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Computational methods for NIPT-based aneuploidy and microdeletion screening

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Institute of Clinical Medicine, Faculty of Medicine, University of Tartu, Estonia

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Contribution of the author to the original publications:

Paper I Co-developed the computational data analysis methods and framework. Contributed to improving the hidden Markov model's (HMM) applicability in detecting trisomy risk. Participated in conceptualising and implementing the secondary computational analysis layer applied to the HMM output. Devised and implemented usage of a support vector machine (SVM) in the second layer. Participated in study design, manuscript draft editing and reviewing.

Paper II Conceptualised the idea of comparing different computational NIPT aneuploidy detection tools, curated sequencing data, developed formal analysis framework for interpreting Z-scores of different computational NIPT tools, developed and implemented methodology, developed and used computational pipeline, developed methodology to simulate data, simulated data, validated and visualised results, wrote the original draft and participated in reviewing and editing. Composed answers to reviewers questions.

Paper III Developed methodology and implemented the BinDel software package, containerised BinDel, performed data analysis, developed methodology to simulate data, simulated data, validated and visualised results, wrote the original draft and participated in reviewing and editing.

ABBREVIATIONS

AC	amniocentesis
AR	allelic ratio
BAM	Binary Alignment Map
cfDNA	cell-free DNA
cffDNA	cell-free fetal DNA
CVS	chorionic villus sampling
FN	false negative
FP	false positive
FF	fetal fraction
FCT	first-trimester combined test
GATK	Genome Analysis Toolkit
HMM	hidden Markov model
β-hCG	human β subunit of chorionic gonadotropin
LCR	low-copy repeats
MD	microdeletion
NF1	neurofibromatosis 1
NIPT	non-invasive prenatal genetic testing
PCA	principal component analysis
PPV	positive predictive value
PAPP-A	protein-A
RC	read count
RCAR	read count and allelic ratio
RPS	reads per sample
SD	standard deviation
SNP	single nucleotide polymorphism
SVM	support vector machine
TP	true positive
T13	trisomy 13
T18	trisomy 18
T21	trisomy 21
WGS	whole-genome sequencing

1. INTRODUCTION

Non-invasive prenatal genetic testing (NIPT) is a screening test that relies on circulating cell-free DNA (cfDNA) and sequencing techniques to detect the risk of fetal aneuploidies. The presence of fetal (placenta) DNA in maternal blood samples was initially reported in 1997, and subsequently, in the years 2011–2012, large-scale NIPT validation studies were conducted and published based on sequencing of cell-free fetal DNA (Bianchi et al., 2012; Lo et al., 1998; Palomaki et al., 2011). Since NIPT relies on cell-free DNA, it not only allows screening for common aneuploidies such as trisomy 21 but also offers the capability to analyse any part of the genome, including areas associated with fetal microdeletions. For example, one frequent microdeletion is the 22q11.2 heterozygous deletion, causing the DiGeorge syndrome, characterised by adverse outcomes that can include schizophrenia, intellectual disabilities, cardiovascular anomalies or hearing deficits (Bassett et al., 2005; Dar et al., 2022). Even though the 22q11.2 microdeletion can lead to a range of phenotypes, including DiGeorge syndrome, velocardiofacial syndrome, or Sedlackova syndrome, the term DiGeorge syndrome is used throughout this thesis to avoid ambiguity (McDonald-McGinn et al., 1993).

NIPT can be based on whole-genome sequencing (WGS) or targeted analysis, which relies on computational sequencing data analysis to assess fetal aneuploidy or microdeletion risk. The analysis of both targeted and WGS-based NIPT data necessitates the utilisation of dedicated algorithms and software tools. Multiple general computational tools exist that are suitable for pre-processing the sequencing data, but the NIPT-specific analysis tools that follow the pre-processing and output the fetal aneuploidy or microdeletion risk are relatively limited. Furthermore, there is a scarcity of information regarding the accuracy of computational NIPT software and algorithms under diverse sample conditions, including variations in sequencing coverage and fetal fraction (FF) and the availability of clinical validation for these tools.

The accurate computational analysis in NIPT depends on a multitude of factors, including the computational tool, sequencing coverage and fetal DNA fraction. While low-coverage WGS-based NIPT assays do not distinguish whether sequencing reads are of fetal or maternal origin, a sufficient proportion of FF and sequencing coverage is crucial to obtain a true result, the latter affecting both the cost, availability and the accuracy of NIPT.

Although NIPT has been studied, the influence of computational tools on its screening accuracy has not been systematically evaluated, and the use of NIPT for assessing the risk of pathogenic microdeletions remains insufficiently explored.

The aim of the doctoral thesis was to create a computational framework for targeted high-coverage sequencing-based NIPT, clinically validate five commonly used and published WGS-NIPT software tools, evaluate the published computational NIPT tools' performance and accuracy considering various sequencing depths and the proportion of cell-free fetal DNA, develop and clinically validate a computational tool for detecting fetal microdeletions risk in WGS-NIPT.

2. REVIEW OF THE LITERATURE

2.1 Fetal aneuploidies

During fertilisation, the chromosomes from both the egg and sperm combine to form the embryo. Each parent is expected to contribute one copy of each chromosome, resulting in a complete set. However, when an incorrect number of chromosomes is contributed by the sperm or egg, a condition known as aneuploidy occurs (**Figure 1**) (Charalambous et al., 2023). Aneuploidies can result in a viable embryo, but the child may be born with congenital conditions, such as Down syndrome, which is caused by an extra copy of chromosome 21 and also referred to as trisomy 21 (T21) (Charalambous et al., 2023).

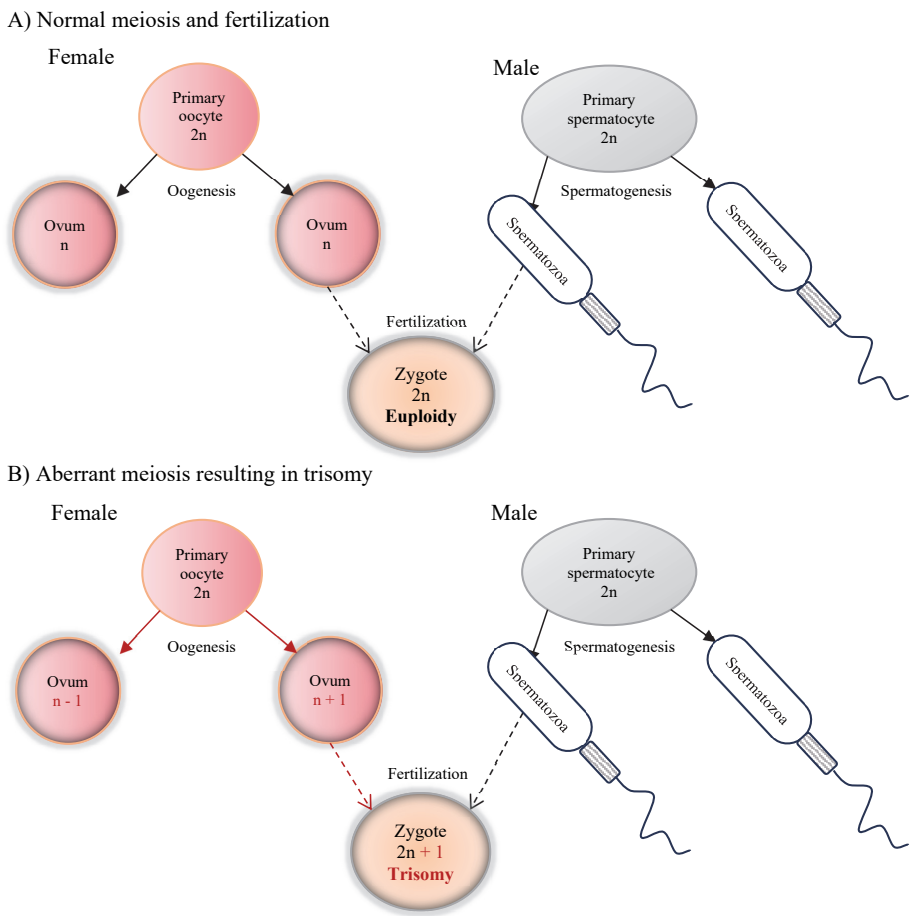


Figure 1. Panel A depicts the process of meiosis and the subsequent fertilisation. Panel B illustrates an instance where a maternal pair of chromosomes fails to separate evenly during meiosis II, leading to a zygote with trisomy after fertilisation. Trisomy can also arise from paternal inheritance or chromosomal translocation, which is not addressed in this figure.

Down syndrome, occurring in approximately 1 of 800 births worldwide, represents the most prevalent form of intellectual disability caused by dosage imbalance of genes due to the triplicate (trisomic) state of all or a critical portion of chromosome 21 (Asim et al., 2015; Bull, 2020; Reimand et al., 2006). Individuals with Down syndrome are predisposed to a spectrum of health issues, including congenital heart disease, Alzheimer's disease or leukaemia (Asim et al., 2015). There is considerable variability between individuals with Down syndrome. For example, cognitive impairment is present in all individuals with Down syndrome, but the extent of it varies widely (Antonarakis et al., 2004). Simultaneously, congenital heart defect occurs only in ∼40% of individuals with Down syndrome (Antonarakis et al., 2004). The median approximate life expectancy of people with Down syndrome in developed countries is approximately 60 years (Bittles & Glasson, 2004).

The second most common autosomal aneuploidy is trisomy 18, causing Edwards syndrome, with prevalence ranging from 1:6,000 to 1:8,000 live births (Cereda & Carey, 2012). The overall prevalence is higher (~1:2,500) due to fetal loss and pregnancy termination after prenatal diagnosis (Cereda & Carey, 2012). Trisomy 18 is associated with multisystem anomalies, with a predilection for affecting the musculoskeletal and cardiac systems (Roberts et al., 2016). Frequently observed musculoskeletal anomalies encompass clenched fists, rocker-bottom feet, and low-set or malformed ears. In the cardiac system, malformations predominantly manifest as ventricular septal defects (Roberts et al., 2016). In one study, the median survival time was 14.5 days for people with trisomy 18 (Rasmussen et al., 2003).

Trisomy 13, causing the Patau syndrome, is the third most common trisomy in live-born infants, with a prevalence of 1 in 16,000 newborns (Heft et al., 2019; Patau et al., 1960). Patau syndrome can cause cryptorchidism, abnormal auricles, polydactyly of hands and/or feet, microphthalmia, micrognathia, low-set ears, aplasia cutis/scalp defects and microcephaly (Petry et al., 2013). The median survival time in one study was seven days for people with trisomy 13 (Rasmussen et al., 2003). With trisomy 13, 18 and 21, the frequency increases with maternal age (Heft et al., 2019).

2.2 Fetal microdeletions

Chromosomal microdeletions represent a subset of copy-number variants (CNV). CNVs are defined as the gain or loss of a DNA segment compared to the reference human genome (Watson et al., 2014). Microdeletions (MDs), which involve the loss of a DNA segment (**Figure 2**), typically result from a process characterised by nonallelic homologous recombination during meiosis between duplicated blocks of sequences known as low-copy repeats (LCRs), which flank the microdeletion event (Watson et al., 2014).

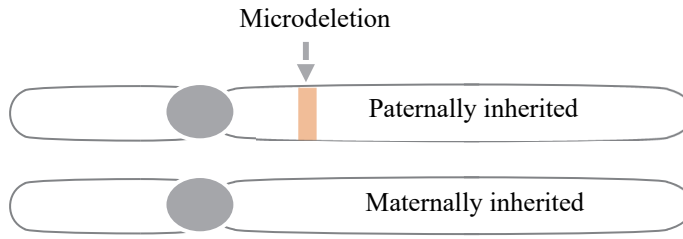
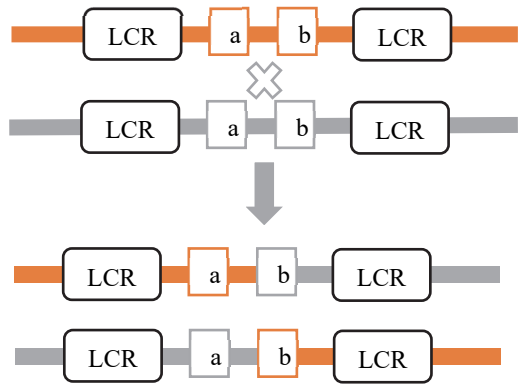


Figure 2. The figure illustrates heterozygous microdeletion on the long arm of chromosome 22, associated with DiGeorge syndrome. One chromosome is inherited maternally, and the other is paternally inherited. Microdeletion can originate from either parent.

Genomic regions prone to microdeletions due to the homologous LCRs can misalign during sperm or egg meiosis so that when crossing-over occurs, the exchange of genetic material is not the same, with one copy of the chromosome getting an extra segment (microduplication) and the other copy losing a segment (microdeletion) (**Figure 3**) (Antshel et al., 2005). Microdeletions can also be inherited or occur early in fetal development and are not connected to maternal age (Heft et al., 2019). Certain genomic regions are more prone to microdeletions and are associated with genomic disorders and neurodevelopmental traits (**Table 1**) (Smajlagić et al., 2021; Stankiewicz & Lupski, 2002).

A) Meiosis I: crossing over



B) Meiosis I: misalignment resulting in unequal crossing cover

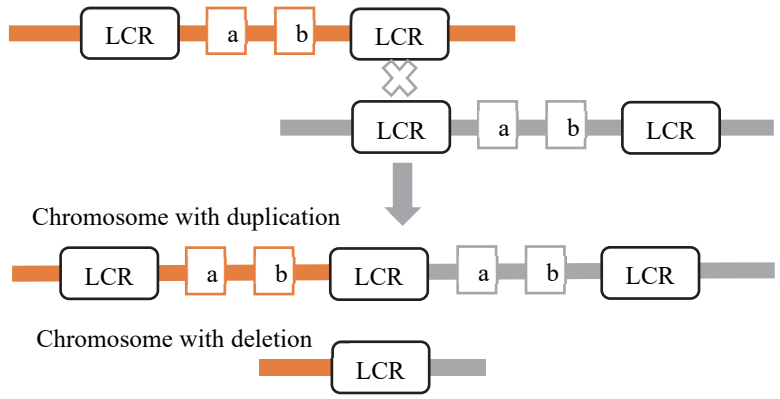


Figure 3. The figure depicts crossing over during meiosis I, wherein homologous chromosomes exchange genetic material. Specifically, the figure portrays the arrangement of two low-copy repeats (LCRs) flanking a genomic locus containing genes designated as 'a' and 'b'. Panel A illustrates the conventional process of crossing over in meiosis I, wherein homologous chromosomes undergo successful recombination without errors. Conversely, panel B delineates a scenario where misalignment occurs between homologous chromosomes facilitated by the presence of LCRs, resulting in an illegitimate crossing over during meiosis. Consequently, one of the chromosome copies undergoes duplication, manifesting as two instances of genes 'a' and 'b', and the other copy undergoes a deletion, leading to the complete loss of genes 'a' and 'b'. Adopted and modified from Watson et al., 2014.

Table 1. A selection of microdeletion syndromes alongside their corresponding frequencies, clinical symptoms, and associated microdeletion genomic regions.

Syndrome	Microdeletion cytogenetic location	Frequency	Clinical symptoms [a]
Angelman syndrome	15q11.2	1:12,000–20,000 (Heft et al., 2019)	Severe cognitive disability, motor dysfunction, speech impairment, hyperactivity, and frequent seizures (Margolis et al., 2015).
Chromosome 1p36 deletion syndrome	1p36	1:5,000–10,000 (Heft et al., 2019)	Intellectual disability, seizures, vision and hearing problems, orofacial clefting, and heart defects (Jordan et al., 2015).
Cri-du-chat syndrome	5p	1:20,000–50,000 (Heft et al., 2019)	High-pitched monochromatic cry, severe psychomotor and mental retardation (Cerruti Mainardi, 2006).
DiGeorge syndrome	22q11.2	1:4,000 (Heft et al., 2019)	Intellectual disability, immunodeficiency, cardiac issues, skeletal defects, and developmental delay (McDonald-Mcginn & Sullivan, 2011).
Jacobsen syndrome	11q23.3	1:100,000 births (Mattina et al., 2009)	Growth and psychomotor retardation, skull deformities, heart, kidney, and gastrointestinal tract malformations (Mattina et al., 2009).
Neurofibromatosis type 1 (NF1)	17q11.2	1:2,500–3,000 (Williams et al., 2009)	Susceptibility to the development of both benign and malignant tumours (Ferner & Gutmann, 2013).
Prader-Willi syndrome	15q11.2	1:10,000–30,000 (Heft et al., 2019)	Severe infantile hypotonia, hypogonadism, obesity, developmental delay and mild intellectual disability (Cassidy et al., 2012).
Smith-Magenis syndrome	17p11.2	1:15,000–1:25,000 (Rinaldi et al., 2022)	Broad face, abnormally shaped ears, congenital heart defects, genitourinary malformations, hearing loss, scoliosis, and impaired production of antibodies (Rinaldi et al., 2022).
Williams-Beuren syndrome	7q11.23	1:7,500 (Strømme et al., 2002)	Cardiovascular anomalies, hypercalcemia, diabetes mellitus, mild-to-moderate intellectual disability (Pober, 2010)
Wolf-Hirschhorn syndrome	4p16.3	1:20,000–50,000 (Battaglia et al., 2015)	Widely spaced eyes, postnatal growth deficiency, intellectual disability, seizures, and antibody deficiency (Battaglia et al., 2015)

[a] The table provides a selection of clinical symptoms, but the list is not exhaustive.

2.2.1 22q11.2 Deletion Syndrome

Chromosome 22q11.2 deletion can result in different overlapping syndromes like DiGeorge syndrome (DGS), velocardiofacial syndrome (VCFS), conotruncal anomaly face syndrome (CTAF), some cases of autosomal dominant Opitz G/BBB syndrome, and Cayler cardiofacial syndrome (asymmetric crying facies) (McDonald-McGinn et al., 1993). Among these, the DiGeorge syndrome occurs in approximately 1 in 4,000 births (overall prevalence of 1:1,524) (Dar et al., 2022; McDonald-Mcginn & Sullivan, 2011).

The majority of 22q11 deletions result from de novo meiotic unequal crossing-over events between homologous chromosomes, with fewer than 10% inherited from an affected parent (**Figure 3**) (Baumer et al., 1998; McDonald-McGinn et al., 2001; Saitta et al., 2004).

DiGeorge syndrome is characterised by adverse infant and childhood outcomes that can include immune deficiency, cardiac, renal and eye anomalies, hypoparathyroidism, skeletal defects, and developmental delay (McDonald-Mcginn & Sullivan, 2011). A Canadian study has shown that the individuals with DiGeorge syndrome who survived to adulthood were at elevated risk of premature death at an average age of 41.5 compared to the Canadian general population expectations of age 80.4 (Bassett et al., 2009).

This DiGeorge microdeletion region is abundant in LCRs, which facilitate misalignment and unequal crossing over during meiosis, resulting in deletions of varying sizes. The most common deletion observed in patients, known as the A-D deletion, accounts for 84%-90% of cases (**Figure 4, Table 2)** (Campbell et al., 2018; Guris et al., 2006). This deletion spans approximately 3 Mb and results in the loss of around 30 genes (Saitta et al., 2004; Yagi et al., 2003). The haploinsufficiency of TBX1 and CRKL is a significant contributor to the phenotype (Guris et al., 2006; Yagi et al., 2003). Another common deletion, the A-B deletion, is approximately 1.5 Mb in length (Saitta et al., 2004). Although less common, the 22q11 region can also harbour other deletions of different sizes (**Figure 4, Table 2**) (Campbell et al., 2018). Mapping unique sequencing reads in the DiGeorge microdeletion region is particularly challenging due to the presence of neurofibromatosis 1 (NF1) microdeletion pseudogenes (Jett & Friedman, 2010; Luijten et al., 2000, 2001). These pseudogenes are present not only in the 22q11 microdeletion region but also in Prader-Willi and Angelman microdeletion region (Jett & Friedman, 2010; Luijten et al., 2000, 2001). This overlap complicates accurately identifying and characterising the deletions specific to DiGeorge syndrome.

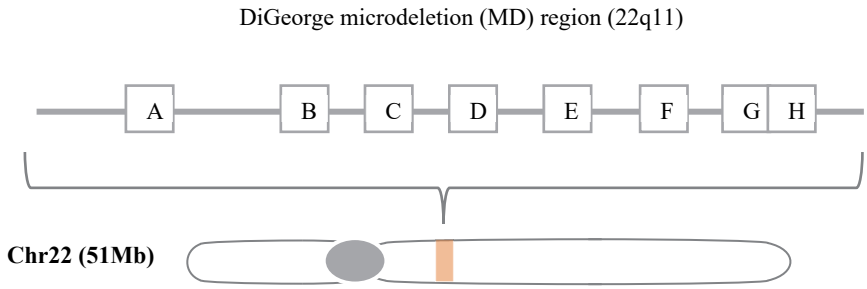


Figure 4. The DiGeorge microdeletion region on chromosome 22 is characterised by multiple low-copy repeats (LCRs), designated as A through H. These LCRs create a genomic landscape susceptible to recombination events, allowing various combinations of microdeletions of varying sizes, including A-D, A-B, or C-D deletions. The deletions can vary in size and encompass different genes within the region, contributing to the heterogeneity observed in individuals affected by DiGeorge syndrome.

Table 2. Microdeletions within the DiGeorge syndrome-related region on chromosome 22 exhibit significant size variability among affected individuals.

Microdeletion	Percentage of affected individuals
A-D	84 %
A-B	5 %
A-C	2 %
B-D	4 %
C-D	1%
C-E, D-E, D-F, D-H, E-F	2%
Non-LCR mediated	2 %

LCR, low-copy repeat. Adapted from Campbell et al., 2018.

Microdeletions collectively exhibit a notable prevalence. Even the most common 22q11.2 microdeletion solely has a more frequent prevalence of 1:1,524 than trisomy 18, causing the Edwards syndrome (~1:2,500) (Cereda & Carey, 2012; Dar et al., 2022). Other common microdeletion syndromes include monosomy 1p36 (1 in 5,000–10,000), Prader-Willi syndrome (1 in 10,000–30,000), Angelman syndrome (1 in 12,000–20,000) and cri-du-chat syndrome (1 in 20,000–50,000) (Heft et al., 2019).

Despite microdeletions' rarity on an individual basis, the cumulative impact of microdeletions is substantial, particularly concerning adverse outcomes during infancy and childhood and no association with increased maternal age (Dar et al., 2022). The high clinical importance of microdeletions necessitates reliable first-trimester fetal screening methods to enable informed decision-making processes and facilitate appropriate medical interventions.

2.3 First-trimester prenatal testing

Given the clinical outcomes of aneuploidies and microdeletions, including viable embryos, early detection during first-trimester screening is essential. Several screening strategies are available, with non-invasive prenatal testing (NIPT) emerging as a recommended method over traditional screening methods due to improved accuracy over conventional screening methods by the American College for Medical Geneticists (Dungan et al., 2023). NIPT uses cell-free DNA (cfDNA) from maternal blood samples to screen for fetal aneuploidies and microdeletions (**Figure 5**) (Mayes et al., 2016). For example, in Belgium and the Netherlands, NIPT is offered as a first-tier screening test to all pregnant women as part of the national prenatal screening program (Lannoo et al., 2023). In Estonia, NIPT serves as a second-tier screening.

In Estonia, for the first-tier screening, the more traditional first-trimester combined test (FCT, also known as One Stop Clinic for Assessment of Risk – OSCAR) is used, which combines the maternal age, ultrasound measurement of fetal nuchal translucency thickness, maternal concentrations of human β subunit of chorionic gonadotropin (β-hCG), and pregnancy-associated plasma protein-A (PAPP-A) (Spencer et al., 1999). FCT is non-invasive and can screen for trisomies 13, 18, and 21 (Geipel et al., 2010). FCT does not screen for microdeletions, but ultrasound sonography can sporadically identify associated findings specific to certain microdeletion syndromes (Lin et al., 2021).

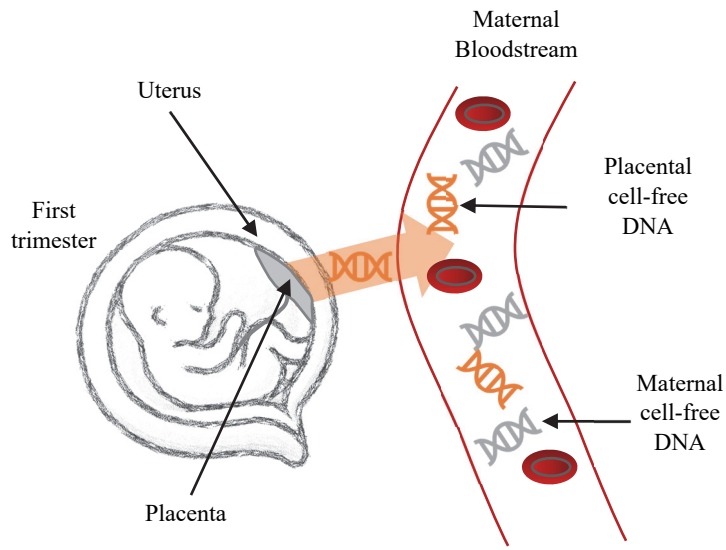


Figure 5. Within the maternal bloodstream, a mixture of genetic material comprises maternal cell-free DNA alongside placental DNA. Placental DNA primarily stems from apoptotic processes within the trophoblastic cells, which are subsequently released into the maternal bloodstream for utilisation. NIPT analysis relies on maternal peripheral blood sample analysis, where the cell-free DNA is extracted, sequenced using high-throughput sequencing, and computationally analysed.

In case of a high-risk finding in NIPT or FCT, a diagnostic invasive confirmation with chorionic villus sampling (CVS) or amniocentesis (AC) is needed. CVS is an invasive procedure where a needle goes through the abdominal wall and uterus or a cannula through the cervix, and placental tissue is collected and karyotyped (Alldred et al., 2017; De Jong et al., 2011). CVS has a risk of a miscarriage of 0.22% (Akolekar et al., 2015). AC is an invasive procedure where a needle is inserted through the abdominal wall into the uterus to sample and karyotype amniotic fluid surrounding the fetus (Alldred et al., 2017; De Jong et al., 2011). The procedure-related risk of miscarriage is 0.11% (Akolekar et al., 2015).

2.4 Non-invasive prenatal testing

Cell-free DNA, which comprises short DNA fragments freely circulating in the bloodstream, was first discovered in 1948 (Mandel & Metais, 1948). A seminal development occurred in 1997 when Lo et al. successfully demonstrated the presence of male fetal DNA in the blood sample of pregnant women (Lo et al., 1997). This pivotal discovery of fetal-derived DNA within maternal blood has catalysed the emergence of a novel generation of prenatal screening methodologies that rely on the analysis of cell-free fetal DNA (cffDNA). Maternal plasma cfDNA contains both maternal and fetal sources of cfDNA (**Figure 5**) (Hui & Bianchi, 2020). The source of fetal DNA is the trophoblast (making the NIPT screening test, not a diagnostic test) (Faas et al., 2012). In DNA sequencing, the proportion of fetal DNA fraction (FF) is the percentage of total maternal plasma cfDNA that is of fetoplacental origin (Hui & Bianchi, 2020). FF is a critical sample-level quality control determinant, as with too low FF, the computational aneuploidy/microdeletion calling nor euploidy confirmation could not be performed with confidence.

NIPT can be based on targeted or whole-genome sequencing and relies on computational sequencing data analysis to assess fetal aneuploidy or microdeletion risk (**Figure 6**). In targeted sequencing-based NIPT, specific genomic regions of interest are selectively amplified or captured before sequencing (Liao et al., 2011). This allows for detecting fetal alleles and the inference of possible fetal aneuploidy (Liao et al., 2012). The targeted strategy reduces the total number of sequencing reads analysed, reducing overall costs (Norwitz & Levy, 2013). At the same time, not all genome is covered, only loci of interest. Therefore, it is only possible to analyse the regions covered during the sequencing in the data analysis. Departing from targeted NIPT, in whole-genome sequencing (WGS) based NIPT, the entire DNA sequence of the genome is interrogated. Instead of targeting specific loci and investigating the count and identity of each allele from each sequence read, the WGS-based NIPT counts sequencing reads across the entire genome and compares these counts to those from a group of healthy, euploid patients.

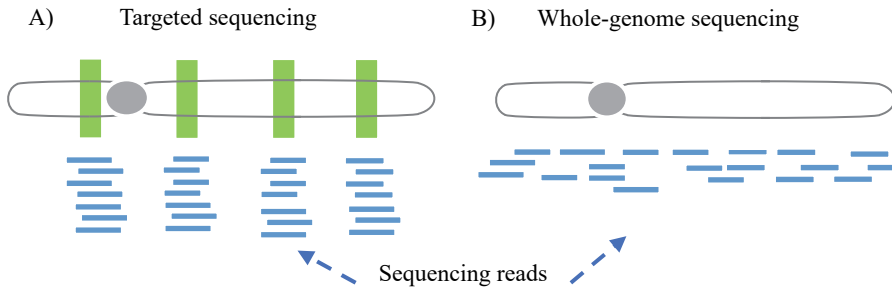


Figure 6. Targeted sequencing involves the selective capture and amplification of specific genomic regions of interest before sequencing (A). This approach allows for the focused analysis of particular regions, such as known disease-associated genes or regions of interest. In contrast, whole-genome sequencing encompasses examining the entire DNA sequence without specifically targeting particular loci (B). By sequencing the entire genome whole-genome sequencing offers a comprehensive interrogation of the entire genome.

2.5 Bioinformatics in routine NIPT screening

In WGS-NIPT, the bioinformatics pipeline involves several key steps to accurately screen fetal aneuploidy and microdeletion risk (**Figure 7**). First, cfDNA is extracted from the maternal blood sample, which contains a mixture of maternal and fetal (placental) DNA. This cfDNA is then sequenced using high-throughput sequencing, generating large volumes of raw sequencing reads. Next, the sequencing reads are aligned to a reference human genome to determine the origin of each read. Following alignment, the bioinformatics pipeline quantifies fetal DNA signals from maternal background noise and performs screening for fetal genetic variations, including aneuploidies and microdeletions. Finally, the results produced by the bioinformatics pipeline are interpreted and reported to the clinician.

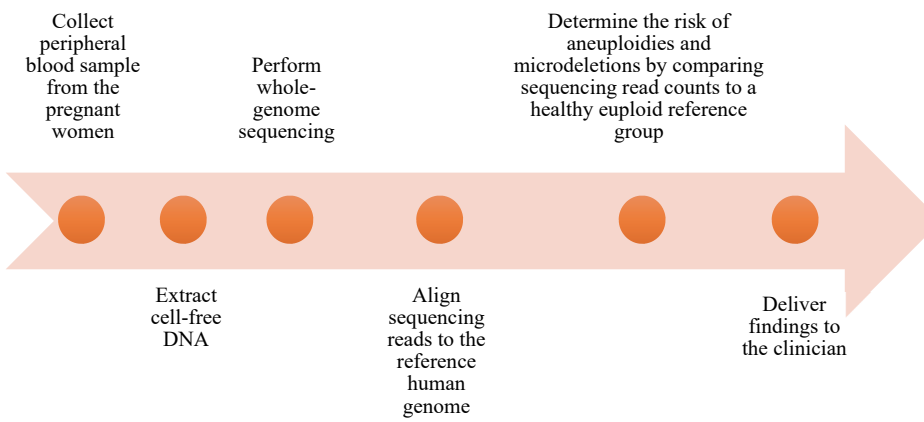


Figure 7. Overview of the whole-genome sequencing-based non-invasive prenatal testing workflow from blood collection to results reporting.

While aneuploidy screening in WGS-based NIPT is relatively straightforward due to the long length of chromosomes, the screening for clinically important microdeletions poses further challenges. The ability to accurately detect microdeletion (MD) risk in NIPT is affected by four main factors: FF, the size of the MD region, sequencing coverage, and the variability in bin read counts in the MD region (Zhao et al., 2015). The higher the FF, the greater the likelihood of detecting potential fetal MDs despite the presence of a significant maternal DNA 'background'. The same principle applies to the MD region length: the longer the MD region, the greater the anticipated coverage by sequencing reads, thereby enhancing the likelihood of detecting any deficiency in sequencing reads (Kucharik et al., 2020; Zhao et al., 2015). MD region length affecting MD screening accuracy has also shown in a study where the detection rate for short microdeletion causing Williams-Beuren syndrome was the lowest compared to the longer microdeletions causing DiGeorge, renal cysts and diabetes, Angelman/Prader-Willi, 1p36 microdeletion, cri-du-chat, and chromosome 9p deletion syndrome (Tian et al., 2023).

Furthermore, MD regions possess different GC% (guanine-cytosine content) compositions and frequently reside proximal to homologous LCRs, which do not facilitate unambiguously unique sequencing read mapping, influencing the standard deviation of the bin read counts, further challenging the accurate microdeletion screening (Watson et al., 2014; Zhao et al., 2015). Last, the low incidence rates require balance in minimising false-positive, false-negative, and maximising true-positive microdeletion calls. Given NIPT's computational intensity and sequencing data complexity, the development of robust algorithms and sophisticated software tools is needed.

2.5.1 Estimating fetal fraction in NIPT

The fetal fraction indicates the signal of interest and is a critical sample-level quality control determinant as different computational tools have different sensitivities for fetal aneuploidy and microdeletion calling. Fetal fraction is influenced by several factors, including gestational age, maternal age, maternal body mass index, and certain medications (Deng et al., 2023; Kuhlmann-Capek et al., 2019). Numerous computational methodologies have been devised to estimate the fetal DNA fraction. One direct approach involves a single nucleotide variation-based method, where the fetal DNA fraction is computed by assessing the ratio of fetal-specific alleles to the total alleles (Peng & Jiang, 2017). To identify fetal-specific alleles, additional parental genotyping or high-depth targeted sequencing as high as ~120× is needed (Jiang et al., 2012; Peng & Jiang, 2017; Sparks et al., 2012).

The available options become more constrained in the context of low-coverage sequencing-based NIPT. It has been shown that size distributions of maternal and fetal cfDNA in maternal plasma differ due to the different fragmentation patterns, fetal DNA being generally shorter than maternal DNA (Chan et al., 2004). Using size distribution differences, Yu et al. developed a method to estimate fetal fraction based on the ratio between the count of fragments ranging from 100 to

150 bp and from 163 to 169 bp (Yu et al., 2014). However, the associated assay requires relatively long sequencing reads, thereby increasing the cost of routine NIPT.

In the context of low-coverage short-read sequencing-based NIPT, estimating fetal fraction tends to be less straightforward. For male fetuses, the FF estimation is relatively straightforward as maternal cfDNA does not contain Y chromosome material, but the male fetal cfDNA contains. Therefore, the Y chromosome ratio to autosomes can be calculated. However, chromosome Y is not completely unique; even in a female fetus pregnancy, some reads align to some parts of the Y chromosome (van Beek et al., 2017). Several computational tools are available to estimate fetal fraction in male fetus pregnancies using reads mapped to the Y chromosome. One such tool is DEFRAG (van Beek et al., 2017). DEFRAG calculates the fetal fraction by taking the fraction of sequencing reads mapped to the Y chromosome and subtracting the average number of reads mapped to the Y chromosome in previously seen female fetus pregnancies (van Beek et al., 2017). This result is then divided by the average fraction of reads in the Y chromosome previously seen from male control samples (van Beek et al., 2017).

In 2015, Kim et al. published a SeqFF, a pre-trained machine learning-based computational tool that estimates fetal fraction using sequence read counts (Kim et al., 2015). In contrast to earlier approaches, SeqFF enables the estimation of fetal fraction for pregnancies with both female and male fetuses without needing prior training (Kim et al., 2015). As a result, SeqFF facilitates immediate and training-data-free utilisation (Kim et al., 2015). In short, SeqFF divides sequenced reads into genomic bins and, with pre-trained coefficients for genomic bins, estimates the fetal fraction (Kim et al., 2015). Elastic net and reduced-rank regression were used to estimate coefficients during the training (Kim et al., 2015). The SeqFF authors suggest that SeqFF leverages the differential representation of the regional sequence of read counts of maternal/fetal ccfDNA, suggesting that fetal ccfDNA may be more likely to originate from genomic regions with increased euchromatic DNA structure, resulting in observed differential fragment length distribution and nonuniformity in coverage relative to maternal ccfDNA (Kim et al., 2015).

Although SeqFF can be readily employed without any additional configuration, it is designed under the assumption that the end-user follows a laboratory protocol and data processing procedure similar to that used during the model's training, as the end-user cannot retrain the model. Deviating from the initial laboratory procedures can reduce accuracy in estimating fetal fraction. In 2019, Raman et al. published a trainable computational fetal fraction estimator PREFACE, relying on neural networks and principal component analysis (PCA), applying biological principles similar to SeqFF (Raman et al., 2019). In contrast to SeqFF, PREFACE necessitates pre-training before its initial use. While PREFACE is a powerful method, it mandates a substantial number of training samples. For example, the authors employed a training set of approximately 5,000 samples in the study (Raman et al., 2019).

2.5.2 Computational approaches for aneuploidy and microdeletion screening in NIPT

The aneuploidy or microdeletion screening of targeted and WGS-based NIPT data necessitates using dedicated algorithms and software tools. For example, in targeted NIPT, there exists software such as FORTE (Fetal Fraction Optimized Risk of Trisomy Evaluation) or NATUS (Next-Generation Aneuploidy Test Using SNPs)(Norwitz & Levy, 2013).

In WGS-based NIPT, multiple analytical aneuploidy risk detection tools have emerged, including RAPIDR, NIPTmer, NIPTeR, and sub-chromosomal aberration (including microdeletion or -duplication) risk detection tools such as Wisecondor or WisecondorX (Johansson et al., 2018; Lo et al., 2014; Raman et al., 2019; Sauk et al., 2018; Straver et al., 2014). In the context of WGS-based NIPT, computational methods for aneuploidy or microdeletion screening generally follow a sequence of steps. Initially, sequencing reads are partitioned into genomic bins to calculate read counts per bin. These read counts undergo normalisation to mitigate various biases inherent in genomic sequencing data. Subsequently, the normalised bins are aggregated to create segments representing entire or specific chromosome segments. The next step involves comparing these normalised values to a reference group of euploid samples, identifying differences that are indicative of aneuploidies or subchromosomal changes in the sample under analysis (**Figure 8**).

The specific algorithms and additional steps in this process may vary between tools. For instance, RAPIDR employs genomic binning, read counting, GC% normalisation to address GC-nucleotide-rich regions, summarisation by chromosome, and computes chromosomal differences relative to a euploid reference (Lo et al., 2014). NIPTeR, in comparison, introduces additional features like chi-squared-based variation reduction to reduce the impact of regions with unexpected variability and alternative methods for quantifying deviations from the euploid reference group (Johansson et al., 2018). WisecondorX employs a Circular Binary Segmentation algorithm to analyse aneuploidies and screen for copy number variations (**Figure 8**)(Olshen et al., 2004; Raman et al., 2019). NIPTmer, however, relies on counting pre-defined sets of unique k-mers on a per-chromosome basis from raw sequencing data, followed by a linear regression model to compare predicted and observed k-mer counts, marking a distinct departure from the methodologies previously described (Sauk et al., 2018).

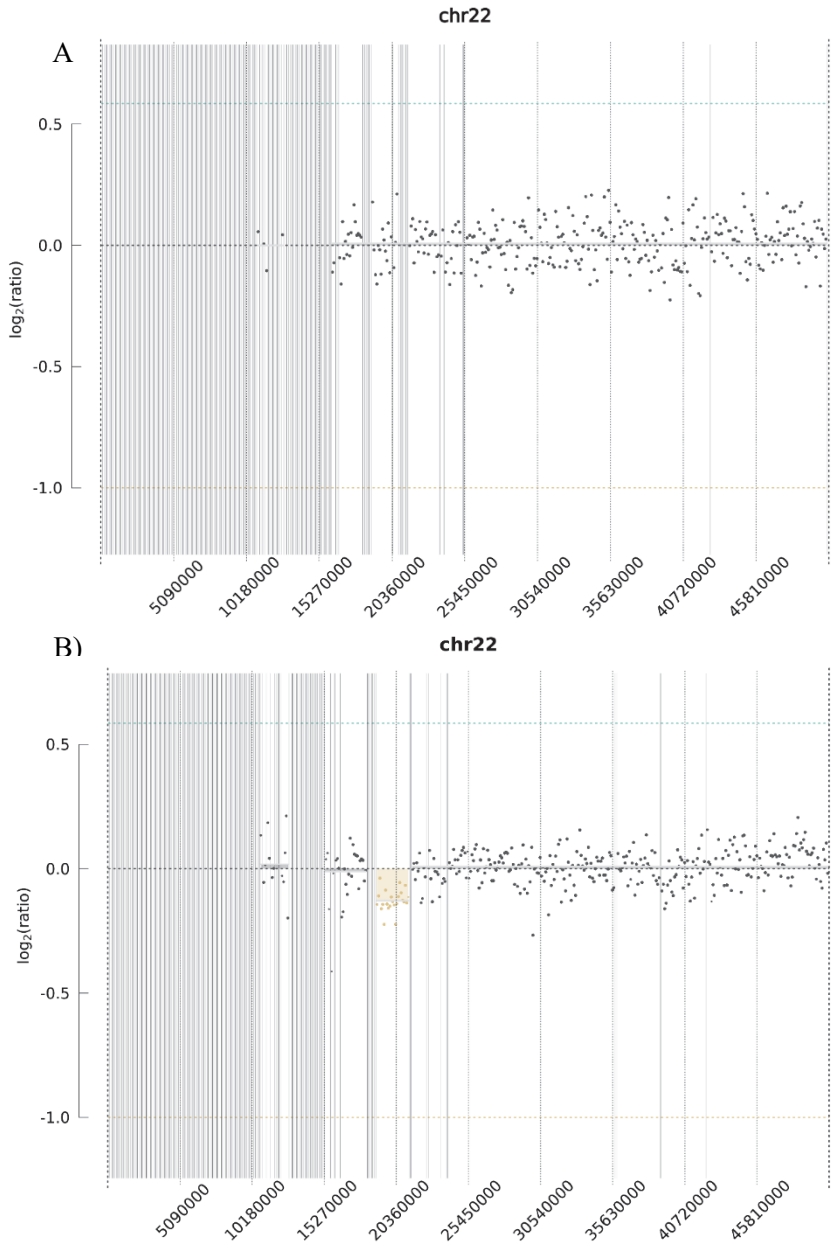


Figure 8. Visualisation of WisecondorX analysis for chromosome 22. The figure illustrates genomic bins and their ratio between observed and expected copy numbers. WisecondorX has not detected any aberration (A). WisecondorX called and highlighted aberration in the DiGeorge microdeletion region for SeraCare Life Sciences Inc circulating cell-free DNA like a mixture of human genomic DNA that consists of matched maternal and fetus DNA with DiGeorge syndrome (lot #10571706) (B). According to the SeraCare Life Sciences Inc specification, 22q11.21 loss is at least 2.8Mb in size from 18,706,046 to 21,540,288 base pairs and fetal fraction 8.50%.

2.6 Rationale for studies

NIPT has emerged as a reliable and safe method for screening common fetal chromosomal abnormalities, such as trisomies, using cell-free DNA obtained from maternal blood. NIPT relies heavily on computational analysis of this DNA. The targeted NIPT approach reduces the number of sequencing reads required, thereby lowering costs and making the method more accessible to a broader patient population. However, the number of available algorithms for analysing targeted sequencing data and calculating trisomy risk remains limited.

WGS-based NIPT is increasingly used and benefits from a range of existing computational tools for trisomy risk estimation, the combined effect of variables such as fetal fraction, sequencing depth, and algorithm choice on screening accuracy has not been systematically evaluated using the same clinically validated sample set.

Finally, while pathogenic microdeletions can lead to phenotypes as severe as those caused by whole-chromosome trisomies, their risk estimation with WGS-based NIPT remains challenging and the availability of appropriate computational tools remains limited.

3. AIMS OF THE STUDY

1. Develop computational methods required for targeted NIPT-based fetal trisomy risk detection and systematically evaluate fetal trisomy risk calling in simulated targeted NIPT sequencing data.
2. Assess the accuracy of fetal trisomy risk assessment using low-coverage WGS-based NIPT data. Determine how sequencing depth and estimated fetal fraction affect trisomy risk calling in low-coverage WGS-based NIPT.
3. Examine the feasibility of accurate fetal microdeletion risk estimation from low-coverage WGS NIPT data. Develop the necessary methodologies and a software tool that implements them to accurately assess fetal microdeletion risk and validate its performance on clinically confirmed microdeletion samples.

4. MATERIALS AND METHODS

4.1 Study subjects and samples

4.1.1 Clinical patients (Papers II–III)

For the evaluation of computational trisomy screening tools, 1092 samples were used, of which 669 were used as reference sets for evaluated computational tools. For these 669 samples, euploidy had been confirmed by the NIPTIFY screening test (Celvia CC) and postnatal evaluation. The remaining 423 samples were used for the validation and were from a previously published study by Žilina et al. (Žilina et al., 2019). Of 423, 258 were high-risk pregnancies that had undergone diagnostic invasive prenatal analysis (Žilina et al., 2019). There were 19 samples with confirmed fetal trisomy 21 (T21), eight with trisomy 18 (T18) and three with trisomy 13 (Žilina et al., 2019). Samples were sequenced with the Illumina NextSeq 500 platform, producing 85 bp single-end reads with an average per-sample coverage of 0.32× at the University of Tartu, Institute of Genomics Core Facility as described previously (Žilina et al., 2019).

We used 300 samples reported previously as euploid fetus pregnancies by NIPTIFY screening test and postnatal evaluation for BinDel fetal microdeletion simulation analysis. Of these 300 samples, 200 were retained for the BinDel reference set, and 100 were used to simulate fetal microdeletions. Samples were processed with modifications similar to previously published guidelines from Katholieke Universiteit Leuven (Bayindir et al., 2015). Peripheral blood samples were collected in Streck cell-free DNA BCT tubes, and plasma was separated with standard dual centrifugation. Cell-free DNA was extracted from 3 ml of plasma using the ThermoFisher Scientific MagMAX Cell-Free DNA Isolation Kit. Subsequently, whole-genome libraries were prepared using the Fragmented DNA Compact Sequencing Assay NIPT protocol with 12 cycles for the final PCR enrichment step. After library quantification, 36 samples were equimolarly pooled, and the quality and quantity of the pool were evaluated using the Agilent Technologies 2200 TapeStation. WGS was carried out on the Illumina Inc. NextSeq 550 instrument with 85 bp single-end reads (average coverage of 0.32×), resulting in the read count between 8M to 18.5M reads per sample (RPS), with a median of 12.7M RPS.

In the BinDel and WisecondorX analysis, Katholieke Universiteit Leuven samples received as single-end FASTQ files were used. We used 200 known fetal euploid samples for the BinDel and WisecondorX (v1.2.4) reference set. For initial validation, 84 samples containing 50 euploids and 34 samples with clinically confirmed MDs were analysed. Samples with clinically confirmed MDs originated from 22 unique patients, from which some cfDNAs were sequenced 2–3 times in different libraries (**Table 3**). Microdeletion origin, region, sample fetal fraction and gestational age are presented in **Table 3**. We applied BinDel and WisecondorX on 84 samples, reported high-risk calls to Katholieke Universiteit Leuven, and received the results.

Table 3. BinDel validation sample set information.

State / Syndrome	Patient	Origin	FF (%) [a]	Gestational age when genomic aberration was proven by microarray analysis [b]
DiGeorge (A-D)	1	Fetal	10.0	32 weeks
DiGeorge (A-D)	1	Fetal	8.8	32 weeks
Angelman	2	Fetal	16.2	Postnatal
Angelman	2	Fetal	8.7	Postnatal
DiGeorge (A-B)	3	Fetal	8.6	Postnatal
DiGeorge (A-D)	4	Maternal and fetal	11.9	16 weeks (NIPT reported maternal)
DiGeorge (A-D)	4	Maternal and fetal	10.0	16 weeks (NIPT reported maternal)
Williams-Beuren	5	Fetal	8.9	Postnatal
DiGeorge (A-D)	6	Fetal	7.8	Postnatal
DiGeorge (A-D)	6	Fetal	9.8	Postnatal
NF1	7	Maternal and fetal	7.9	Postnatal
DiGeorge (A-D)	8	Fetal	12.1	21 weeks
DiGeorge (A-D)	8	Fetal	8.1	21 weeks
Smith-Magenis	9	Fetal	16.4	15 weeks (NIPT reported)
Prader-Willi	10	Fetal	12.2	15 weeks (NIPT reported)
Prader-Willi	10	Fetal	10.9	15 weeks (NIPT reported)
Angelman	11	Fetal	10.1	Postnatal
DiGeorge (A-D)	12	Fetal	7.4	32 weeks
DiGeorge (A-D)	12	Fetal	6.2	32 weeks
DiGeorge (A-D)	13	Fetal	6.1	17 weeks

State / Syndrome	Patient	Origin	FF (%) [a]	Gestational age when genomic aberration was proven by microarray analysis [b]
Williams-Beuren	14	Fetal	7.4	postnatal
DiGeorge (A-D)	15	Fetal	5.7	Stillbirth (31 weeks)
DiGeorge (A-D)	16	Maternal and fetal	5.8	15 weeks (NIPT reported maternal)
DiGeorge (A-D)	16	Maternal and fetal	9.1	15 weeks (NIPT reported maternal)
Smith-Magenis	17	Fetal	12.0	Postnatal
Smith-Magenis	17	Fetal	10.3	Postnatal
DiGeorge (A-B)	18	Maternal	12.2	16 weeks (NIPT reported maternal)
DiGeorge (A-D)	19	Fetal	16.5	17 weeks (NIPT reported)
DiGeorge (A-B)	19	Fetal	15.8	17 weeks (NIPT reported)
DiGeorge (A-B)	19	Fetal	13.4	17 weeks (NIPT reported)
DiGeorge (A-D)	20	Fetal	5.9	Postnatal
DiGeorge (A-D)	20	Fetal	7.8	Postnatal
Williams-Beuren	21	Fetal	6.4	Postnatal
DiGeorge (A-D)	22	Fetal	7.4	Postnatal

[a] The fetal fraction (FF) values provided in the table correspond to the first trimester of pregnancy.
[b] Microdeletions were detected during screening only if reported as “NIPT reported”.
Adapted from Paluoja et al., 2025.

4.1.2 Simulated NIPT samples (Paper I-III)

A total of 1,800 datasets, each containing 100 training and 10,000 test samples simulating pregnant women's cfDNA targeted sequencing data, were used for the development of the computational framework for targeted sequencing-based NIPT. Each sample incorporated 1,000 targeted loci per chromosome of interest. Datasets varied in fetal condition (euploidy, maternally or paternally originated trisomy), sequencing depth (500–15,000 reads per locus) and FF (1–20%).

To measure the impact of sequencing depth on the accuracy of WGS-based NIPT computational tools, we systematically subsampled raw sequencing read data from the average of 20M reads to 1.25M reads per sample. Specifically, each sample was subsampled into six different groups of subsamples, including 20M, 15M, 10M, 5M, 2.5M, and 1.25M (**Table 4**). Subsampling to 5–15M was achieved by leaving the appropriate number of NextSeq 500 output lanes out. For lower coverages, one lane (5M) was taken and subsampled with samtools view (Danecek et al., 2021). Next, we applied a computational tool to infer Z-scores. As all computational NIPT tools required the creation of a euploid reference set to which the sample under analysis is later compared, we also created a corresponding reference set for each analysed sequencing coverage. To illustrate, with a 5M RPS coverage, both validation and reference samples were subsampled to 5M RPS and analysed as a 5M RPS group. The fetal DNA fraction and Z-score estimates for chromosomes 13, 18, and 21 for each sample on each coverage were calculated. If the tool failed to operate on certain coverages, then analysis of that coverage for the failed tool was left out.

Table 4. Analysed sample groups.

M RPS [a]	Sample Group [b]	SD [c]	Min [d]	Average [e]	Max [f]	N [g]
20	T21 (clinically validated)	4,277,096	14,347,516	24,163,449	32,626,785	19
15	T21 (clinically validated)	3,148,835	10,937,064	18,083,151	24,334,564	19
10	T21 (clinically validated)	2,081,671	7,209,906	11,958,958	16,127,678	19
5	T21 (clinically validated)	1,048,024	3,670,427	6,023,952	8,173,000	19
2.5	T21 (clinically validated)	523,966	1,833,823	3,011,415	4,086,277	19
1.25	T21 (clinically validated)	262,260	915,450	1,505,824	2,042,374	19
20	T18 (clinically validated)	2,791,633	18,450,798	23,124,624	27,336,184	8
15	T18 (clinically validated)	2,071,817	14,024,066	17,350,621	20,485,890	8
10	T18 (clinically validated)	1,395,351	9,235,616	11,476,668	13,625,081	8
5	T18 (clinically validated)	697,871	4,685,815	5,794,650	6,891,991	8
2.5	T18 (clinically validated)	348,455	2,344,985	2,898,169	3,446,589	8
1.25	T18 (clinically validated)	174,390	1,172,245	1,449,377	1,722,745	8
20	T13 (clinically validated)	2,980,546	19,042,280	22,183,555	24,971,957	3
15	T13 (clinically validated)	2,257,362	14,500,538	16,725,422	19,013,917	3
10	T13 (clinically validated)	1,469,534	9,567,867	11,039,287	12,506,927	3
5	T13 (clinically validated)	742,824	4,870,521	5,591,835	6,354,446	3
2.5	T13 (clinically validated)	370,783	2,435,984	2,796,437	3,176,752	3
1.25	T13 (clinically validated)	184,533	1,219,133	1,398,883	1,587,854	3
20	Validation Low Risk	3,594,972	13,643,477	23,747,773	32,396,033	393
15	Validation Low Risk	2,688,192	10,345,586	17,800,264	24,374,332	393

M RPS [a]	Sample Group [b]	SD [c]	Min [d]	Average [e]	Max [f]	N [g]
10	Validation Low Risk	1,791,357	6,839,734	11,799,169	16,181,461	393
5	Validation Low Risk	896,917	3,453,849	5,944,443	8,061,718	393
2.5	Validation Low Risk	448,456	1,726,568	2,972,178	4,028,665	393
1.25	Validation Low Risk	224,239	863,244	1,486,192	2,014,776	393
20	Reference	6,032,848	11,534,450	23,506,647	55,026,706	669
15	Reference	4,531,992	8,656,925	17,654,442	41,383,148	669
10	Reference	3,013,960	5,736,648	11,695,518	27,432,704	669
5	Reference	1,516,731	2,919,971	5,942,981	13,920,933	669
2.5	Reference	758,325	1,458,860	2,971,523	6,957,681	669
1.25	Reference	379,135	729,274	1,485,737	3,476,940	669

T21, trisomy 21; T18, trisomy 18; T13, trisomy 13. The table presents the average read count in millions of reads coverage group[a], condition[b], coverage standard deviation[c], minimum[d], average[e] and maximum[f] read count and the number of samples[g]. Adapted from Paluoja et al., 2021.

To observe low sequencing coverage-driven uncertainty in Z-score inference, we used ten high FF (10.23–18.57%) low-risk (NIPTeR chromosome 21 Z-score -0.0996–0.0942) WGS-NIPT (22M–30M) samples. We generated a single pooled low-risk sample with 247M reads by concatenating and sorting these ten samples. Next, the pooled sample was randomly subsampled 2,000 times to groups of 2.5, 5, 10 and 20M RPS, and for each subsample, we applied NIPTeR to infer Z-scores. Using the inferred Z-scores, we calculated deviations by subtracting the average group Z-score from the calculated Z-scores. Finally, deviations were added to the original validation sample Z-scores, leading to 2,358,000 simulated low-risk Z-scores and the number of false positive and true negative trisomy calls were counted. The same methodology was also applied to eight T21 samples (20M–33M, FF 10.43%–14.21%, Z-score 13.77–23.64), resulting in the pooled sample with 203M reads. For T21 analysis, we also created 6, 7, 8 and 9M RPS reference groups.

We analysed sequencing depth's impact on the accuracy of fetal fraction estimation. First, we estimated FF for the 20M RPS group. As the number of samples exhibiting low FF estimations (0–4%) was limited to 12 samples, we divided samples by FF into groups of 0–5% (n = 28) and 5–15% (n = 302). This stratification allows for more accurate correlation estimates on samples with low FF. Next, we subsampled raw sequencing read data from the average of 20M reads to 1.25M reads per sample and compared the original 20M FF estimates with reduced coverage estimates by calculating Pearson correlation.

We simulated heterozygous fetal MDs in WGS-NIPT using GATK (Genome Analysis Toolkit (Van der Auwera et al., 2020)) for BinDel development and evaluation. First, we extracted and downsampled the reads within the MD region to a temporary BAM (Binary Alignment Map) file, replicating heterozygous deletions while considering the target fetal fraction. Next, we extracted the reads outside the MD region from the original file and transferred them to another temporary (BAM) file. Finally, these temporary BAM files were then merged. We applied this simulation process for every combination of 12 FF, 12 MD, and 100 euploid fetus samples.

4.2 Computational methods

4.2.1 Sample pre-processing (Paper II–III)

For alignment-based computational NIPT tools, all samples were aligned against human reference assembly GRCh37 (RAPIDR) or GRCh38 (BinDel, NIPTeR, WisecondorX). Sequencing reads originating from identical DNA fragments were flagged as duplicates and sorted. K-mer-based NIPTmer required no special preprocessing.

4.2.2 Support vector machine as a framework for HMM output interpretation (Paper I)

Support vector machine (SVM) is a supervised learning model that finds a hyperplane that separates samples by category during a training phase. In the case of the developed computational framework, the hyperplane is found based on the hidden Markov model (HMM) state proportions, read count and FF such that it maximally separates samples by their state (euploid, maternal trisomy or paternal trisomy). Once trained, SVM can predict the state of an unseen sample by observing which side of the hyperplane the sample falls based on the read count, FF, and HMM state proportions.

4.2.3 Uniform framework for defining trisomy risk (Paper II)

While all evaluated fetal trisomy detection tools outputted a per-chromosome Z-score for assessing the trisomy risk, each algorithm also had specific differences. For example, RAPIDR also outputs trisomy calls, WisecondorX calculates in addition to the Z-score the $\log_2$ ratio of the observed number and expected number of reads, NIPTmer specifies a trisomy risk calling cut-off, GIPseq provides decision rules for interpretation of the output, and NIPTeR avoids defining cut-off threshold (Bayindir et al., 2015; Johansson et al., 2018; Lo et al., 2014; Raman et al., 2019; Sauk et al., 2018). To ensure uniform scoring between different computational tools, we used Z-scores as all the computational NIPT tools supported outputting it in the analysis.

However, a trisomy risk-calling Z-score cut-off threshold is needed to decide trisomy risk. We defined a generally usable framework, relying on a percent point (quantile) function. The framework allows choosing a probability such that, presuming a euploid sample, sets a cut-off value such that the Z-score of a euploid sample would be less than or equal to the cut-off with the chosen probability. The cut-off threshold can be calculated by presuming the Z-score distribution follows a standard normal distribution, or it can be calculated by empirically observing the mean and SD of the euploid sample reference group. The first cut-off is referenced as a theoretical cut-off Z_t and the second as the empirical cut-off Z_e. A probability of 0.9999 was used in all calculations: $P_{99.99}(P[Z_{sample} \leq z_{cut\ off\ threshold}]) = 0.9999$.

4.2.4 Computational tools (Papers II–III)

The most relevant analysis aspects for each evaluated computational tool are described below.

NIPTeR v1.0.2 was used. Each sample was binned and GC% corrected with bin_bam_sample (separate_strands set to TRUE) and gc_correct (method ‘bin’, ref_genome ‘hg38’), respectively. A reference group was created with the as_control_group. Each sample under analysis was further chi-corrected with chi_correct, and the Z-score was calculated with calculate_z_score. We also cal-

culated using prepare_ncv (max_elements set to 9) and calculate_ncv_score normalised chromosome values (denoted NIPTeR NCV), which minimises variation between sequencing runs (Johansson et al., 2018; Sehnert et al., 2011).

WisecondorX v1.1.5 was evaluated. Each sample was converted to .npz format with the convert command. The reference group was created with newref -nipt -refsize 669 -binsize 100000. Z-scores were calculated using the predict command. We also tested Wisecondor (Paco_0.1) but left it out due to the existence of newer WisecondorX and due to Wisecondor not providing Z-scores for the entire chromosomes.

NIPTmer was obtained from the University of Tartu Department of Bioinformatics webpage but was updated to the latest version from GitHub due to software issues. Pre-built lists based on the GRCh37 reference genome were used as NIPTmer is not an alignment-based method, and GRCh37 lists are known to work (Žilina et al., 2019).

GIPseq was run by KU Leuven (Bayindir et al., 2015). We uploaded samples to the KU Leuven Google Cloud bucket for both the reference set creation and analysis and received the raw output of the pipeline. Since the GIPseq NIPT pipeline provided sophisticated output not limited to euploid and trisomy states, the pipeline was not directly comparable with other evaluated tools. GIPseq author's interpretations (euploid or trisomy) for each sample were used to assess the number of false-negative and false-positive trisomy cases. The read count effect on the Z-scores was analysed using the theoretical Z-score cut-off for comparability between different computational tools.

We also planned to include DASAF R in the analysis, but the corresponding web links to the software in the publication were inoperable, and the software was not included (Liu et al., 2016).

RAPIDR v0.1.1 was used. GRCh37 human reference genome was used as RAPIDR does not support GRCh38. Function makeGCContentData was used for calculating the GC% content before binning reference samples with makeBinnedCountsFile (using a default bin size of 20000). Finally, the R object representing the reference set was created with createReferenceSetFromCounts (gcCorrect and filterBin set to TRUE). Although RAPIDR provides trisomy calls (based on a fixed Z-score cut-off of three) alongside Z-scores, it was not used for uniform computational tool comparison (Lo et al., 2014).

SeqFF was used to estimate FF (Kim et al., 2015). Before applying SeqFF, samples were aligned against the human reference assembly GRCh37.

BinDel, developed as part of this doctoral thesis, is a computational tool for estimating fetal microdeletion risk in whole-genome sequencing-based NIPT. The tool is publicly available at: https://github.com/seqinfo/BinDel. Microdeletion high risk was called if the BinDel risk probability was 50% with PCA99% for shorter MD regions (NF1, Williams-Beuren) or 80% with PCA95% for longer target MD regions. PCAX% denotes that the number of PCA components are chosen to capture X% of the cumulative variance based on BinDel the reference group. BinDel supports varying genomic bin sizes. After analysing different bin sizes

and considering random sequencing read placement effects, differences in sequencing read placement patterns across various microdeletion regions, and the number of bins per MD region, 300kb bin size was used (Paluoja et al., 2021). However, shorter bin sizes were used in regions where 300kb led to too few bins (**Table 5**). The coordinates were mainly derived from the DECIPHER, ClinGen and OMIM databases (Amberger et al., 2015; Firth et al., 2009; Rehm et al., 2015).

Table 5. A priori chosen target microdeletion regions, their coordinates and bin sizes used in the analysis.

Coordinate (chromosome:start-end)	Microdeletion region and database used	Genomic bin size (kb)
1:1–27 600 000	1p36 OMIM (Amberger et al., 2015)	300
1:10 001–12 780 116	1p36 DECIPHER (Firth et al., 2009)	300
1:898 703–6 229 913	1p36 terminal region, ClinGen dosage ID: ISCA-37434 (Firth et al., 2009; Rehm et al., 2015)	300
3:192 600 000–198 295 559	Chromosome 3q29 (Amberger et al., 2015)	300
4:1–4 500 000	Wolf-Hirschhorn OMIM (Amberger et al., 2015)	300
4:337 779–2 009 235	Wolf-Hirschhorn terminal region, ClinGen dosage ID: ISCA-37429 (Firth et al., 2009; Rehm et al., 2015)	300
4:1 567 470–2 108 509	Wolf-Hirschhorn DECIPHER (Firth et al., 2009)	100
5:10 001–12 533 192	Cri-du-chat DECIPHER (Firth et al., 2009)	300
7:72 700 001–77 900 000	Williams-Beuren OMIM (Amberger et al., 2015)	300
7:73 330 452–74 728 334	Williams-Beuren DECIPHER (Firth et al., 2009)	300
8:116 700 000–126 300 000	Langer-Giedion OMIM (Amberger et al., 2015)	300
11:114 600 000–135 086 622	Jacobsen OMIM (Amberger et al., 2015)	300
15:22 677 345–28 134 728	Prader-Willi/Angelman Type I. Start coordinate DECIPHER CNV syndrome, end coordinate ClinGen (dosage ID ISCA-37478) (Firth et al., 2009; Rehm et al., 2015)	300
15:23 374 765–28 134 728	Prader-Willi/Angelman Type II. Start coordinate DECIPHER CNV syndrome, end coordinate ClinGen (dosage ID ISCA-37404) (Firth et al., 2009; Rehm et al., 2015)	300
17:16 869 758–20 318 836	Smith-Magenis DECIPHER (Firth et al., 2009)	300
17:30 780 079–31 936 302	NF1 DECIPHER (Firth et al., 2009)	200
20:80 106–1 311 812	20p13. Coordinates from a custom-ordered DNA Mix (lot #10560229) specification from SeraCare Life Sciences Inc with fetus DNA having a pathogenic loss of the terminal region of 20p13 and a pathogenic 3q29 duplication.	300
22:18 924 718–20 299 685	DiGeorge A-B, ClinGen dosage ID: ISCA-37433 (Firth et al., 2009; Rehm et al., 2015)	300
22:18 924 718–21 111 383	DiGeorge A-D, ClinGen dosage ID: ISCA-37446 (Firth et al., 2009; Rehm et al., 2015)	300
22:20 377 696–21 111 383	DiGeorge B/C-D, ClinGen dosage ID: ISCA-37516 (Firth et al., 2009; Rehm et al., 2015)	100

Adapted from Paluoja et al., 2025.

4.3 Ethics

This study was performed with the written informed consent from the participants and with the approval of the Research Ethics Committee of the University of Tartu (#315/T-13, #352/M-12) and the Ethics Committee Research UZ / KU Leuven (S66817).

5. RESULTS

5.1 Paper I: Computational fetal trisomy risk detection with targeted sequencing-based NIPT

Our objective was to integrate a targeted NIPT strategy aimed at screening common fetal trisomies. Targeted NIPT strategy lowers overall costs but also facilitates increased sample throughput. However, the majority of available computational methods for sequencing-based NIPT rely on low-coverage WGS and are unsuitable for targeted NIPT. We developed a novel computational framework for a targeted high-coverage sequencing-based NIPT to screen fetal trisomy.

5.1.1 Analytical framework for HMM output interpretation (Paper I)

The developed HMM-based approaches aimed at detecting fetal chromosomal trisomies within targeted sequencing assays exhibited limitations in sensitivity under specific scenarios. For example, the combined read count and allelic ratio model (RCAR) failed to detect any paternally inherited trisomies across varying fetal fractions (**Table 6**). Conversely, within a fetal fraction range of 1–5%, the sensitivity for maternally inherited trisomies was also only 0.47 (**Table 6**). Although the read count (RC) model showed a high sensitivity of 1.0 across all fetal fractions, its robustness relied on the accurate FF estimation (**Table 7**). Since determining fetal fraction based solely on the read count in targeted NIPT is unfeasible, instances may arise when FF cannot be estimated. In such cases, assuming a presumed FF of 10% while the actual FF falls within the 1–5% range, the RC model sensitivity dropped from 1.0 to 0.11 (**Table 7**).

Table 6. Fetal trisomy classification accuracy with read count and allelic ratio model.

Fetal fraction	Specificity	Sensitivity (maternal trisomy)	Sensitivity (paternal trisomy)
1–5%	1.00	0.47	0.00
6–10%	1.00	0.96	0.00
11–15%	1.00	1.00	0.00
16–20%	1.00	1.00	0.00

Each value represents an average classification accuracy over 1,200,000 simulated cell-free DNA samples with fetal (maternally or paternally inherited) trisomy at the different sequencing depth intervals (500–15,000 reads). Adopted and modified from Teder et al., 2019.

Table 7. Fetal trisomy classification accuracy with read count computational model.

Fetal fraction	Specificity		Sensitivity	
	Fetal fraction is known.	The model presumes a fetal fraction of 10%.	Fetal fraction is known.	The model presumes a fetal fraction of 10%.
1–5%	1.00	1.00	1.00	0.11
6–10%	1.00	1.00	1.00	1.00
11–15%	1.00	1.00	1.00	1.00
16–20%	1.00	1.00	1.00	1.00

Each value is calculated over 1,200,000 simulated cell-free DNA samples with fetal trisomy at the different sequencing depth intervals (uniformly 500 - 15,000 reads). Adopted and modified from Teder et al., 2019.

The underlying reason for the notably reduced sensitivity observed in the HMM-based solution is caused by the interpretation of HMM output scores. Sample trisomy is determined by the highest frequency of locus states (e.g., trisomy or euploid). In this approach, the sample is designated as trisomy if the count of trisomic loci surpasses that of euploid loci. However, this methodology presents certain limitations. For instance, when the trisomic and euploid loci counts are nearly equivalent, the final sample state is susceptible to random fluctuations.

Moreover, the inability to detect any paternally inherited trisomies relates to the overlapping emission distributions of maternally and paternally originated trisomies within the HMM model (**Table 6**). The HMM model was designed to favour maternally originated trisomies due to their relatively higher prevalence in the population. As a result of this bias, combined with the decision-making process based on the most prevalent state of loci, no paternally inherited trisomies were identified (**Table 6**).

Having recognised the necessity for a more advanced decision-making system to classify the sample state, we developed the concept of integrating an additional classification layer upon the HMM output scores that would also consider the distribution of loci counts associated with euploid, maternally, and paternally inherited trisomies when determining the sample state. The additional layer determines the decision boundary distinguishing samples with euploid, maternally, and paternally inherited trisomies during the training phase that could later be applied to unseen samples. For implementation, we used a support vector machine (SVM) as it finds a decision boundary (hyperplane) such that the distance (margin) between different states (in the feature space) is maximised, replacing previous highest frequency based classification system with SVM outputted state (based on the decision boundary).

5.1.2 Evaluation of the analytical framework of HMM output interpretation (Paper I)

Supplemental SVM considerably increased trisomy detection sensitivity across all three models and marginally decreased specificity in some cases (**Table 8, Table 9, Table 10**). Notably, the SVM increased paternally inherited trisomy detection sensitivity from zero to one across all FF for the allelic ratio (AR) and RCAR models (**Table 8, Table 9**). For the AR and RCAR models, the SVM also considerably improved the detection of maternally inherited trisomy, albeit, for RCAR, there was a decrease of 0.01 specificity on FF 1–5% and for the AR model, a decrease between 0.02–0.03 on all tested FF (**Table 8, Table 9**).

Table 8. Fetal trisomy classification accuracy with allelic ratio computational model and with supplemental SVM.

Fetal fraction	Specificity		Sensitivity (maternal trisomy)		Sensitivity (paternal trisomy)	
	HMM	SVM	HMM	SVM	HMM	SVM
1–5%	1.00	0.98	0.02	0.10	0.00	1.00
6–10%	1.00	0.97	0.63	0.78	0.00	1.00
11–15%	1.00	0.97	0.89	0.97	0.00	1.00
16–20%	1.00	0.98	0.96	1.00	0.00	1.00

SVM, support vector machine; HMM, hidden Markov model. Each value represents an average classification accuracy over 1,200,000 simulated cell-free DNA samples with fetal (maternally or paternally inherited) trisomy at the different sequencing depth intervals (uniformly 500–15,000 reads). Adopted and modified from Teder et al., 2019.

Table 9. Fetal trisomy classification accuracy with RCAR computational model and supplemental SVM.

Fetal fraction	Specificity		Sensitivity (maternal trisomy)		Sensitivity (paternal trisomy)	
	HMM	SVM	HMM	SVM	HMM	SVM
1–5%	1.00	0.99	0.47	0.73	0.00	1.00
6–10%	1.00	1.00	0.96	1.00	0.00	1.00
11–15%	1.00	1.00	1.00	1.00	0.00	1.00
16–20%	1.00	1.00	1.00	1.00	0.00	1.00

RCAR, combined read count and allelic ratio model; SVM, support vector machine; HMM, hidden Markov model. Each value represents an average classification accuracy over 1,200,000 simulated cell-free DNA samples with fetal (maternally or paternally inherited) trisomy at the different sequencing depth intervals (500–15,000 reads). Adopted and modified from (Teder et al., 2019).

Table 10. Fetal trisomy classification accuracy with read count computational model and supplemental SVM.

Fetal fraction	Specificity				Sensitivity			
	Fetal fraction is calculated beforehand and provided as input		The model presumes a fetal fraction of 10%		Fetal fraction is calculated beforehand and provided as input		The model presumes a fetal fraction of 10%	
	HMM	SVM	HMM	SVM	HMM	SVM	HMM	SVM
1–5%	1.00	1.00	1.00	0.98	1.00	1.00	0.11	0.92
6–10%	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00
11–15%	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
16–20%	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

SVM, support vector machine; HMM, hidden Markov model. Each value is calculated over 1,200,000 simulated cell-free DNA samples with fetal trisomy at the different sequencing depth intervals (uniformly 500 - 15,000 reads). Adopted and modified from Teder et al., 2019.

RC model benefits from the SVM only if the FF is not known. If FF is known, then SVM does not affect sensitivity or specificity. If FF is unknown, then using SVM on low FF of 1–5%, sensitivity increases by 8-fold, and specificity decreases between 0.01 and 0.02 across all tested FF (**Table 10**).

The targeted strategy with HMM combined with supplemental SVM would work well, but the need for pre-sequencing loci selection does not allow straightforward NIPT panel expansion. Consequently, we transitioned to using WGS-based NIPT. Although WGS-based NIPT, when paired with publicly available computational analysis tools, enables comprehensive genome analysis, determining cost-effective sequencing depth while keeping clinically acceptable trisomy detection accuracy must be determined.

5.2 Paper II: Fetal trisomy risk screening in low-coverage WGS-based NIPT

While WGS-based NIPT is a powerful screening method with several published computational NIPT analysis tools available, there is no comprehensive accuracy comparison of these tools on the same clinically validated data. We applied computational NIPT aneuploidy analysis tools on the same clinically validated samples across various sequencing coverages and fetal DNA fractions to determine clinically usable sequencing depth(s) and computational tool(s).

5.2.1 Sequencing depth effect on trisomy detection (Paper II)

One central parameter in WGS is sequencing depth. To assess the sequencing depth effect on fetal trisomy risk assessment accuracy, we compared GIPseq, NIPTeR, NIPTmer, RAPIDR and WisecondorX on the same clinically validated NIPT samples.

Our findings indicated predominantly accurate results when sequencing coverages exceeded 5M reads per sample (RPS) (**Table 11**). In contrast, differentiation trends among compared algorithms became apparent at sequencing depths lower than 5M RPS, particularly with increased false-negative trisomy calls driven by systematically more conservatively estimated Z-scores (**Figure 9)**. For example, in one instance, on very low read coverage of 1.25M RPS, GIPseq detected 13 more cases of trisomy than RAPIDR (**Figure 10**).

Table 11. Number of undetected trisomy 13, 18 and 21 samples at 5M reads per sample per computational NIPT tool.

Number of cases	GIPseq	NIPTeR	NIPTeR NCV	NIPTmer	RAPIDR	WisecondorX
T21: 19	0	0	0	2	4	0
T18: 8	1	1	0	1	8	0
T13: 3	0	0	0	0	2	0

Combined from Paluoja et al., 2021.

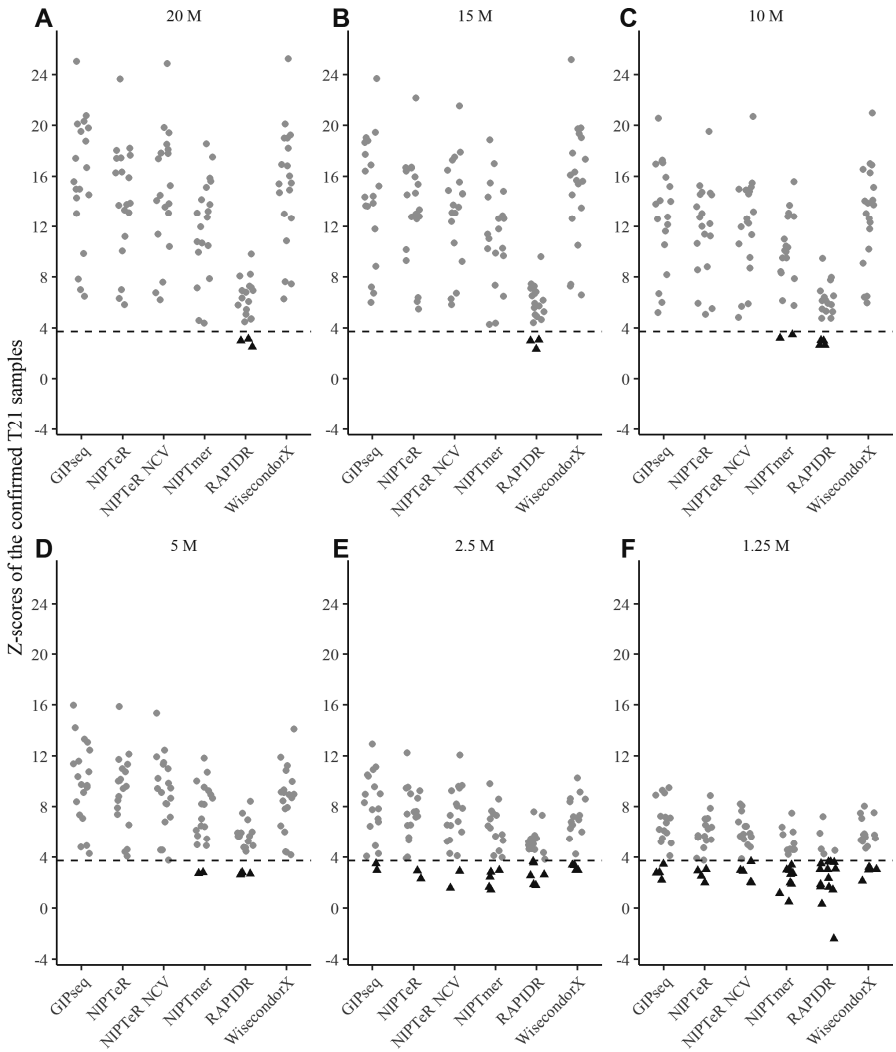


Figure 9. Z-scores of clinically validated T21 (trisomy 21) samples across a range of sequencing depths. Z-scores of known trisomy samples at sequencing depths of 20M RPS (A), 15M RPS (B), 10M RPS (C), 5M RPS (D), 2.5M RPS (E), and 1.25M RPS (F) are shown. Undetected (false negative) trisomies falling below the Zt cut-off thresholds (black dashed line in each graph) are represented as black triangles. Adopted from Paluoja et al., 2021.

Although systematically decreased Z-scores contribute to the rise in false-negative trisomy calls due to the distancing from the trisomy cut-off threshold, our observations also highlight that low sequencing coverages increase false-positive trisomy calls (**Figure 10B**). We demonstrated that lower trisomy detection accuracy at lower sequencing depths is influenced by the naturally occurring arbitrary placement of sequencing reads, introducing uncertainty in the Z-score estimation of the studied sample (**Figure 11)**.

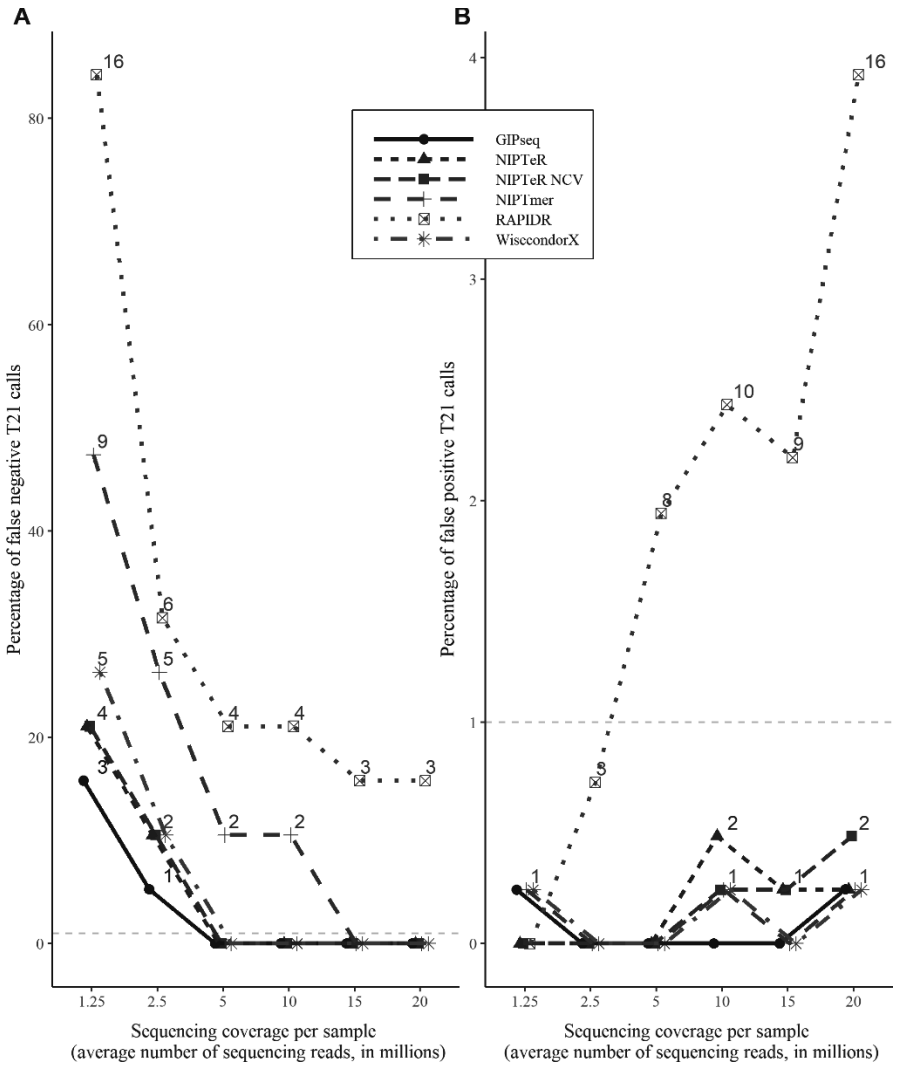


Figure 10. Trisomy detection accuracy of tested NIPT software tools across different sequencing depths. (A) Percentages and absolute numbers of undetected trisomy cases among known trisomies. (B) False-positive T21 results among known euploid samples. The horizontal dashed line in each graph marks the 1% cut-off level often used in clinical screening. Adopted from Paluoja et al., 2021.

Furthermore, we noted that when certain computational tools were applied to their own euploid reference group across different sequencing depths, the Z-score distribution deviated from the anticipated theoretical distribution, translating into a variance in the empirical cut-off threshold (**Figure 12**). For instance, without accounting for this variance, WisecondorX introduced two additional undetected trisomies at a sequencing depth of 2.5M RPS compared to the cut-off threshold considering this variance (**Figure 9E, Figure 10**).

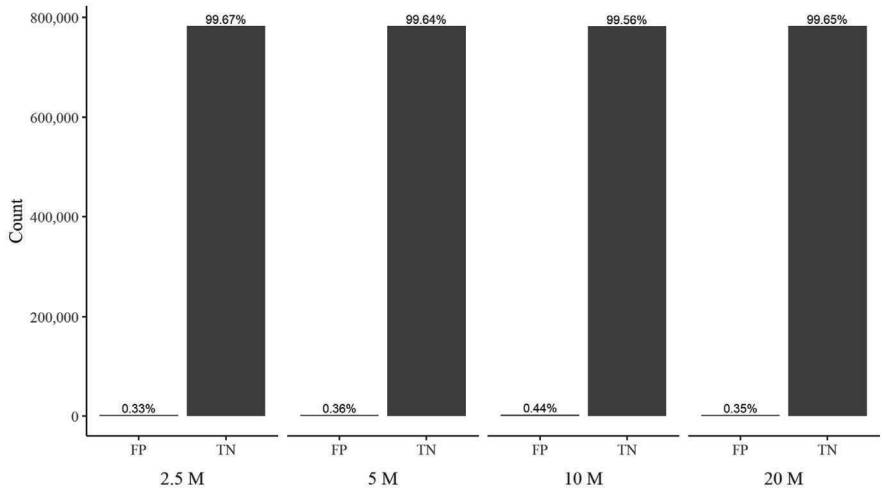


Figure 11. Effect of the natural sequencing read placement uncertainty on euploid sample group number of false-positive (FP) trisomy 21 cases. 0.33%–0.44% of Z-scores depending on the subsample group were detected as FP trisomy 21 cases. Adopted from Paluoja et al., 2021.

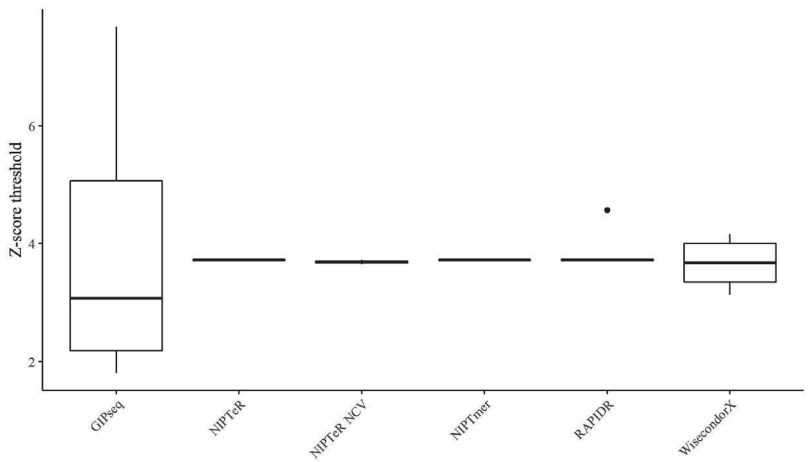


Figure 12. Chromosome 21 Z_e cut-off threshold variability**.** Adopted from Paluoja et al., 2021.

Finally, we also noted variations in accuracy specific to chromosomes and algorithms. For example, RAPIDR did not detect any of the trisomy 18 samples or NIPTeR and NIPTeR-NCV detected trisomy 21 cases equally accurately, but NCV showed better trisomy 18 detection accuracy compared to the NIPTeR in lower sequencing coverage conditions (**Table 11, Figure 13**).

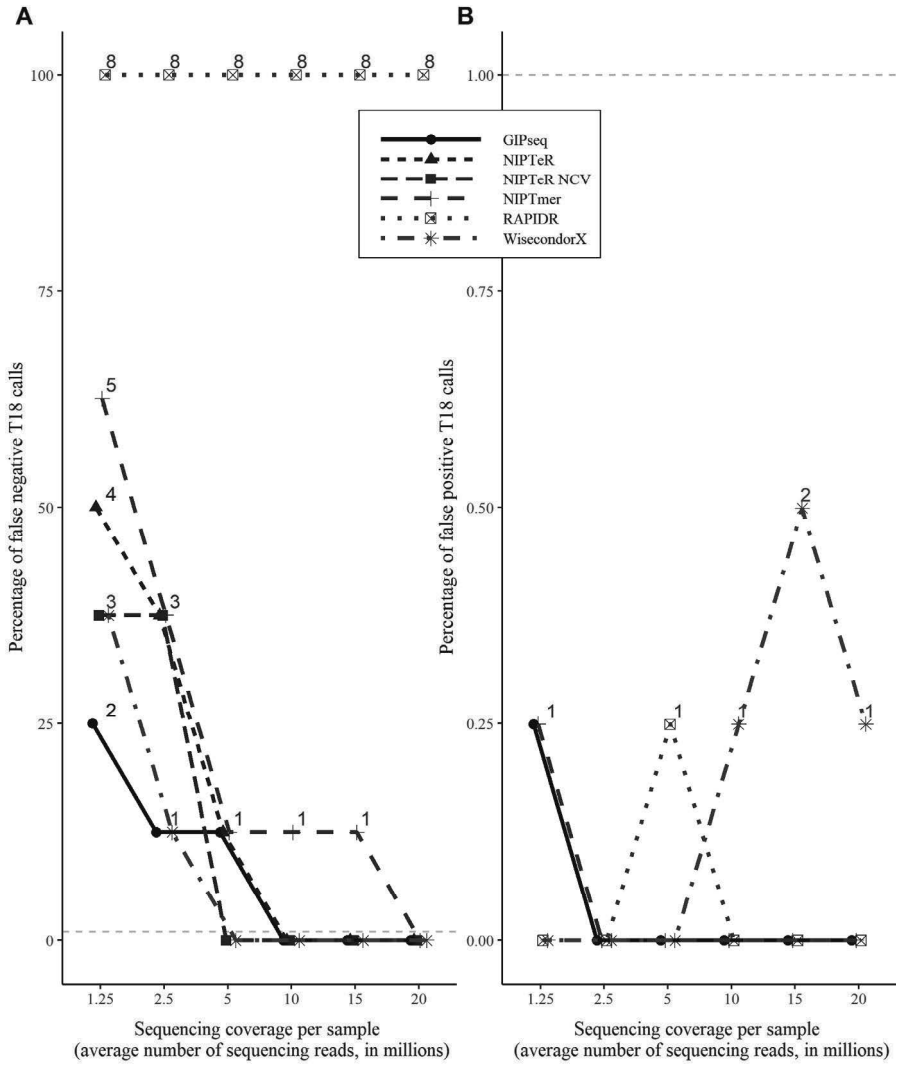


Figure 13. The percentage of false-negative (A) and false-positive (B) cases of trisomy 18 on different emulated sequencing coverages. (A) depicts percentages and the absolute number of false negative trisomy cases out of all known trisomy cases and (B) illustrates false-positive trisomy 18 calls out of all samples obtained with each NIPT software tool in case of various sequencing coverages. The horizontal dashed line marks the 1% cut-off, often used in case of clinical screening tests. Adopted from Paluoja et al., 2021.

5.2.2 Fetal DNA fraction effect on trisomy detection (Paper II)

As expected, we observed that fetal DNA fraction had a significant effect on trisomy detection accuracy, especially in the case of the lowest sequencing depth of 1.25M RPS, where all trisomy 21 samples with an FF under 7% remained

undetected (**Figure 14**). NIPTeR NCV and WisecondorX were the only tested tools with no false negative calls at extremely low FF of 3.65% at 5M RPS sequencing depth (**Figure 14, Table 11**). At 10M RPS with an FF of 3.65%, GIPseq and NIPTeR had no false negative trisomy calls. NIPTmer achieved a no-false-negative outcome with a 3.65% FF only at a sequencing depth of 20M RPS.

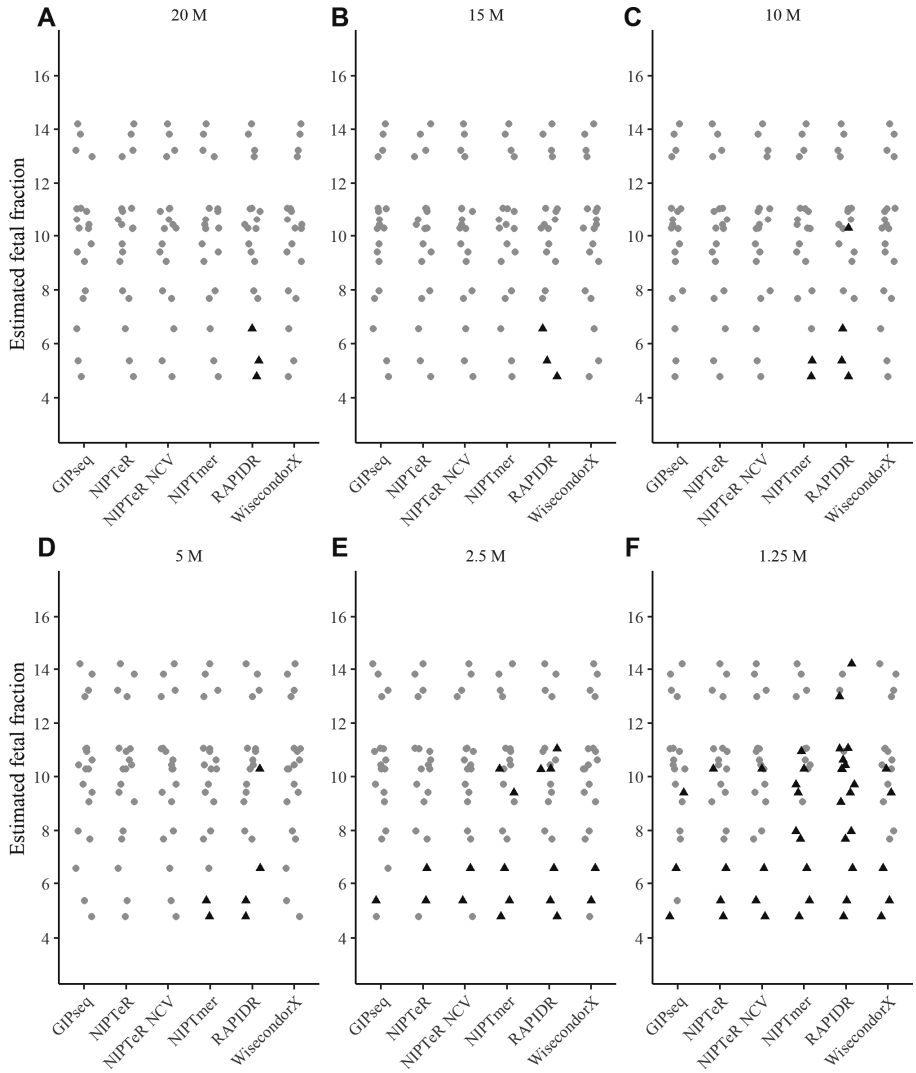


Figure 14. The effect of fetal DNA fraction on the detection of trisomy 21 across different sequencing coverages**.** Z_e cut-off was used for identifying the presence of the trisomy (internal classification in the case of GIPseq). Black triangles represent undetected trisomy cases. The 20M RPS group served as the standard for FF calculations. Data obtained with sequencing depths of 20M RPS (A), 15M RPS (B), 10M RPS (C), 5M RPS (D), 2.5M RPS (E), and 1.25M RPS (F) are shown. Adopted from (Paluoja et al., 2021).

We noted inconsistencies in SeqFF FF estimates at lower sequencing depths than estimates obtained in the reference coverage condition (~20M RPS) (**Figure 15**). For FFs ranging from 0% to 5%, the Pearson correlation value between 1.25M RPS FF estimates and corresponding 20M RPS FF estimates was only 0.217 (**Figure 15E**). At higher FF values (true FF, 5%–15%), the influence of sequencing depth was more subtle. For example, we obtained a correlation value of 0.959 between 10M RPS and 20M RPS but only 0.636 between 1.25M RPS and 20M RPS fetal fraction estimates. The observed correlation patterns between FF estimates at low sequencing depths and those at higher sequencing depths suggest a systematic tendency for FF to be overestimated under low-sequencing-depth conditions.

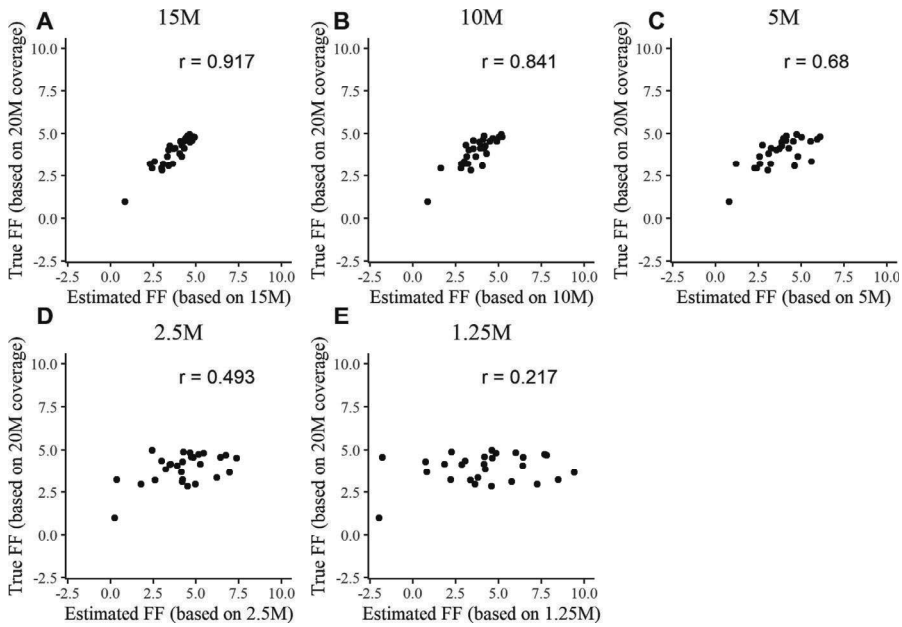


Figure 15. Correlation between the 'true' (based on 20M RPS data) and low sequencing depth based fetal fraction (FF) estimates. Pearson correlation data are shown for 20M RPS FF 0–5% estimates and estimates obtained at sequencing depths of 15M RPS (A), 10M RPS (B), 5M RPS (C), 2.5M RPS (D), and 1.25M RPS (E). Adopted from Paluoja et al., 2021.

WGS-based NIPT facilitates analysis beyond screening whole-chromosome aberrations. WGS allows examining any genomic region, including those spanning only a few million base pairs or less and recognised to be at risk of having pathogenic (micro)deletions that can lead to phenotypic severity comparable to trisomies. Unlike trisomy screening, with the availability of multiple computational tools, the options for microdeletion screening are scarce, and existing computational tool(s) are not validated on the same clinically confirmed NIPT fetal microdeletion samples under various fetal fraction and sequencing depth conditions.

5.3 Paper III: Fetal microdeletion risk screening in low-coverage WGS-based NIPT

Despite WGS-based NIPT covering the entire genome, only a fraction of the sequencing data originates from the fetal/placental source. Combining a limited amount of signal originating from the fetal/placental source with relatively short microdeletion regions and with the lack of microdeletion-screening-specific computational tools, we examined the feasibility of accurately estimating fetal microdeletion risk from low-coverage WGS NIPT data. We developed the necessary methodologies and a software tool, BinDel, with a microdeletion region-specific normalisation to assess and validate fetal microdeletion risk screening and compared it with a universal alternative computational tool.

5.3.1 Methodology and a software tool BinDel to estimate fetal microdeletion risk (Paper III)

In the clinical context, not all microdeletions are pathogenic. We concentrated solely on a predefined list of 12 microdeletion regions with known pathogenic clinical significance, as reporting variants of uncertain significance would lead to unnecessary invasive diagnostic procedures during pregnancy (**Figure 16**). Since clinically relevant pathogenic MDs typically have well-defined genomic coordinates, we designed the microdeletion risk detection algorithm to use these coordinates in risk detection, unlike heuristic/exploratory algorithms that do not consider and use external known biological information. Exploratory algorithms may not estimate MD risk if they interpret the MD as noise. However, the coordinate-based approach guarantees that the target MD region always receives an MD risk estimate.

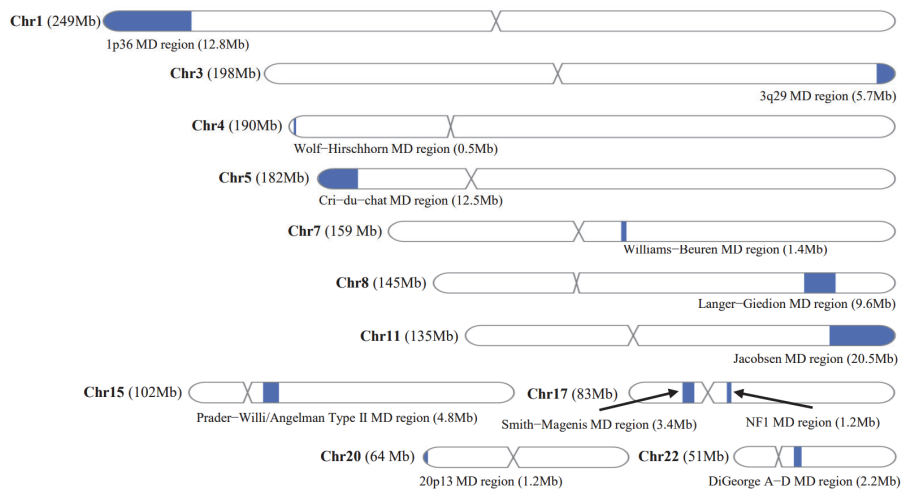


Figure 16. A priori chosen clinically relevant microdeletions (MDs) and their locations on the genome. Microdeletion regions are indicated with blue sections on the chromosomes. Adapted from Paluoja et al., 2025.

As we designed the BinDel for WGS data, which in NIPT relies on dividing sequencing reads to genomic bin counts, we made the genomic bins individually configurable as microdeletions are often in locations that are challenging to analyse. After the division of aligned sequencing reads into a priori selected and named genomic bins (certain bins denote MD regions, others non-MD regions), the genomic bins undergo GC% correction and are further normalised based on the total sequencing read count and bin lengths. The normalised values are divided by PCA dimensionality-reduced versions. Next, a normalised Z-score for each genomic bin using the euploid fetus reference set is calculated and aggregated per target region (see **Materials and Methods**). The Mahalanobis distance for each MD region from the euploid fetus reference group is calculated using the aggregated features, and for uniform interpretation between different genomic regions, the distances are transformed into chi-squared distribution p-values and subsequently -log10 transformed. This step restricts the estimated MD risk output to a range between 0% and 100%, ensuring a consistent interpretation of MD risk levels.

5.3.2 Calibrating BinDel (Paper III)

When developing BinDel, we observed that the number of PCA components used in the normalisation process affects the sensitivity and specificity of MD risk detection. To quantify the PCA effect on specificity and sensitivity, we used euploid and simulated fetal microdeletions as outlined in the **Materials and Methods** section. We observed that a higher number of PCA components (PCA99%) increased MD detection sensitivity, particularly in short MD regions like NF1 (1.2Mb) (**Figure 17A1**). However, this does not apply universally across all MD regions. For example, in the Williams-Beuren MD region, the overall sensitivity and specificity were either equal or slightly lower compared to the lower number of components setting (PCA95%) (**Figure 17B1, B2**). It is also important to note that using a higher number of PCA components also increased false-positive MD risk calls in certain regions, such as DiGeorge A-D (**Figure 17A2, C2**).

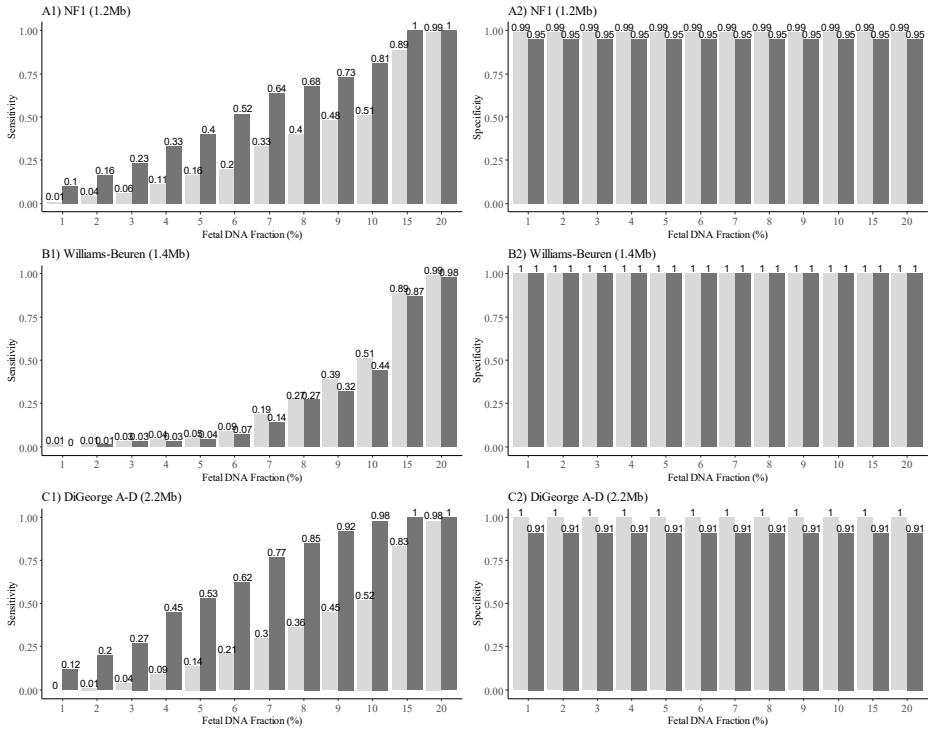


Figure 17. BinDel detection sensitivity and specificity with *in silico* simulated microdeletions using different numbers of PCA components. Sensitivity estimates are indicated by grey and dark grey bars, denoting PCA95% and PCA99%, respectively. The analysis focuses on three MD regions: NF1 (A1, A2), Williams-Beuren (B1, B2), and DiGeorge A-D (C1, C2) associated microdeletion regions. Adapted from Paluoja et al., 2025.

5.3.3 Fetal fraction and region length effect on BinDel performance using computationally simulated fetal microdeletions (Paper III)

We investigated the impact of FF on MD calling accuracy with computationally simulated fetal microdeletions in 1p36, 20p13del, 3q29, Cri-du-chat, DiGeorge, Jacobsen, Langer-Giedion, NF1, Prader-Willi/Angelman, Smith-Magenis, Williams-Beuren and Wolf-Hirschhorn MD regions.

As anticipated, higher FF considerably increased the MD detection sensitivity (**Figure 18A-D, relates to Figure 17**). However, notably, the observed patterns of increased sensitivity exhibited variations among distinct MD regions. For example, when comparing the Williams-Beuren or NF1 MD region to the NF1 MD region, they exhibited different MD risk-calling across all FF levels despite having a similar MD length (**Figure 18A-D)**.

Furthermore, longer MD regions exhibited higher MD risk detection sensitivity, particularly in cases with low FF (**Figure 18A**). However, this trend did not linearly persist across all MD regions. For instance, upon comparing the Langer-Giedion and 1p36 MD regions, the longer 1p36 MD region exhibited lower sensitivity than the shorter Langer-Giedion MD region (**Figure 18A**).

Based on our microdeletion simulation detection results, as a general guideline, screening accuracy notably decreased when the fetal DNA fraction fell below 10% and the microdeletion region length was under 5 Mb (**Figure 18C**). In a clinical setting, these microdeletion screening limits would be influenced by various factors, including sequencing depth, usage of fetal fraction enrichment, and the strategies used to balance false-positive and false-negative microdeletion calls.

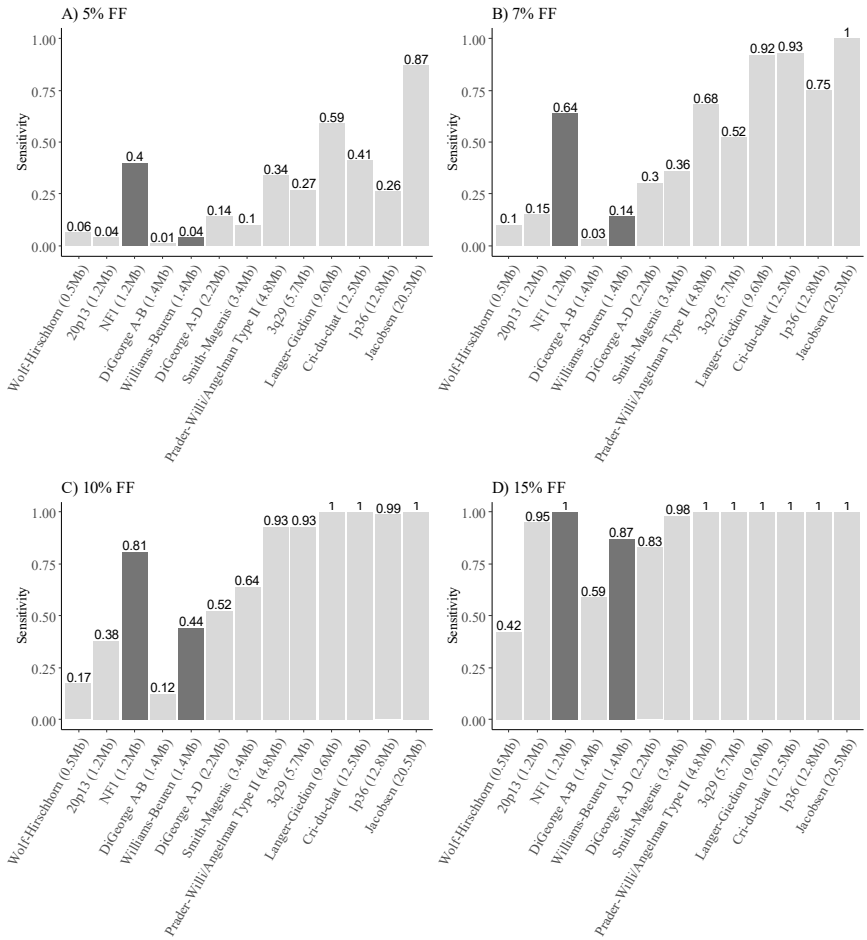


Figure 18. Microdeletion associated region length effect on the BinDel sensitivity of detecting microdeletion risk. Fetal DNA fractions of 5% (A), 7% (B), 10% (C) and 15% (D) were used for *in silico* simulated microdeletions. Sensitivity estimates are indicated by grey and dark grey bars, denoting PCA95% and PCA99%, respectively. Adapted from Paluoja et al., 2025.

5.3.4 Evaluation of BinDel with clinically validated microdeletion samples (Paper III)

We conducted an analysis with BinDel for 84 samples containing 34 clinically confirmed MD and 50 confirmed euploid NIPT samples. These 34 samples had microdeletions in DiGeorge, Prader-Willi/Angelman, Smith-Magenis, Williams-Beuren and NF1 MD regions. BinDel detected 73.5% (25 out of 34) clinically validated microdeletions (**Table 12**).

However, as also in clinical practice, microdeletions are always not fetal-only, as is also the case with the validation sample set. When excluding maternal and fetal or one solely maternal deletion and considering samples only with fetal microdeletions, BinDel correctly detected 69% (19 out of 28) of fetal MD samples (**Table 12**).

We observed that samples missed with the microdeletions were either in the Williams-Beuren microdeletion region or had an average lower FF (8.9%) compared to those where fetal MDs were correctly called (10.4%).

BinDel had 15 false-positive microdeletion calls, whereas the majority of them were concentrated in a few challenging regions, like the NF1 microdeletion region (**Table 12**).

To assess the BinDel results in context, we also inferred MD calls with WisecondorX, a universal WGS copy number detection tool intended for broader genomic applications (Raman et al., 2019). WisecondorX detected 47% (16 out of 34) MD samples or 35.7% (10 out of 28) fetal-only MDs (**Table 12**). WisecondorX had no false-positive MD calls.

Table 12. The number of true positive (TP), false positive (FP), false negative (FN), and positive predictive values (PPV) of microdeletion high-risk calls of BinDel and WisecondorX software packages on clinically confirmed 34 microdeletion and 50 euploid NIPT samples.

Microdeletion region, origin and number of samples (n)	BinDel				WisecondorX			
	TP	FP	FN	PPV	TP	FP	FN	PPV
DiGeorge, fetal, n = 17	12	0	5	1	4	0	13	1
DiGeorge, maternal and fetal, n = 4	4	0	0	1	4	0	0	1
DiGeorge, maternal, n = 1	1	0	0	1	1	0	0	1
Prader-Willi/Angelman, fetal, n = 5	5	2	0	0.71	4	0	1	1
Smith-Magenis, fetal, n = 3	2	0	1	1	2	0	1	1
Williams-Beuren, fetal, n = 3	0	3	3	0	0	0	3	0
NF1, maternal and fetal, n = 1	1	10	0	0.09	1	0	0	1

NF1, neurofibromatosis 1; Adapted from Paluoja et al., 2025.

5.3.5 Improving BinDel and reanalysing with clinically validated microdeletion samples (Paper III)

The analysis revealed two significant concerns. First, there was an unsuitable number of false-positive MD calls in NF1 and Williams-Beuren MD region for clinical usage, where we used PCA99% combined with a lower cut-off threshold (see **Materials and Methods**) (**Table 12**). Secondly, no true-positive MD calls were found in the Williams-Beuren MD region (**Table 12**).

Based on these insights, we systematically re-evaluated the BinDel data normalisation and MD calling process to improve region-specific microdeletion risk detection. First, we established if the high-risk MD cut-off threshold was optimal and if conservative PCA95% would yield higher specificity without compromising sensitivity. Our investigation revealed that elevating the cut-off threshold for calling MD across all microdeletion regions to 90% and using PCA95% reduced the total number of false-positive MD calls from 15 to 1 (false positive rate of 0.02) without affecting the number of true-positive MD calls (**Table 13**). The single false-positive MD call was in the Prader-Willi/Angelman MD region.

Table 13. Microdeletion cut-off threshold effect on BinDel accuracy.

Microdeletion high-risk cut-off threshold (%)	TP	FP	FN	PPV
10	27	19	7	0.59
20	26	9	8	0.74
30	25	5	9	0.83
40	25	5	9	0.83
50	25	4	9	0.86
60	25	3	9	0.89
70	25	3	9	0.89
80	25	3	9	0.89
90	25	1	9	0.96

The number of true-positive (TP), false-positive (FP), false-negative (FN), and positive predictive values (PPV) of microdeletion high-risk calls by BinDel with a PCA95% on different microdeletion high-risk cut-off thresholds. Adapted from Paluoja et al., 2025.

However, while using PCA95% and a uniform cut-off threshold of 90% across all MD regions lowered the false positive rate to 0.02, it did not resolve the absence of correct microdeletion detections in the Williams-Beuren MD region. As also indicated by the simulations and witnessed by Tian *et al.*, the Williams-Beuren MD region shows a lower microdeletion detection rate than other microdeletion regions with similar lengths (**Figure 18)** (Tian et al., 2023).

Observing the non-uniform MD detection sensitivity across MD regions of comparable sizes, we implemented a region-specific novel methodology (**Table 12, Figure 18**). Building upon our prior observation that PCA normalisation across all genomic bins increased MD detection sensitivity, we subsequently re-implemented the same normalisation technique. However, instead of uniformly applying it to all genomic bins, after the initial PCA normalisation, we applied PCA normalisation once more, but on a per MD region basis, factoring in the cumulative variance within each specific MD region (**Figure 19**). We also considered MD region risk score distribution to set the per-MD region cut-off threshold. We measured BinDel accuracy on validation sample set with combinations of PCA95%, PCA99%, cut-offs 1%-100% and cumulative regional PCA of 5%, 10%, 15%, 25% and 50% (only for PCA95%).

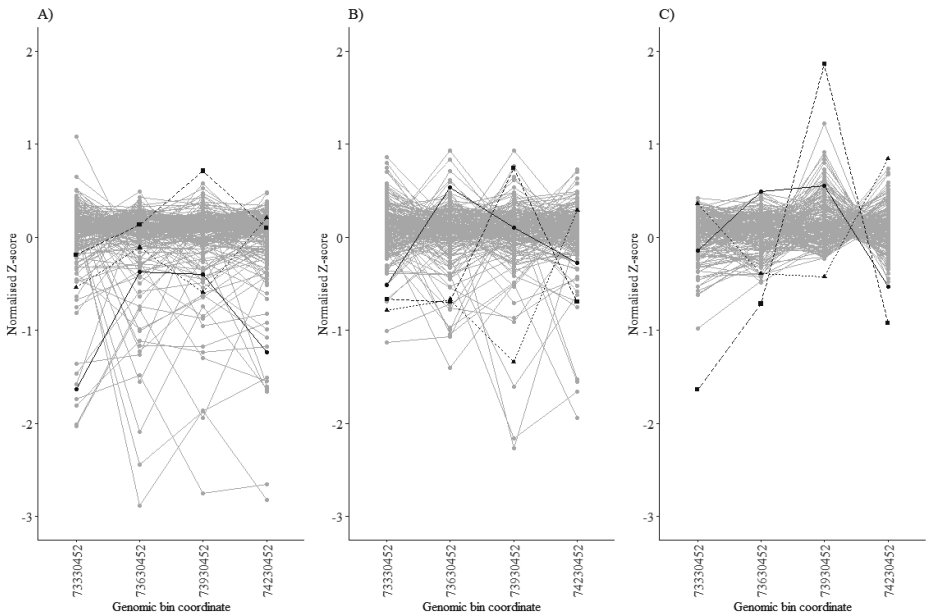


Figure 19. BinDel normalisation effects on genomic bin values in Williams-Beuren microdeletion region. (A) depicts normalised Z-scores (see **Methods**) without utilising the PCA normalisation method. (B) illustrates the impact of PCA95% normalisation on normalised Z-scores. (C) displays the combined effects of PCA95% and regional PCA (cumulative variance of 50%) normalised Z-scores. Each line in the figure represents a sample, where grey lines denote euploid reference samples, and black lines represent validation samples with fetal genomic microdeletion in the Williams-Beuren syndrome genomic region. Adapted from Paluoja et al., 2025.

The novel MD-region-specific approach detected 2 out of 3 samples with a fetal deletion in the Williams-Beuren MD region. Furthermore, this approach overall detected 30 out of 34 (88.2%) MD samples with a total of only three false positive MD calls, marking a 14.7% increase in detection sensitivity compared to the initial validation analysis (**Table 12, Table 14)**. Notably, the two false-positive MD calls emerged in the Williams-Beuren MD region (**Table 14)**. In a clinical setting, error-prone microdeletion regions with high false-positive rates, such as the Williams-Beuren MD region, can be excluded from the analysis. Instead, the focus could be on more common microdeletions, like those associated with the DiGeorge and NF1 syndromes, where BinDel demonstrated no false-positive calls (**Table 12**, **Table 14**). We observed that the average FF for four samples not correctly identified as MD samples was 8% compared to 10% for 30 correctly identified MD samples.

Table 14. The number of true-positive (TP), false-positive (FP) and positive predictive values (PPV) of microdeletion calls by BinDel with a region-specific approach.

Microdeletion, origin and number of samples (n)	Microdeletion high-risk cut-off threshold (%)	Cumula-tive PCA (%)	Cumulative regional PCA (%)	TP	FP	PPV
DiGeorge fetal, fetal and maternal, maternal n = 22	9	95	Not used	19[a]	0	1
Prader-Willi/ Angelman fetal n = 5	87	95	Not used	5	1[d]	0.8
Smith-Magenis fetal n = 3	4	95	Not used	3[b]	0	1
Williams-Beuren fetal n = 3	10	95	50	2[c]	2[f]	0.5
NF1 maternal and fetal n = 1	87	95	Not used	1	0[e]	1

The number of true-positive high-risk microdeletion calls increased compared to the initial analysis in DiGeorge[a], Smith-Magenis[b] and Williams-Beuren[c] microdeletion regions. False-positive microdeletion calls are reduced in Prader-Willi/Angelman[d], NF1[e] and Williams-Beuren[f] microdeletion regions. Adapted from Paluoja et al., 2025.

6. DISCUSSION

In computational NIPT, algorithms necessitating training (distinct from reference set requirements) are less commonly used due to the need to obtain a well-balanced set of positive and negative trisomy controls necessary for unbiased training. This challenge is further compounded by the high dimensionality of model features resulting from the multitude of genomic loci involved, requiring a substantial-sized training set. However, the trainability aspect enables the estimation of cut-off points and the determination of sample states based on learned data distributions, eliminating the need to devise trisomy-calling rules manually.

Interestingly, there is a scarcity of readily available, pre-packaged computational NIPT tools in the public domain for targeted NIPT. While some, such as FORTE or NATUS, are described, they are not readily available or pre-packaged for use in the public domain (Norwitz & Levy, 2013). Due to this scarcity, developing and implementing a computational framework for targeted NIPT became necessary. An optimal methodology should ideally be without excessive complexity while maintaining high accuracy. While the HMM approach displayed promise (**Results section Analytical framework for HMM output interpretation (Paper I)**) and alone did not have excessive complexity and was not affected by the dimensionality of the input, it did not have high accuracy in all cases due to the criterion used to interpret HMM output, which relied solely on the locus state with the highest frequency (trisomy or euploid). Introducing other available classification algorithms, such as random forests instead of HMM, was not considered due to their possible susceptibility to high dimensionality (the number of loci involved). In the clinical setting, prioritising simplicity over accuracy is not tenable, so we introduced a second classification layer. Since the two-level framework supported training with simulated data and the HMM reduced the dimensionality to only locus counts associated with euploid, maternally, and paternally inherited trisomies, we opted for SVM as it is known to work well when there is a clear distinction between different states. However, other machine learning-based classifiers, such as random forests or nearest neighbours, could also work well due to different states' low dimensionality and clear distinction. Combining HMM with supplementary SVM showed potential (**Results section Evaluation of the analytical framework of HMM output interpretation (Paper I)),** but the need for pre-sequencing loci selection hindered the straightforward expansion of the NIPT panel. Consequently, we shifted our focus to utilising WGS-based NIPT as a more viable alternative.

Interestingly, unlike in targeted NIPT, in the WGS-NIPT, numerous software options were readily accessible for WGS-based NIPT, obviating the need to develop and implement a computational tool. However, no study existed that had evaluated these available computational tools on the same clinically validated data. We conducted a comparative study to make an informed decision in choosing the most accurate computational WGS-NIPT tool. While prioritising accuracy

as the primary criterion, the tool needed to exhibit precision even at lower sequencing coverage levels, as the sequencing depth directly influences the cost-effectiveness of NIPT. We saw that the algorithm's choice significantly affects the number of false-negative trisomy calls, especially at sequencing depths lower than 5M RPS (**Results section Sequencing depth effect on trisomy detection (Paper II)**). This is somewhat expected, considering that the tested tools operate based on partitioning sequencing reads into genomic bins (or counting k-mers), followed by comparative analysis with the euploid sample (reference) group bins. In instances of reduced coverage, the discrepancy between euploid bins and reference bins diminishes, resulting in more conservative trisomy risk scores (**Results section Sequencing depth effect on trisomy detection (Paper II)**). Unexpectedly, inconsistencies in SeqFF FF estimates were also observed at lower sequencing depths (**Results section Fetal DNA fraction effect on trisomy detection (Paper II)**), particularly in scenarios where the true FF was low, despite these findings aligning with logical expectations given that SeqFF is pre-trained on sample-set with higher sequencing coverage (Kim et al., 2015). Although FF, sequencing depth, and algorithm selection are typically the primary factors influencing trisomy screening accuracy, we also observed variations in the distributions of outputted Z-scores among software tools when these tools were employed on euploid reference set samples across different sequencing depths. Variations in Z-score distributions across different sequencing coverages imply that employing a single, uniform cut-off threshold independent of the specific tool may diminish the accuracy of trisomy detection. This discrepancy was notably apparent in one instance involving the WisecondorX tool by introducing two undetected trisomies at a sequencing depth of 2.5M RPS (**Results section Fetal DNA fraction effect on trisomy detection (Paper II)**). Interestingly, variations in Z-score distributions were observed only with some tools, implying differences in the internals of the algorithms related to Z-score calculations and the steps preceding. Overall, this study allowed us to make an informed decision in choosing NIPTeR (NCV) as the optimal trisomy screening algorithm by considering the overall accuracy under different sequencing depths, fetal fractions, and software licensing, also allowing non-scientific use.

NIPTeR, however, is not designed to infer microdeletion risk scores (Johansson et al., 2018). While over 50 copy number variation tools exist, the number of tools designed for NIPT is minimal (Gabrielaite et al., 2021). For example, Wisecondor and WisecondorX were among the few readily available and prepackaged tools available at the time of writing that could infer fetal aberrations, including microdeletions from WGS-based NIPT (Raman, Dheedene, et al., 2019; Straver et al., 2014). However, these tools are not explicitly designed for fetal microdeletion screening and use a heuristic approach in screening for fetal aberrations. Moreover, existing studies examining the impact of factors like FF or MD region length on microdeletion screening, as demonstrated by Kucharik et al., are few and constrained to simulations, mixing DNA samples, or do not provide readily available computational tools for public use (Kucharik et al., 2020). Considering the lack of a dedicated low-coverage WGS-based NIPT fetal

microdeletion computational screening tool, we developed BinDel (**Results section Evaluation of BinDel with clinically validated microdeletion samples (Paper III)**).

Given the infrequency of pathogenic microdeletions, conducting an extensive study using naturally occurring fetal microdeletions (similar to our approach with trisomies) is challenging due to the rarity of such samples. For instance, in 2024, Estonia reported 9,523 live births (Eesti Meditsiiniline Sünniregister, 2025). This scarcity indicates the occurrence of only a few fetal microdeletions annually in such a population context (e.g. DiGeorge syndrome occurring in approximately 1 in 4,000 births) (McDonald-Mcginn & Sullivan, 2011). Furthermore, only NIPT samples procured and sequenced during the first trimester are suitable for computational research. This criterion introduces complexities, such as alterations in laboratory protocols that can render previously processed samples unusable for research purposes. Moreover, our sample set highlighted the diversity of microdeletions, exemplified by the presence of various forms. For instance, within our validation set, we encountered fetal-only, fetal and maternal, and a single maternal-only microdeletion or 22q11.2 (DiGeorge MD region) deletion occurring as forms of A-B and A-D. Additionally, our observations indicated differences for microdeletions in similar length. For example, the Williams-Beuren MD region exhibited a lower detection accuracy compared to other regions of similar length, such as the NF1 MD region (**Results section Evaluation of BinDel with clinically validated microdeletion samples (Paper III)**). We also observed that detection accuracy is influenced by the fetal fraction (FF). Our simulation results showed a significant decline in detection accuracy when the FF dropped below 10% and the affected region was shorter than 5 Mb. In a real-world clinical setting, increasing FF through laboratory enrichment techniques and retesting with a new sample in cases of elevated MD risk may help reduce false-positive calls and minimise the need for invasive diagnostic procedures. Furthermore, MD regions with high false-positive rates or low population prevalence may be excluded from routine NIPT analysis. Instead, clinical screening could focus on more common microdeletions, such as the DiGeorge MD region, for which BinDel showed no false positives and demonstrated significantly higher sensitivity compared to WisecondorX. These findings, supported by our validation study, underscore the feasibility of detecting MD syndrome risk through NIPT and highlight its potential to broaden the spectrum of fetal genetic conditions detectable in the first trimester.

This study was limited by the number of clinically validated samples with pathogenic microdeletions and trisomies, particularly for certain regions such as those associated with Williams-Beuren or NF1 syndromes, and for some aneuploidies like trisomy 13. Additionally, we were constrained to low sequencing coverage to maintain the cost-effectiveness of NIPT as a broadly accessible screening method. However, lower coverage reduces the sensitivity of computational tools, especially in cases with low fetal fraction. Finally, there is a lack of publicly available NIPT-specific microdeletion screening tools for comparison.

While tools like WisecondorX are available, they are not explicitly designed for detecting pathogenic microdeletion risk.

Overall, while frequently understated, the algorithms deployed significantly influence the accuracy and cost-effectiveness of NIPT.

7. CONCLUSION

Aim 1: First, we developed computational methods for targeted NIPT-based trisomy risk detection and systematically evaluated trisomy risk calling, as the majority of available computational methods for sequencing-based NIPT rely on WGS and cannot be used for targeted NIPT. The developed computational method relied on HMM incorporated with an analytical framework for HMM output interpretation using SVM. Notably, the supplemental SVM elevated paternally inherited trisomy detection sensitivity from zero to one. For some HMM-based models, the SVM also considerably improved the detection of maternally inherited trisomy, with a specificity decrease between 0.01 and 0.03 in some cases.

Aim 2: We determined how sequencing depth and estimated FF affect trisomy risk calling in low-coverage WGS-based NIPT by assessing the accuracy of WisecondorX, NIPTeR, NIPTmer, RAPIDR, and GIPseq trisomy risk calling across various sequencing depths and fetal DNA fractions. While all of the compared tools were affected by an increased number of undetected trisomy cases when sequencing depth was lowered, our findings generally indicated accurate results when sequencing depth exceeded 5M reads per sample. However, the least accurate algorithm would miss almost one-third of trisomy cases at lower sequencing depths, such as at and below 2.5M reads per sample. We also described previously undocumented aneuploidy risk uncertainty that can increase undetected trisomy cases. Finally, as expected, we observed that FF significantly affected trisomy detection accuracy. For instance, at the lowest sequencing depth of 1.25M RPS, all trisomy 21 samples with an FF under 7% remained undetected.

Aim 3: We investigated the feasibility of estimating fetal microdeletion risk from low-coverage WGS NIPT data. We developed the necessary methodologies, including BinDel, a software tool. Using simulated fetal microdeletions, we observed that higher FF considerably increased the MD detection sensitivity in 12 clinically pathogenic microdeletion regions, including 1p36 deletion, 3q29, Wolf-Hirschhorn, Cri-du-chat, Williams-Beuren, Langer-Giedion, Jacobsen, Prader-Willi/Angelman, Smith-Magenis, neurofibromatosis type 1, 20p13 and DiGeorge syndrome related microdeletion regions. While longer MD regions exhibited higher MD risk detection sensitivity, there were variations in detection sensitivity among distinct MD regions despite having a similar MD length. Finally, in the analysis of clinically confirmed 34 microdeletions and 50 euploid fetal samples, BinDel correctly identified 25 samples with microdeletions. However, 15 false-positive microdeletion calls were concentrated in a few challenging regions. The number of false-positive microdeletion calls led us to improve BinDel. After the improvement in the algorithm, 30 microdeletion samples were correctly

determined, with a total number of false-positive microdeletion calls reduced to three. The improvement confirmed BinDel's usefulness for fetal microdeletion risk screening in NIPT, opening the possibility of integrating BinDel successfully into routine NIPT protocol.

SUMMARY IN ESTONIAN

Arvutuslikud metoodikad loote trisoomiate ja mikrodeletsioonide riski tuvastamiseks mitteinvasiivses sõeluuringus

Mitteinvasiinve sünnieelne loote DNA uuring ehk NIPT (*non-invasive prenatal testing*) on sõeluuringu meetod, mis võimaldab ohutut ja täpset sünnieelset mitteinvasiivset kromosoomhaiguste riski hindamist. NIPT põhineb lapseootel naise veres ringleva loote (platsenta) rakuvaba DNA laboratoorsel ja arvutuslikul analüüsil. Kuigi loote (platsenta) DNA esinemine raseda vereproovis avastati juba 1997. aastal, siis aastatel 2011–2012 publitseeriti NIPT esimesed suuremahulised valideerimisuuringud (Bianchi et al., 2012; Lo et al., 1998; Palomaki et al., 2011).

Lisaks loote kromosoomi koopiaarvu muutustele võimaldab NIPT uuring tuvastada mikrodeletsiooni riski. Mikrodeletsiooni sündroom on lühikese kromosoomiosa kaost põhjustatud kromosoomihaigus, mille kliiniline raskusaste sõltub deleteerunud regioonist. Näiteks üks sagedane mikrodeletsioon on 22q11.2 heterosügootne deletsioon, mis põhjustab DiGeorge'i sündroomi, mida iseloomustavad mitmesugused kaasasündinud arenguhäired, nagu südamerikked või vaimupuue (McDonald-Mcginn & Sullivan, 2011).

Võtmeroll NIPT uuringus on DNA sekveneerimisandmete analüüsi arvutuslikel algoritmidel, mis otseselt väärindavad NIPT andmeid.

Uurimistöö eesmärgid

1. Töötada välja ning süstemaatiliselt hinnata arvutuslike metoodikate täpsust loote triosoomia riski määramisel suunatud NIPT sekveneerimisanalüüsi tarbeks.
2. Hinnata loote trisoomia riski määramise täpsust madala katvusega ülegenoomse NIPT sekveneerimisanalüüsi jaoks. Teha kindlaks sekveneerimissügavuse ja loote DNA fraktsiooni mõju trisoomiariski määramise täpsusele.
3. Uurida loote patogeensete mikrodeletsioonide riski määramise võimalikkust madala katvusega NIPT sekveneerimisanalüüsi tarbeks. Töötada välja vajalikud metoodikad ja neid rakendav tarkvara loote mikrodeletsiooniriski täpseks hindamiseks ja valideerida täpsus kliiniliselt kinnitunud mikrodeletsiooni proovidega.

Materjal ja metoodika

Esimeses uurimuses välja töötatud peidetud Markovi mudeli (HMM) põhinev arvutuslik metoodika loote trisoomia tuvastamiseks kõrge katvusega sekveneeritud NIPT tarbeks oli paljulubav, ent määras loote seisundi (euploidne, isa või ema päritolu trisoomia) kõige sagedasema HMM poolt klassifitseeritud lookuse oleku (euploidne, isa või ema päritolu trisoomia) järgi. Selline trisoomia riski määramine ei suutnud tuvastada isapoolse päritoluga trisoomiat ja ei arvestanud

keerukamate jaotustega (piirjuhud, kus euploidseks ja trisoomiaks klassifitseeritud lookuste arv oli peaaegu võrdne). Uurimuse raames töötati välja arvutusliku raamistik adresseerimaks HMM limitatsioone kasutades simuleeritud raseda rakuvaba DNA andmeid, mis sisaldasid nii isapoolse kui ka emapoolse päritoluga loote trisoomiat arvestades erinevad lootefraktsioone.

Teises uurimuses määrati WisecondorX, NIPTeR, NIPTmer, RAPIDR ja Leuveni Ülikooli analüütiliste töövoogude täpsus samal kliiniliselt valideeritud andmestikul teisendatult erinevale sekveneerimise sügavustele (katvustele). Kliiniliselt valideeritud kohort koosnes 423 raseda (üksikrasedus) sekveneeritud rakuvaba DNA proovist, mis oli eraldatud veeniverest. Sekveneerimisandmed sisaldasid 19 Downi, kaheksa Edwardsi ning kolme Patau sündroomiga juhtu. Teisendatud sekveneerimise katvustega NIPT andmete komplektid sisaldasid 1.25–20 miljonit lugemit patsiendi NIPT sekveneerimisandmete kohta. Ühtseks analüüsi tulemuste tõlgendamiseks defineeriti protsendipunkti funktsioonil põhineva raamistik trisoomia määramiseks. Määrati rakuvaba loote DNA hulga mõju analüütiliste töövoogude täpsusele.

Kolmandas uurimuses töötati välja bioinformaatiline analüüsitarkvara BinDel määramaks loote patogeenseid kliiniliselt olulisi mikrodeletsioone madala katvusega kogu genoomi NIPT sekveneerimisandmetel. BinDel rakendab mitmeid sekveneerimise lugemite normaliseerimise etappe, uudset algoritmilist mikrodeletsioonide signaali võimendamist, sh kaheastmelist peakomponentide analüüsil põhinevat normaliseerimist, suunatud analüüsi ning riski tõenäosuse arvutamist Z-skoori asemel. Määrasime rakuvaba loote DNA fraktsiooni ja mikrodeletsiooni pikkuse mõju mikrodeletsioonide tuvastamise täpsusele kasutades bioinformaatiliselt indutseeritud mikrodeletsioonidega NIPT proove. Koostöö Belgias asuva ülikooli Katholieke Universiteit Leuven'iga valideeriti BinDel 34 teadaoleva kliiniliselt kinnitunud patogeense mikrodeletsiooniga ja 50 euploidse NIPT proovi vastu. Kohort sisaldas DiGeorge, Angelman/Prader-Willi, neurofibromatoos tüüp 1 (NF1), Smith-Magenis ja Williams-Beureni sündroomiga proove.

Uurimistöö kokkuvõte ja järeldused

Esimese uurimuse tulemusena töötati välja kaheastmeline arvutuslik metoodika trisoomiariski tuvastamiseks suunatud NIPT sekveneerimisanalüüsi tarbeks. Väljatöötatud arvutuslik metoodika tugines peidetud Markovi mudelil (HMM), mille väljundit interpeteeriti tugivektor-masinaga (SVM). Täiendav SVM rakendatuna HMM mudelile tõstis isalt päritud trisoomia tuvastamise tundlikkuse nullilt üheni. Teatud HMM-põhiste mudelite puhul parandas SVM märkimisväärselt ka emalt päritud trisoomia tuvastamist vähese spetsiifilisuse langemisega.

Teise uurimuse tulemusena tuvastati analüütilised töövood, mille minimaalne sekveneerimise katvus ilma tuvastamata trisoomiateta oli viis miljonit lugemit proovi kohta. Lisaks näidati, et analüüs alla viie miljoni sekveneerimise lugemiga oli võimalik kõrge loote DNA fraktsiooni korral, ent kätkes tuvastamata trisoomia riski. Kõigil analüüsitud NIPT tööriistadel esines teatud sekveneerimise lugemite

piirist tuvastamata trisoomiaid. Avastamata trisoomia riski oli võimalik vähendada, kui riski määramisel arvestada euploidsete proovide võrdlushulga Z-skooride jaotusega. Veel täheldati, et loote rakuvaba DNA hulga hinnangutel esines vähenenud korrelatsioon sama vereproovi madalamatel katvustel, mis juhib ettevaatlikusele loote DNA fraktsiooni hinnangute tõlgendamisel.

Kolmanda uurimuse raames valmis bioinformaatiline analüüsitarkvara BinDel määramaks madala katvusega ülegenoomse sekveneerimise NIPT andmetest loote kliiniliselt patogeensete mikrodeletsioonide esinemise riske. Simuleeritud loote mikrodeletsioonide abil määrasime loote DNA fraktsiooni ja mikrodeletsioonipiirkonna pikkuse mõju mikrodeletsiooniriski tuvastamise täpsusele 12 kliiniliselt patogeenses mikrodeletsiooni piirkonnas, sealhulgas 1p36 deletsiooni, 3q29, Wolf-Hirschhorn, Cri-du-chat, Williams-Beuren, Langer-Giedion, Jacobsen, Prader-Willi/Angelman, Smith-Magenis, NF1, 20p13 ja DiGeorge'i sündroomiga seotud genoomsetes piirkondades. BinDeli esialgne analüüs, kuhu oli kombineeritud 34 kliiniliselt kinnitunud mikrodeletsiooniga ja 50 euploidse lootega NIPT proovi, tuvastas BinDel õigesti 25 mikrodeletsiooniga proovi. 15 terve lootega proovi määrati ekslikult kõrge riskiga proovideks. Järgnes BinDeli edasiarendus, mille tulemusel määrati korrektselt 30 mikrodeletsiooniga proovi ning valepositiivsete mikrodeletsioonide risk vähenes kolmele.

Uuringu peamine piirang oli kliiniliselt valideeritud patogeensete mikrodeletsioonidega ja trisoomiatega sekveneeritud rakuvaba DNA proovide piiratud hulk. Lisaks olid sekveneerimise andmed madala katvusega, sest sekveneerimise katvus mõjutab NIPT kulutõhusust. Madalam katvus aga vähendab arvutuslike tööriistade tundlikkust tuvastada mikrodeletsioonide või trisoomiate riski ja seda eriti madala loote fraktsiooni korral. Samuti puuduvad NIPT andmetest spetsiaalselt mikrodeletsioonide riski tuvastamiseks loodud avalikult kättesaadavad arvutuslikud tööriistad, millega tulemusi võrrelda. Kuigi tööriistad nagu WisecondorX on kättesaadavad, ei ole need spetsiaalselt loodud patogeensete mikrodeletsioonide riski tuvastamiseks.

Kokkuvõttes mõjutavad kasutatavad algoritmid märkimisväärselt NIPT täpsust ja kulutõhusust ning seeläbi NIPT kättesaadavust tervishoiuteenuse osana.

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PUBLICATIONS

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List of publications

1. **Paluoja, Priit**; Jatsenko, Tatjana; Teder, Hindrek; Krjutškov, Kaarel; Vermeesch, Joris Robert; Salumets, Andres; Palta, Priit (2025). BinDel: Detecting Clinically Relevant Fetal Genomic Microdeletions Using Low-Coverage Whole-Genome Sequencing-Based NIPT. Prenatal Diagnosis. DOI: 10.1002/pd.6758.
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Publikatsioonid

1. **Paluoja, Priit**; Jatsenko, Tatjana; Teder, Hindrek; Krjutškov, Kaarel; Vermeesch, Joris Robert; Salumets, Andres; Palta, Priit (2025). BinDel: Detecting Clinically Relevant Fetal Genomic Microdeletions Using Low-Coverage Whole-Genome Sequencing-Based NIPT. Prenatal Diagnosis. DOI: 10.1002/pd.6758.
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