusest, NT-mõõdust, seerumi biokeemiast ja ultraheli lisamarkeritest, on võimalik tõsta T21 DR üle 90% ja langetada vale-positiivsust alla 3% [Nicolaides *et al.* 2005].

Eestis alustati sünnieelsete sõeluuringutega 1995. aastal, mil peamiseks riskimarkeriks oli ema vanus. Alates 1999. aastast on olnud kasutusel teise trimestri (14.–18. rasedusnädala) seerumskriining [Sitska *et al.* 2008]. Kuni 2015. aasta lõpuni oli Eestis kasutusel järjestikune skriiningmeetod, mille T21 DR oli 88,3% ja vale-positiivsus 3,4% [Muru *et al.* 2010]. Alates 2016. aastast on vastavalt uuele sünnieelse diagnostika juhendile esimese trimestri KS Eestis esmane loote kromosoomhaiguste sõeluuring [Ustav *et al.* 2016].

Sünnieelse sõeltestimise edasiseks arenguks oli vaja uusi tehnoloogilisi lahendusi. Loote DNA esinemist ema plasmas kirjeldati esimest korda 1997.

aastal [Lo *et al.* 1997]. See algatas sünnieelse sõeltestimise valdkonnas uue, mitteinvasiivse sünnieelse testimise (*non-invasive prenatal testing*, NIPT) ajastu. Ulatuslikud loote rakuvaba DNA sekveneerimisel põhinevad valideerimisuuringud avaldati juba 10 aastat tagasi. Nendes uuringutes T21 DR oli peaaegu 100% koos väga madala (alla 0,5%) vale-positiivsusega [Bianchi *et al.* 2012; Palomaki *et al.* 2011]. Üks uus NIPT-platvorm on hiljuti välja arendatud ka Eestis [Žilina *et al.* 2019].

Järgmine samm pärast kõrge riskiga sõeluuringu tulemust on uuritava loote lõplik diagnoos: kromosoomhaiguse kinnitamine või välistamine. Kogu kromosoomi aneuploidia diagnoosimise kuldstandardiks on olnud loote rakkude, mida on võimalik saada vaid invasiivse protseduuri abil, karüotüpiseerimine. Klassikaline karüotüpiseerimine oli kromosoomhaiguse diagnoosimisel pikka aega esmaseks testiks. Selle meetodi peamine puudus on aga selle madal (5–10 Mb) eraldusvõime, mis ei võimalda diagnoosida submikroskoopilisi kromosomaalseid muutusi.

Kromosomaalne mikrokiibi analüüs (KMA, *chromosomal microarray analysis*) ehk „molekulaarne karüotüüp" on DNA-põhine diagnostikameetod, mis tuvastab kogu genoomi ulatuses DNA kadusid või lisakoopiaid ehk koopiaarvu muutusi (*copy number variants*, CNV) ning on 100 korda suurema lahutusvõimega kui klassikaline karüotüpiseerimine [Shearer *et al.* 2007]. Käesoleval hetkel jätkub arutelu KMA universaalse kasutamise üle sünnieelses diagnostikas. Ameerika sünnitusabi ja günekoloogide kolledž (ACOG) teatas 2013. aastal, et KMA-d tuleks kasutada sünnieelses diagnostikas esmase diagnostilise testina ultraheli anomaaliatega loodetel [ACOG 2013]. On näidatud, et patogeensete CNV-de esinemissagedus võib sünnieelselt olla kuni üks juht 270 raseduse kohta ning ei sõltu ema vanusest [Srebniak *et al.* 2018]. Seetõttu praegusel NIPT-ajastul võivad kliiniliselt olulised submikroskoopilised kromosoomianomaaliad olla aladiagnoositud invasiivsete protseduuride sageduse olulise languse tõttu. Eesti Haigekassa rahastab KMA-d alates 2011. aastast. Selle kliinilist kasutust ja diagnostilist efektiivsust on senini uuritud peamiselt lastel, diagnostilise lisaväärtusega 25% [Žilina *et al.* 2014].

Sünnieelse diagnostika suurim väljakutse on ultraheli uuringute käigus leitud loote kaasasündinud anomaaliate täpse tekkepõhjuse väljaselgitamine. Klassikalise karüotüpiseerimine ja KMA abil leitakse täpne etiloogiline tegur kromosomaalsel tasemel umbes 27,4%-l nendel juhtudel [Fiorentino *et al.* 2013]. Seega ei saa ülejäänud neist uuritud loodetest lõplikku diagnoosi [Monaghan *et al.* 2020]. Järgmise põlvkonna sekveneerimismeetodid (*next-generation sequencing*, NGS) sünnieelses diagnostikas on erinevad: sihitud sekveneerimine fenotüübispetsiifiliste geenipaneelidega, suuremahulised geenipaneelid, eksoomi sekveneerimine (*exome sequencing*, ES) ja isegi genoomi sekveneerimine (*genome sequencing*, GS) [Ferretti *et al.* 2019]. Mitmed uuringud on näidanud, et täiendavat diagnostilist informatsiooni võib leida 8,5–81%-l juhtudel [Chandler *et al.* 2018; Lord *et al.* 2019]. Loote NGS analüüsi korral on kõige keerulisem fenotüübi ja genotüübi korrelatsiooni tuvastamine. Eestis on NGS-i diagnostilist efektiivsust hinnatud senini ainult laste ja täiskasvanute populatsioonis ning 501

prooviga kohordis leiti 26,3%-line diagnostiline lisaväärtus [Pajusalu *et al.* 2018].

Uurimistöö eesmärgid

1. Hinnata esimese trimestri kombineeritud sõeluuringu efektiivsust Eestis;
2. Hinnata kromosomaalse mikrokiibi analüüsi kasutust ja selle efektiivsust kõrge riskiga rasedustel loote kromosoomhaiguste avastamiseks;
3. Juurutada järgmise põlvkonna sekveneerimismeetodid ja hinnata nende efektiivsust kõrge riskiga või kombineeritud arenguriketega loodetel;
4. Kirjeldada Simpson-Golabi-Behmeli sündroomi sünnieelset fenotüüpi;
5. Kirjeldada *TXNDC15*-geeni uut patogeenset varianti sünnieelselt avastatud Meckel-Gruberi sündroomiga lootel.

Uuringugruppide ja meetodite lühikirjeldus

Esimese trimestri KS uuringugrupp ja meetodid

Uuring viidi läbi kahe aasta jooksul Ida-Tallinna Keskhaigla (ITK) ja Tartu Ülikooli Kliinikumi (TÜK) naistekliinikus (perioodil 01. jaanuar 2017 kuni 31. detsember 2018). Lõplik uuringugrupp koosnes 14 566 üksiklootega rasedast. Kõigile nendele naistele tehti esimese trimestri KS ja nad andsid oma nõusoleku selleks rutiinse sünnieelse sõeluuringu osana. Andmed analüüsiti anonüümselt. Vereanalüüs võeti 9.–13. rasedusnädalal kahe seerummarkeri määramiseks: f-βhCG ja PAPP-A. Analüüse tehti TÜK-is ja ITK-s erinevatel analüsaatoritel, vastavalt Roche Cobas (Roche Diagnostics, Basel, Switzerland) ja KRYPTOR compact PLUS (Thermo Fisher Scientific, MA, USA) seadmel. Loote ultraheliuuring tehti 11…13+6 rasedusnädalal vastavalt Fetal Medicine Foundationi (FMF) soovitustele (Appendix 1) ja Eesti sünnieelse diagnostika juhendi protokollile. Ultraheliuuringu käigus täpsustati raseduse suurus, mõõdeti loote NT ning hinnati loote arengut. Vajadusel hinnati ka ultraheli lisamarkereid: ninaluu puudumine, kolmikhõlmalise klapi verevoolu ja venoosjuha pulsatiilsuse indeks. Ultraheliuuringu ja seerummarkerite tulemuste alusel kalkuleeriti risk kolme trisoomia (T21, T18 ja T13) esinemiseks lootel. Riski kalkulatsiooniks kasutati TÜK naistekliinikus Astraia (Astraia Software gmbh, Munich, Germany) tarkvara ja ITK naistekliinikus Viewpoint 6 for OB/GYN (GE Healthcare, IL, USA) tarkvara. Kõigile kõrge riskiga rasedatele pakuti diagnostilist invasiivset protseduuri, koorionibiopsiat või amniotsenteesi vastavalt Eestis kehtivale sünnieelse diagnostika juhendile. KS-i kõrge riskitulemuse saanud 517 rasedast 92 valisid teiseks sõeluuringuks NIPT-testi, kuna nad soovisid võimalusel vältida invasiivset kromosoomhaiguste diagnostikat. Uuringuperioodil olid Eestis kättesaadavad kaks NIPT-testi: PANORAMA ™ test (Natera Inc., SanCarlos, CA, USA) ja NIPTIFY® test (Tervisetehnoloogiate Arenduskeskus AS, Tartu, Eesti), mis on Eestis välja arendatud NIPT-platvorm. Invasiivne

diagnostika tehti vaid nendele rasedatele, kelle NIPT-testi tulemus näitas kõrgenenud riski trisoomiate esinemiseks. Materjal loote tsütogeneetilise analüüsi tegemiseks kõrge riskiga rasedatele saadi koorionibiopsia või amniotsenteesi teel. Saadud lootematerjal kultiveeriti vastavalt kliinilisele diagnostilisele protokollile. Loote karüotüüpi uuriti G-vöödistuse meetodil ja kromosoome analüüsiti 450–550 vöödi tasemel vastavalt rahvusvahelise tsütogeneetika nomenklatuurile (ISCN 2016). Igas proovis analüüsiti vähemalt 12 metafaasi; kahtlustatud mosaiiksuse korral analüüsiti kokku 30 metafaasi.

Võimalike vale-negatiivsete KS-testitulemuste avastamiseks koguti sünnieelselt diagnoosimata trisoomiaga sündinud laste kohta informatsiooni TÜK Kliinilise geneetika keskuse andmebaasidest.

KMA uuringugrupp ja meetodid

KMA diagnostilise efektiivsuse hindamiseks värvati 14 566-st esimese trimestri KS-i läbinutest 334 rasedat. Moodustati kaks rühma. A-gruppi kuulus 184 naist, kellest kõigil tuvastati pärast esimese trimestri KS-i kõrge trisoomide risk, kuid NT-mõõt oli normaalne (alla 3,5 mm) ja ultraheli anomaaliaid ei olnud. Kõiki naisi A-grupis nõustati enne protseduuri ja saadi täiendav kirjalik teadlik nõusolek KMA tegemiseks loote DNA-st. B-gruppi kuulus 150 naist, kes kõik vastasid KMA kui esmase diagnostilise testi kriteeriumidele. KMA tehakse Eestis esmase diagnostilise testina pärast invasiivset protseduuri, kui on täidetud üks järgmistest kliinilistest näidustustest: NT suurem kui 3,5 mm, loote väärarendid, perekonna anamnees või teadaolev tasakaalustatud translokatsioon ühel vanemal. Kõik B-rühma naised andsid kirjaliku nõusoleku invasiivse protseduuri tegemiseks.

Loote DNA oli KMA tegemiseks eraldatud kas otseselt koorionibiopsia või amniotsenteesi proovist või kultiveeritud rakkude kultuurist. KMA tehti, kasutades Illumina HumanCytoSNP-12 BeadChips (Illumina Inc., SanDiego, CA, USA) ning QuantiSNP v2.3 tarkvara [Colella *et al.* 2007]. Tuvastatud koopiaarvu muutused klassifitseeriti nelja klassi: patogeensed, tõenäoliselt patogeensed, VOUS (ebaselge kliinilise tähendusega leid) ja healoomulised. Leidude tõlgendamisel kasutati mitmeid veebipõhiseid andmebaase nagu Online Mendelian Inheritance in Man (OMIM), *human genome browsers* (UCSC and Ensembl), DECIPHER ja the Database of Genomic Variants (DGV). PubMed andmebaasi kasutati avaldatud eelretsenseeritud artiklite otsinguks. Raporteeritud leidude korral analüüsiti lisaks vanemate DNA-d, mida eraldati vere lümfotsüütidest, pärandumise täpsustamiseks.

NGS uuringugrupp ja meetodid

Uuringuperioodi jooksul valiti NGS paneelanalüüsi rühma 28 juhtumit. Kõik juhtumid värvati esimese trimestri KS uuringugrupist. Lisamise kriteeriumid olid järgmised: loote aju anomaaliad, mitteimmuunne loote hüdrops, kombinee-

ritud südamerikked ja kombineeritud looteanomaaliad, mis andis alust kahtlustada geneetilist sündroomi. NGS-i paneelanalüüsi tegemise otsuse tegi meditsiinigeneetik, kes ka patsienti nõustas. NGS-analüüsi tegemiseks loote DNA-l saadi patsiendilt täiendav kirjalik informeeritud nõusolek. Ainult kaks rasedust lõppesid elussünniga, kõik ülejäänud katkestati vastavalt meditsiinilistele näidustustele enne 22. nädalat. Loote DNA eraldati kas sünnieelse invasiivse protseduuri käigus saadud materjalist või loote kudedest peale raseduse katkestamist. NGS analüüs tehti, kasutades TruSight One (4813 geeni) või TruSight One Expanded (6699 geeni) geenipaneele. Sekveneerimine tehti, kasutades NextSeq 500 platvormi (Illumina).

Simpson-Golabi-Behmeli sündroomiga looted ja nende uurimismeetodid

SGB sündroom on haruldane X-liiteline retsessiivne haigus, mida on kirjeldatud maailmas vähem kui 100 sünnieelset juhtu. Dikoriaalse kaksikraseduse sega 28 aastane esmasrase, kellel on anamneesis üks varajane iseeneslik raseduse katkemine. Mõlemal kaksikul oli NT üle 3,5mm ja seetõttu teostati ühel kaksikul koorionbiopsia ning KMA ülalpool kirjeldatud meetodil. Ultraheli uuringul 20. rasedusnädalal diagnoositi mõlemal lootel mitmed kaasasündinud anomaaliad: liigkasv, lame näoprofiil, prefrontaalne turse näol, hüperehhogeensed neerud, suguelundite anomaaliad ja aju mõhkkeha düsgenees. Lootele teostati trio- ES koos vanemate materjaliga ning leiti *GPC3*-geeni uus patogeenne hemisügootne variant, NM_004484.3:c.1166+1G>T, mis kinnitas SGB diagnoosi. Järgmise raseduse ajal diagnoositi sama variant *GPC3*-geenis uuesti. Lootel esinesid huule-suulae lõhe, liigkasv ja prefrontaalne turse näol. Väitekirja raames tehtud kirjanduse ülevaade käsitleb ka avaldatud allikaid SGB sündroomi sünnieelsete juhtude kohta [Ridnoi *et al.* 2018].

Meckel-Gruberi sündroomiga loote uurimismeetodid

33 aastane esmasrase suunati kliinilise geneetiku vastuvõtule. Tema loote esimese trimestri UH uuringul 13. rasedusnädalal leiti NT suurenemine 4,1mm-ni, polütsüstilised neerud, entseefalotseele ja postaktsiaalne polüdaktüülia. Antud leiu alusel oli diagnoosi hüpoteesiks Meckel-Gruberi sündroomi (MKS). Suunatud NGS-analüüsil, mis teostati ülal kirjeldatud meetodil, ei leitud MKS-iga seonduvates geenides muutusti. Uus liit-heterosügootne variant *TXNDC15*-geenis diagnoositi ES-i abil. Antud geeni seos MKS-iga oli eelnevalt kirjeldatud ainult ühes rahvusvahelises publikatsioonis.

Peamised tulemused ja järeldused

1. Esimese trimestri KS prospektiivne uuring viidi läbi 14 566 rasedatest koosnevas kohordis kahes Eesti suurimas haiglas.
   1.1. Esimese trimestri KS on efektiivne meetod kolme sagedasema trisoomia sünnieelseks avastamiseks. Trisoomia 21 avastamismäär oli 94%, trisoomia 18 ja 13 avastamismäär oli 100% raseduse esimeses trimestris. Võrreldes eelmise Eestis läbi viidud uuringuga tõusis trisoomia 21 avastamismäär 88,3%-lt 94%-ni.
   1.2. Käesoleva uuringu tulemused näitasid, et üleminek esimese trimestri KS-le on toonud kromosoomhaiguste suurema sünnieelse avastamise ja on ennast õigustanud.
   1.3. Invasiivsete diagnostiliste protseduuride sagedus peale esimese trimestri KS-d oli 2,7% ning on madalam võrreldes avaldatud uuringute ja Eesti eelnevate andmetega.
2. KMA diagnostilist efektiivsust uuriti 334-l normaalse karüotüübiga kõrge riskiga lootel peale esimese trimestri KS-d, ultraheli anomaaliate või kindla geneetilise näidustusega lootel.
   2.1. KMA diagnostiline efektiivsus kõrge riskiga rasedustel oli peale esimese trimestri KS-d või ultraheli anomaaliate korral 3,6%.
   2.2. Alarühmas, kus loodetel oli kõrge riski tulemus trisoomiate suhtes peale esimese trimestri KS-d, kuid normaalne ultraheli leid, on KMA-ga avastatud kliiniliselt oluline koopia-arvu muutus 1,6%-l juhtudest. Antud andmed on kooskõlas varasemate uuringutega ja toetavad KMA kasutust esmase diagnostilise analüüsina kõrge riski tulemusega loodetel peale esimese trimestri KS-d.
   2.3. Patogeense koopia-arvu muutuse sagedus kõrge riskiga rasedustel peale esimese trimestri KS-d on meie andmete puhul 1 juht 62st. Rasedad peavad olema teadlikud KMA teaostamise võimalustest ja vastavalt nõustatud.
   2.4. KMA kasutamine esmase diagnostilise analüüsina annab võrreldes karüotüübiga diagnostilist lisainformatsiooni 6,0%-l juhtudest ultraheli anomaaliate või geneetiliste näidustustega loodetel. Antud andmed on kooskõlas avaldatud uuringutega.
   2.5. Kogu KMA uuringugrupis oli VOUS-leidude sagedus 3,3%, mis on madalam kui enamusel avaldatud KMA uuringutel.
   2.6. Enamik healoomulistest leidudest peale KMA-d olid LSCH-alad ja ei olnud raporteeritud sünnieelselt.
3. NGS analüüsi diagnostilist efektiivsust uuriti 28-l kombineeritud ultraheli anomaaliate ja normaalse KMA tulemusega lootel.
   3.1. NGS analüüsi diagnostiline efektiivsus kombineeritud anomaaliatega loodetel oli 17,9%. Antud tulemus sarnaneb teiste avaldatud uuringutega, kus patogeensete variantide sagedus peale sünnieelset NGS-analüüsi või ES-i oli raporteeritud vahemikus 8,5–81%, sõltuvalt uuringu valimist ja anomaaliate tüüpidest.

3.2. Raporteerisime kahest VOUS-leiust peale NGS-analüüsi *NOTCH1*- ja *PKD1*-geenis, mis sobisid kokku loote fenotüüpidega. *NOTCH1*-geeni variandiga lootel oli diagnoositud kombineeritud südamerike, kuid geeni kandlusega ema oli fenotüüpiliselt terve. *PKD1*-geeni variandiga lootel diagnoositi polütsüstilised neerud, kuid vanemate analüüsi ei saanud teha.

3.3. Raporteerisime kahest kliiniliselt olulisest juhuleiust vähi eelsoodumuse geenides *MLH1* ja *BRCA1*, mis ei olnud seotud loote fenotüübiga, ning need juhuleiud viisid pereliikmete skriininguni.

4. Raporteerisime kolmest SGB-sündroomi juhust kahel järjestikusel rasedusel. Sündroomi sünnieelseid leide analüüsiti detailselt koos varasemalt avaldatud kirjanduse ülevaatega.

4.1. Raporteerisime uuest hemisügootsest *splice-site*-variandist *GPC3*-geenis, NM_004484.3:c.1166+1G>T, mis diagnoositi kolmel lootel kahes järjestikusel rasedusel.

4.2. Sagedasemad sünnieelsed leiud SGB-sündroomil olid loote makrosoomia, lootevee liigsus, siseorganite suurenemine ja neerude anomaaliad, mis on avastatavad detailsel ultraheliuuringul.

4.3. Teiste liigkasvusündroomide diferentsiaaldiagnostika on vajalik, eriti tuleb mõelda Beckwith-Wiedemanni sündroomile.

5. MKS juht diagnoositi sünnieelselt ja kirjeldati detailselt antenataalsete ultraheli leidude, molekulaarsete ES-i leidude ja loote patoanatoomiliste leidude osas.

5.1. Klassifitseerimisime kahte liit-heterosügootset varianti *TXNDC15*-geenis patogeenseks: NM_024715.3:c.211dup p.(Gln71Profs*32) rs780024847 ja NM_024715.3:c.635T>C p.(Leu212Pro) rs760579409. Selle variandi pole varem kirjeldatud haigusseolisena. Selle geeni seost MKS-ga on raporteeritud eelnevalt ainult ühel korral.

5.2. Meie avaldatud juhtumi leitud loote ultrahelianomaaliaid, molekulaarsed variandid ja patoanatoomilised tunnused toetavad eelnevalt avaldatud hüpoteesi, et *TXNDC15* on uudne MKS-ga seonduv geen.

Käesoleva töö praktiliseks väljundiks on sünnieelse diagnostika ja sõeluuringute uute tõenduspõhiste juhendite väljatöötamine Eestis. Uurimistöö näitas selgelt raseduse esimese trimestri kombineeritud sõeluuringu tõhusust kromosoomhaiguste sünnieelsel avastamisel. Samuti näitasime DNA-l põhinevate molekulaarsete analüüside tulemuslikkust rutiinses sünnieelses diagnostikas. Edasised uurimissuunad NIPT ajastul peaksid rohkem hõlmama KMA kasutamist tavadiagnostikas ja lisaks NGS meetodi rakendamist loote arengurikete korral.

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PUBLICATIONS

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2019–…	Ida-Tallinna Keskhaigla, Naistekliinik, sünnieelne osakond, günekoloog-juhtivarst
2016–…	Kliinilise Geneetika Keskus, Kliinilise Meditsiini Instituut, Tartu Ülikool, doktorantuur

Teadus- ja erialane tegevus:
Uurimisvaldkonnad
Kliiniline geneetika, sünnitusabi- ja günekoloogia, diagnostika
Liikmelisus
Eesti Naistearstide Selts (juhatuse liige), Eesti Arstide Liit (liige), ISUOG (liige).

Publikatsioonide nimekiri:

1. **Ridnõi, K**.; Muru, K.; Keernik, M.; Pajusalu, S.; Ustav, E-L.; Tammur, P.; Mölter-Väär, T.; Kahre, T.; Šamarina, U.; Asser, K.; Szirko, F.; Reimand, T.; Õunap, K. **A two-year prospective study assessing the performance of fetal chromosomal microarray analysis and next-generation sequencing in high-risk pregnancies.** Mol Genet Genomic Med. (2021) (accepted).
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