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„Integrating Cell-Free Fetal DNA from Amniotic Fluid in Prenatal Diagnostics: Advancements, Challenges, and Prospects“

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Abstract

Prenatal diagnosis can be challenging, particularly in the early stages of pregnancy, where direct genomic fetal DNA extraction is impossible. However, by integrating cell-free fetal DNA (cff-DNA) from amniotic fluid, results can be expedited, delays can be prevented, and the need for additional punctures can be eliminated in case of cell culture failure.

Our research has identified a correlation between gestational age and the concentration of cff-DNA. While higher concentrations can be achieved in later stages, earlier weeks of pregnancy often require a concentration step for DNA-intensive methods. The study also discovered a significant finding in preventing maternal cell contamination through centrifugation. This method was tested on 34 visibly blood-tinged amniotic fluids and was proven effective through STR-Marker analysis. Maternal cells, such as lymphocytes, can release maternal DNA during lysis during DNA extraction, leading to misinterpretation of results in the worst-case scenario.

The study used different molecular techniques on 162 patient samples, including Array Comparative Genomic Hybridization, Multiplex Ligation-Dependent Probe Amplification, Sanger sequencing, and Whole Exome Sequencing. The results showed that all methods could be successfully adapted to work with cff-DNA with specific protocols, achieving 100% concordance with the gf-DNA routine diagnostic results. Our research presents a new method for extracting cff-DNA from amniotic fluid, enabling immediate prenatal diagnosis before 20 weeks of pregnancy, significantly reducing the time required for diagnosis and improving clinical decision-making, benefiting healthcare professionals and patients.

Kurzfassung

Die pränatale Diagnostik kann in frühen Schwangerschaftsstadien, in denen eine direkte Extraktion von genomischer fetaler DNA nicht möglich ist, eine große Herausforderung sein, um eine schnelle und zuverlässige Diagnose für beunruhigte Eltern zu stellen. Durch die Integration von zellfreier fetaler DNA (cff-DNA) aus dem Fruchtwasser können jedoch Ergebnisse beschleunigt und zusätzliche Punktionen im Falle eines Zellkulturversagens eliminiert werden. Unsere Forschung hat einen Zusammenhang zwischen Schwangerschaftsalter und der Konzentration von cff-DNA festgestellt. Während in späteren Stadien höhere Konzentrationen erreicht werden können, erfordern frühere Schwangerschaftswochen oft einen Konzentrationsschritt für DNA-intensive Methoden. Die Studie entdeckte außerdem, dass mütterliche Zellkontamination durch Zentrifugation bei der Verwendung von cff-DNA minimiert oder sogar beseitigt wird. Mütterliche Zellen können während der Lyse bei der DNA-Extraktion DNA freisetzen, was zu einer Fehlinterpretation der Ergebnisse führt. Dies wurde an 34 sichtbar blutunterlaufenen Fruchtwässern getestet und erwies sich durch die STR-Analyse als wirksam. Die Studie untersuchte 162 Patientenproben mit verschiedenen molekularen Techniken, darunter Mikroarray-basierte komparative gnomische Hybridisierung, Multiplex-ligationsabhängige Sondenamplifikation, Sanger-Sequenzierung und Whole-Exome-Sequenzierung. Die Ergebnisse zeigten, dass alle Methoden erfolgreich an die Arbeit mit cff-DNA angepasst werden konnten und eine 100% Übereinstimmung mit den routinediagnostischen Ergebnissen der gf-DNA erzielten. Unsere Forschung stellt eine neue Methode zur Extraktion von cff-DNA aus dem Fruchtwasser vor, die eine sofortige pränatale Diagnostik vor der 20. Schwangerschaftswoche ermöglicht, auch im Falle einer möglichen maternalen Kontamination. Dies verkürzt die Diagnosezeit deutlich.

Introduction

1.1 Prenatal diagnostics

Prenatal genetic testing is critical for maternal-fetal health and provides vital information about the developing fetus. This diagnostic process aims to increase accuracy while finding solutions for issues related to traditional invasive methods, such as Chorionic Villus Sampling (CVS) and amniocentesis. Expectant parents often go through invasive prenatal diagnostic procedures, such as CVS or amniocentesis, based on indications like abnormal ultrasound findings or familial predisposition.

CVS is a diagnostic technique that involves taking a small tissue sample from the placenta. It was first introduced in China in the mid-1970s and further developed during the 1980s [1], [2]. This procedure is usually performed after 12 weeks of gestation, as it carries a higher risk of abortion if done earlier. It is used to diagnose genetic conditions and chromosomal abnormalities. The choice between CVS and amniocentesis depends on factors such as the timing of the procedure and the potential risks. CVS is often preferred due to its early timing, which allows for earlier intervention and decision-making when there are concerns about genetic disorders. However, CVS has some limitations, such as the potential unreliability due to placental mosaicism and a slightly higher risk of miscarriage compared to amniocentesis [1].

Time plays a significant role in genetic prenatal diagnosis since pregnant women experience uncertainty and psychological distress while awaiting test results after an invasive procedure based on a screening test result. This waiting period can cause a heightened emotional burden for expectant parents. Therefore, shortening the waiting time and providing prompt results are primary objectives in genetic prenatal diagnosis to alleviate the psychological impact on patients [3], [4].

Screening programs have evolved into critical tools that provide essential information during pregnancy and offer expectant mothers assurance about their unborn children's health. However, traditional invasive methods come with inherent challenges and risks. The anxiety-inducing waiting period for test results, coupled with the potential for 'false positive' outcomes, poses a significant concern. Furthermore, the limited therapeutic options for identified chromosomal abnormalities make decision-making complex for expectant parents [5].

Amniocentesis is a prenatal diagnostic procedure that involves the insertion of a needle

through the mother's abdomen to collect a sample of the amniotic fluid surrounding the fetus and was first described in the 1950s [6], [7] This procedure is typically performed after 15 weeks of gestation and is used to diagnose genetic conditions, chromosomal abnormalities, and neural tube defects [1], [7].

Amniocentesis is preferred over CVS in cases where the patient has a history of bleeding disorders or in cases where the placenta is not accessible. However, the procedure does come with some risks, including the possibility of infection, bleeding, and miscarriage. The decision to undergo amniocentesis should be made after a thorough discussion with the healthcare provider regarding the potential risks and benefits [8].

Pregnant women may experience psychological distress while awaiting test results. This uncertainty surrounding fetal health and potential impacts on pregnancy outcomes contribute to their distress. Therefore, promptly performing genetic tests is crucial to alleviate the psychological burden on pregnant women and their families. As we look into the intricacies of prenatal genetic diagnostics, it becomes imperative to address these challenges and explore how advancements aim to mitigate risks and enhance the overall efficacy of these diagnostic procedures. This multifaceted landscape of prenatal genetic diagnostics underscores the delicate balance between advancing medical knowledge, addressing historical challenges, and meeting the evolving needs of expectant parents.

1.2 Fetal and placental development

Fetal development: The development of the fetus and placenta is a complex process that begins with the fertilisation of an egg and continues through various stages of pre-implantation, embryonic, and fetal development. Over 9 months, the fetus grows from a single cell to a fully-formed human being [9], [10].

The process starts when the egg is released from the ovary and moves down the fallopian tube, where a sperm may fertilise it. Once fertilised, the egg divides repeatedly as it travels to the uterus, ultimately forming a solid ball of cells known as a blastocyst. The blastocyst then attaches itself to the top lining of the uterus and completes implantation between days 9 and 10.

The inner cell mass of the blastocyst grows into the embryo, while the outer cells burrow into

the uterine wall to form the placenta. The placenta produces hormones that help maintain the pregnancy and carry oxygen and nutrients from the mother to the fetus. The outer membranes (chorion) and the inner layer (amnion) form the amniotic sac when the blastocyst becomes an embryo. This sac fills with fluid and envelops the developing embryo within it [11].

The embryo develops most organs and body parts within the amniotic sac 3 to 10 weeks after fertilisation, although the brain and spinal cord grow throughout pregnancy. At 8 weeks, the placenta and embryo have been developing for 6 weeks. The placenta has formed villi, which enable material to be exchanged between the mother's blood and the embryo's blood while protecting the embryo from the mother's immune system. The embryo is surrounded by amniotic fluid, providing a secure environment for growth [10], [12]. The amniotic sac is solid and resilient. By 10 weeks, the embryo has transformed into a fetus and continues to mature and develop. At 12 weeks, the fetus has filled the uterus, and by 14 weeks, its sex can be determined. Generally, the pregnant woman can feel the fetus moving at 16-20 weeks. The placenta has grown hair-like projections, increasing the contact area with the uterus, allowing for more efficient nutrient and waste exchange. The placenta is fully formed by 18-20 weeks and continues to grow until delivery. The lungs and brain continue to develop until close to the time of delivery [9], [10], [12], [13].

Placental development: Placental development is an essential process during the first trimester of pregnancy. The placenta, which connects the fetus to the mother through the umbilical cord, plays crucial roles in endocrine, immune, and physiological functions. It is a spongy disc that gradually develops in the uterus, with a diameter of about 20 centimetres. Proper placental development is essential for a successful pregnancy, as defects in early placental development may lead to significant pregnancy disorders such as recurrent miscarriage, fetal growth restriction, pre-eclampsia, and stillbirth [14]–[20]. Early placental development is a complex and carefully orchestrated process. It begins with attaching the blastocyst's polar trophectoderm to the endometrial epithelium around 5-6 days post-fertilisation. Gene expression forms a primary syncytium, penetrating the endometrial cells and transforming them into the decidua, a specialised tissue. The remaining trophectoderm cells become cytotrophoblasts, which fuse with the primary syncytium, covering the entire gestational sac. Fluid-filled spaces develop within this syncytial

mass, forming a lattice of trabeculae that extend into the decidual glands, nourishing the syncytiotrophoblast [21]–[23]. Around 12 days post-fertilisation, cytotrophoblast cells proliferate, forming primary villi penetrating trabeculae. After cytotrophoblast penetration, around 17–18 days post-fertilisation, extraembryonic mesenchymal cells infiltrate the villous core, forming secondary villi. Haemangioblastic clusters differentiate within the mesenchyme, initiating the fetal vascular network and tertiary villi development. The villous tree rapidly expands through progressive branching from the chorionic plate [24]–[26]. Individual cytotrophoblast cells exit the cytotrophoblast shell at the maternal-fetal interface, becoming non-dividing human leukocyte antigen G (HLA-G)-positive extravillous trophoblasts (EVT). These cells undergo an epithelial-mesenchymal transition (EMT), expressing various factors involved in invasion, immunomodulation, and cellular adhesion [15], [27]. Extravillous trophoblasts (EVT) are non-dividing human leukocyte antigen G (HLA-G)-positive cells that play crucial roles in immune cell interaction, defence against pathogens, and vascular remodelling. EVT cells undergo an epithelial-mesenchymal transition (EMT), expressing various factors involved in invasion, immunomodulation, and cellular adhesion. Some EVT cells undergo polyploidisation and induce cellular senescence, resulting in trophoblast giant cells deposited in the maternal decidua and myometrium. One of the pivotal roles of EVT cells is mediating vascular remodelling, contributing to the transformation of spiral arteries into high-conductance vessels. This early development sets the stage for successful fetal and placental development throughout pregnancy [27]–[31].

1.3 Amniotic fluid

The amniotic fluid, which has several functions, protects the fetus during its development. It is a physical barrier for the fetus and umbilical cord, and its antibacterial properties help prevent infection. The fluid also provides nutrients for the fetus' growth, and doctors can use it to monitor the pregnancy's progress and the fetus' outcomes [32], [33]. The development of amniotic fluid is divided into two stages: early gestation and late gestation. The embryonic period, which lasts from fertilisation until 8 weeks, is known as early gestation, and the fetal period, which lasts from 8 weeks until birth, is known as late gestation. During the embryonic period, the amniotic fluid comprises water from maternal serum, coelomic fluid, and the amniotic cavity.

However, during late gestation, the fluid is mainly produced by fetal urine and lung secretions [33]–[35]. In early gestation, the embryo is surrounded by two fluid-filled sacs: the exocoelomic cavity and the amniotic cavity. The exocoelomic cavity divides the extraembryonic mesoderm into the splanchnic mesoderm lining and the somatic mesoderm, creating a transfer area and nutrient reservoir for the developing embryo until it disappears by week 12. The exocoelomic cavity is a vital transfer interface and nutrient reservoir of maternal serum and products derived from the placenta. The amniotic fluid expands the amniotic sac, allowing the fetus to grow without any impediments [31]–[33], [35]. The amniotic cavity takes over when the coelomic fluid disappears. Fetal urine becomes the largest source of amniotic fluid during the second and third trimesters. Amniotic fluid comprises water, electrolytes, signalling molecules, peptides, carbohydrates, lipids, and hormones. It is less viscous and transparent due to its lower protein concentration than yellow coelomic fluid. Lung secretions contribute the most to amniotic fluid composition [33], [34], [36], [37]. During fetal development, it is crucial to maintain a balance between fluid formation and elimination for homeostasis. Fluid formation comes from fetal urine and lung secretions, while fetal swallowing and intramembranous absorption are the main routes for elimination. Fetal skin is permeable to fluid in the early stages of pregnancy. Still, once it becomes fully keratinised later on, it can no longer absorb or transfer fluids as quickly. The main mechanisms for eliminating amniotic fluid are fetal swallowing and the intramembranous pathway [34], [37]–[41]. Abnormal levels of amniotic fluid can lead to poor fetal outcomes, making it crucial for a healthy pregnancy. The amniotic fluid index (AFI) or single deepest pocket (SDP) is used to estimate the amniotic fluid volume and is part of the biophysical profile. An AFI greater than 24 cm or an SDP over 8 cm is considered polyhydramnios, while an AFI under 5 cm or an SDP less than 2 cm is called oligohydramnios. Polyhydramnios can be caused by various disorders, such as gastrointestinal tract obstruction and musculoskeletal disorders, whereas oligohydramnios can lead to complications like renal agenesis and IUGR. Amniotic fluid can also be used to screen for genetic diseases, and amniocentesis can diagnose chromosomal abnormalities such as Down syndrome [33], [42]–[44].

1.4 Emerging of non-invasive circulating cff-DNA for prenatal diagnostics

In recent years, the field of prenatal testing has seen a paradigm shift with the introduction of non-invasive methods, notably circulating cell-free Fetal DNA screening, which has been commercially available since 2011. This innovative technology isolates placental-origin cell-free DNA from maternal serum samples, offering a non-invasive means of detecting genetic disorders. However, the significance of these non-invasive approaches becomes clearer when juxtaposed against traditional invasive techniques like Chorionic Villus Sampling (CVS) and Amniocentesis.

CVS, a first-trimester diagnostic test, collects chorionic villi for genetic analysis. Despite its diagnostic accuracy, concerns about confined placental mosaicism and associated false positives contribute to a decline in CVS frequency. Similarly, amniocentesis, a second or third-trimester diagnostic tool, poses risks such as pregnancy loss [2], [5], [7], [31]. Compared to other prenatal testing methods, cell-free fetal DNA screening has a high detection rate for trisomy 21. However, it is only recommended for women with a high risk of aneuploidy. Benefits of this screening include the accurate identification of fetal sex and Rh status. Challenges include inconclusive results and the need for careful interpretation, highlighting genetic counsellors' importance. Non-invasive methods offer an attractive alternative, but there are still issues, such as false-positive results, maternal chromosomal abnormalities, and the need for further validation, especially in multiple gestations. These non-invasive approaches have led to a reevaluation of the significance of invasive techniques and a shift towards methods that prioritise patient safety and informed decision-making in prenatal testing [7], [45]. Cell-free fetal DNA in maternal circulation provides a non-invasive avenue for prenatal diagnosis, avoiding the risks associated with invasive procedures. However, technical challenges arise due to the low proportion of circulating cell-free fetal DNA in early pregnancy, typically less than 10% of total circulating free DNA. Current clinical applications are limited to identifying alleles unique to the fetus, such as sex-determining genes and specific disorders. Efforts to expand applications to include X-linked and recessive disorders face challenges, with reports indicating inconclusive results in some cases. Optimising circulating cell-free fetal NA yield becomes crucial, especially in situations involving both parents carrying

a mutant allele for recessively-inherited monogenic disorders or fetal aneuploidy diagnosis [35], [46], [47].

1.5 Unlocking the potential of cell-free fetal DNA from amniotic fluid in prenatal diagnosis

Non-invasive prenatal testing (NIPT) has revolutionised prenatal care by providing an accurate and safe way to screen for fetal chromosomal abnormalities. However, certain limitations must be addressed to improve the current NIPT methods. These limitations include false positives and negatives, which can lead to unnecessary anxiety or the potential overlooking of actual fetal chromosomal abnormalities. Additionally, current NIPT methods are generally restricted to screening for a select number of common chromosomal abnormalities, limiting their comprehensive diagnostic capability. There is increasing interest in investigating the potential of cell-free fetal DNA (cff-DNA) taken directly from amniotic fluid to address this issue. This fluid is abundant in cff-DNA released from the fetus and has the potential to overcome some of the restrictions of current NIPT techniques and provide a more precise way of fetal DNA screening. Furthermore, cff-DNA from amniotic fluid may be more accurate than maternal blood, as it has a lower chance of maternal DNA contamination. This approach could potentially screen for a broader range of fetal chromosomal and genetic abnormalities than current NIPT methods.

1.5.1 Unveiling new avenues: Isolating cff-DNA from Amniotic fluid

Recent advancements in isolating cff-DNA from amniotic fluid instead of maternal plasma have opened new vistas for prenatal diagnosis. The amniotic fluid surrounding the developing fetus contains a rich source of genetic material that can be analysed to gain insights into the fetal genetic profile. By isolating and characterising cff-DNA from amniotic fluid samples, valuable genetic information can be obtained for early detection and diagnosis of genetic disorders. Analysing cff-DNA from amniotic fluid offers the possibility of comprehensive genetic testing without the need for cell culturing. This can significantly reduce waiting times and provide prompt results to pregnant women, alleviating their psychological distress.

cff-DNA from amniotic fluid emerges as a promising avenue for NIPT, potentially addressing current limitations and enhancing the accuracy and scope of prenatal diagnostics. However, further research is imperative to evaluate this approach's accuracy, safety, and cost-effectiveness.

1.5.2 Methodoly for prenatal diagnosis

Recent DNA-based techniques have significantly improved prenatal diagnosis, transforming the landscape of genetic analysis. Segregation analysis, Quantitative Real-Time Polymerase Chain Reaction (qPCR), Multiplex Ligation-depended Probe Amplification (MLPA), Array-based Comparative Genomic Hybridisation (Array-CGH), and Next Generation Sequencing-based Whole Exome Sequencing (WES) stand out as revolutionary tools in the field.

While karyotyping has long been the gold standard for prenatal diagnosis, its limitations have become increasingly apparent. Although capable of identifying numerical and structural chromosomal abnormalities within 5-10 megabases, karyotyping falls short in detecting smaller structural alterations and identifying genetic abnormalities beyond chromosomal aberrations. Furthermore, obtaining meta-phase chromosomes, taking 10-14 days, introduces challenges such as occasional cell culture failures, erroneous karyotypes due to artefacts, and pseudo-mosaicism associated with maternal cell contamination.

Array-CGH and NGS have emerged as innovative solutions in response to these limitations. Array-CGH, with its ability to detect gains or losses in the genome, including small CNVs undetectable by conventional karyotyping, has significantly improved diagnostic yields in prenatal diagnosis. Notably, it proves valuable for patients with normal karyotypes but suspected genetic disorders. However, limitations persist, as it cannot examine small deletions, duplications, or repeat expansions, necessitating supplementary methods like Sanger sequencing or fragment analysis. The identified gains or losses can be confirmed through MLPA or qPCR [48].

NGS-based techniques, exemplified by WES, have ushered in a new era in genetic analysis by enabling the identification of genetic variants in the coding regions of the genome. WES showcases remarkable potential in diagnosing rare and novel genetic disorders, overcoming challenges that conventional methods may struggle with [49].

Acquiring high-quality DNA from prenatal samples poses a significant challenge in the pursuit

of precise prenatal genetic investigations. DNA extraction from sources like amniotic fluid (AF) or chorionic villi encounters obstacles such as maternal cell contamination and limited DNA content in the early weeks of pregnancy. Maternal cells in AF or chorionic villi samples pose a risk of contaminating extracted DNA, leading to potential misdiagnosis. Additionally, the reliance on cell culturing, a time-consuming process spanning several days, exacerbates the challenge. The delayed delivery of diagnostic results intensifies the emotional burden on pregnant women and their families.

In addressing these challenges, developing methods to minimise maternal cell contamination and maximise DNA yield from limited prenatal samples is paramount. Such innovations promise to significantly enhance the accuracy and efficiency of prenatal diagnosis, ultimately providing timely and reliable results to pregnant women.

1.6 Aims and objectives of the study

This research assesses the feasibility, accuracy, and efficiency of using cell-free fetal DNA (cff-DNA) extracted from amniotic fluid for genetic prenatal diagnosis. The goal is to establish cff-DNA as a dependable source of genetic information for prenatal diagnosis. It is expected that the outcomes of this study will help advance prenatal medicine and genetic counselling, providing a better understanding of the effectiveness and utility of cff-DNA in genetic testing during pregnancy. A significant challenge is maternal cell contamination, which can distort the analysis of fetal DNA. Maternal cells in amniotic fluid can contaminate the extracted DNA, leading to inaccurate results and the risk of misdiagnosis. Additionally, limited fetal cells in early pregnancy weeks necessitates cell culturing to obtain enough DNA for analysis. This process requires growing cells in the laboratory over several days, resulting in a delay in obtaining diagnostic results and causing distress for pregnant women and their families.

The four primary objectives are:

Investigating the correlation between gestational age and cff-DNA concentration: The study aims to impartially explore the relationship between gestational age in euploid fetuses and the concentration of cff-DNA using quantitative Polymerase Chain Reaction (qPCR). The research

seeks to comprehensively understand this relationship across a broad spectrum of gestational ages.

Significantly reducing turnover time for prenatal diagnosis: The research will use cff-DNA extracted from amniotic fluid to enable early diagnosis, particularly when traditional cff-DNA extraction faces challenges, such as early weeks of gestation or blood-tinged amniotic fluid indicating maternal cell contamination or cell culture challenges.

Mitigating maternal cell contamination via centrifugation: The research aims to screen and mitigate maternal cell contamination via centrifugation in cff-DNA samples from amniotic fluid.

Implementing and validating cff-DNA for prenatal diagnostic methods: The research will validate the applicability and reliability of various genetic analysis methods, including Sanger sequencing, Multiplex Ligation-Dependent Probe Amplification, Array Comparative Genomic Hybridization, and Whole Exome Sequencing, for comprehensive prenatal genetic analysis using cff-DNA.

Marterials and Methods

2.1 Ethics

The utilisation of clinical data and collection of patient samples for this study has received ethical approval from the ethics board of the Medical University of Vienna. Prior to sample collection, all participating pregnant women provided written informed consent, and strict measures were taken to pseudonymise their patient data, ensuring utmost confidentiality. The study imposes no additional risks or complications on the participants, and all experiments adhered to relevant guidelines and regulations.

2.1.1 Data protection

During the routine diagnostics, all participants will be assigned continuous numbers, and their identities will be pseudonymised for further analysis. Access to personal data will be restricted to authorised individuals only, specifically employees of the clinical department at the Institute of Medical Genetics, Medical University of Vienna. The data generated in this study will have an additional access control layer, ensuring that only study personnel can access and review the information. Confidentiality agreements bind all employees involved in the study and have provided their commitment to data protection.

2.1.2 Study population

2.1.2.1 Amniotic Fluid

Samples of amniotic fluid were taken from pregnant women who were already receiving invasive prenatal care at the University Hospital of Vienna (AKH Wien), provided by the Department of Obstetrics and Gynaecology, Division of Obstetrics and Feto-Maternal Medicine. Ultrasound-guided trans-abdominal amniocentesis was used to obtain the samples, which were then centrifuged at 300 xg for 10 minutes and cultured for routine diagnostics. The supernatant from the procedure was collected, centrifuged for 10 minutes at 3234 xg (Centrifuge 5804, Eppendorf), and stored at -20°C until further analysis.

2.1.2.2 Maternal EDTA blood

Maternal plasma is regularly taken from pregnant women during amniocentesis for the purpose of detecting any maternal cell contamination in the fetal sample or for segregation analysis. This plasma, along with the amniotic fluid, is then sent to the University Hospital of Vienna, Department of Obstetrics and Gynecology, Division of Obstetrics and Feto-Maternal Medicine. The maternal plasma of pregnant women who have given written consent is used to ensure that the cell-free fetal DNA is free from maternal cell contamination.

2.2 General molecular biology methods

2.2.1 DNA quantification via Qubit Flex fluorometer

The concentration of DNA was determined using the Qubit Flex fluorometer (Thermo Fisher Scientific) in conjunction with the Qubit™ 1X dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific). Monthly calibration was performed using the supplied DNA standards and manufacturer's protocol to ensure accuracy. For sample measurement, 1 µl of DNA was mixed with 199 µl of Qubit™ 1X dsDNA High Sensitivity Working Solution in duplicate or triplicate (depending on the sample availability), and the samples were incubated for 2 minutes at room-temperature, after which the fluorescence emitted by each sample was recorded. Finally, DNA concentrations were calculated automatically by the Qubit Flex fluorometer using the calibration curve, and the mean concentration was calculated manually. The samples were diluted accordingly if the DNA concentration was outside the specified quantitation range of 0.1 to 120 ng of the kit.

2.2.2 Gel electrophoresis for separation of nucleic acids

Agarose gels with 2% concentration separated DNA fragments based on molecular size through horizontal gel electrophoresis. The agarose was dissolved in 0.5% TBE by boiling it for 2-3 minutes. After cooling it to 50-60 °C, 5 µl of Midori Green Advance (NIPPON Genetics) was added to every 90 ml. The gel was poured into the gel tray of the PerfectBlue Mini M electrophoresis system (VWR), and PCR products amplified via DreamTaq Green PCR Master

Mix (2X) (Thermo Scientific) were directly loaded onto the gel since the master mix contains two tracking dyes. The electrophoresis chamber was filled with 0.5% TBE buffer and separated by gel electrophoresis at a steady voltage of 100 V for 25-45 minutes. A ready-to-use GeneRuler 100 bp DNA Ladder (Thermo Scientific) was loaded into one well to determine the size of the DNA fragments. The DNA fragments were observed and photographed under UV irradiation in the stand-alone imaging system E-Box CX5 (Vilber) due to the intercalating of the fluorescent dye Midori green into the double strand of DNA.

2.2.3 Tapestation - Automated electrophoresis solution for fragment size distribution and sample quality control

The quality and size distribution of cell-free fetal DNA (cff-DNA) samples were assessed using the 4150 TapeStation System (Agilent). Initially, the cell-free DNA ScreenTape and Reagents by Agilent were used to analyse cff-DNA ranging from 50 to 800 bp. For cff-DNA, 1.5 µl of the sample was loaded onto a Cell-free DNA ScreenTape (Agilent). Later, 1 µl of cff-DNA was loaded onto the D5000 ScreenTape and Reagents (Agilent), which can analyse DNA ranging from 35 to 5,000 base pairs. For quality control of Whole exome sequencing, 2 µl libraries were loaded onto High Sensitivity D1000 ScreenTape by Agilent, which can analyse DNA within 35 to 1000 bp. After loading the sample onto the respective ScreenTape, electrophoresis was conducted. The resulting fragment size distribution was analysed using the TapeStation software (Agilent).

2.3 Polymerase Chain Reactions (PCRs)

2.3.1 Quantitative real-time PCR for establishing the best cell-free fetal DNA extraction kit: CFX96 Connect Real-Time PCR Detection System

The best kit for extracting cell-free fetal DNA (cff-DNA) with the highest yield was identified through quantitative real-time PCR using the CFX96 Connect Real-Time PCR Detection System (Bio-Rad). The master mix was created by combining 5 µl of cff-DNA with 1.6 µl of the PKRD1 primer pool (0.2 µl final concentration of the forward and reverse primer), 10 µl of 2x GoTaq qPCR Master Mix from Promega, and adding nuclease-free water up to a total volume of 20

µl. The reference standard was created by diluting the human female reference DNA (Promega) from 32 to 1 ng/ l. All samples, including a no template control (NTC) and the reference standard, were loaded in duplicate wells. The PKRD1 reference primers are listed in Table 2.1, and the thermal program (including dissociation curve) indicated in Table 2.2 was used for qPCR. The results were analysed using the CFX Maestro Software Bio-Rad) to compare unknown concentrations to the standard curve and extrapolate a value.

Table 2.1: PRKD1 reference primer sequences and melting temperature.

Primer	Sequence	TM (°C)
Q_PRKD1_F	GTGTGTGGTGAAGGCTTGTTCTCT	64.0
Q_PRKD1_R	GAAGTGGAACCTTGAGGAGGTGAT	64.0

Target Region: chr14:30239158-30239326 (169bp)

Table 2.2: qPCR cycling program for CFX96 Connect Real-Time PCR Detection System (Bio-Rad).

Temperature (°C)	Time	Cycles
95	10 min	1
95 60	15 sec 1 min	39x
95	15 sec	1
60	1 min	1
Melt Curve		
65°C	to 95°C	Increment 0.5°C

2.3.2 Quantitative real-time PCR to exploring the correlation between cff-DNA concentration and gestational Age: StepOne™ Real-Time PCR System

On 139 patient samples, a qPCR was conducted on cell-free fetal DNA (cff-DNA) to investigate a yield concentration correlation at different gestational ages in euploid fetuses. To ensure comparability, the cff-DNA was extracted from 5 ml amniotic fluid supernatant using a standard extraction protocol described in section 2.5 over consecutive days. The analysis used qPCR with the StepOne™ Real-Time PCR System (Applied Biosystems) and Luna Universal qPCR Master

Mix (2x). The master mix was created by combining 2 µl of cff-DNA with 0.8 µl of the GAPDH primer pool (0.2 µl final concentration of the forward and reverse primer), 10 µl of 2x Luna Universal qPCR Master Mix (2x), and adding nuclease-free water up to a total volume of 20 µl. The samples were analysed over three consecutive days, with a no template control (NTC) and two reference standards included in duplicate wells for each sample. The reference standard, consisting of female and male reference DNA (Promega), was loaded in a serial dilution ranging from 32 to 0.5 ng/ l, and the same standard was used for all plates for all 139 patient samples. The GAPDH reference primers are listed in Table 2.3, and the thermal program (including dissociation curve) is indicated in Table 2.4. After the qPCR analysis, a dissociation curve analysis was performed to assess the specificity of the amplified products by gradually increasing the temperature from 60°C to 95°C with an increment of 0.3°C. The StepOne Software v2.3 (Applied Biosystems) was used to analyse the results. The unknown concentrations were compared to the standard curve, and a value was extrapolated. Concentrations were also measured using the Qubit Flex Fluorometer (Thermo Scientific) as described in Table 2.2.1.

Table 2.3: GAPDH reference primer sequences and melting temperature.

Primer	Sequence	TA (°C)
Q_GAPDH_F2	ACATGTTCCAATATGATTCCA	55.3 C
Q_GAPDH_R2	TGGACTCCACGACGTACTCAG	61.3

Target Region: chr12:6536688+6536978 (291bp)

Table 2.4: Quantitative PCR analysis for evaluating different cff-DNA concentrations throughout different weeks of gestation.

Temperature (°C)	Time	Cycles
95	1 min	1
95 60	15 sec 30 sec	40x
95	15 sec	1
60	1 min	1
Melt Curve		
60°C	to 95°C	Increment 0.3°C

2.3.3 PCR for specificity assessment of follow-up experiment to evaluate the best DNA extraction kit for cff-DNA from amniotic fluid supernatant

PCR was used to evaluate the specificity of cff-DNA from four different DNA extraction kits by targeting Exon 11 and Exon 12 of the *CFTR* gene. The primer sequences for each exon are provided in table 2.5 and 2.6. The PCR amplification was carried out using SimpliAmp Thermal Cycler (Applied Biosystems) and DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific). For amplification, 3 µl of the cff-DNA sample was mixed with 12.5 µl of DreamTaq Master Mix (2x), 1 µl of each primer (0.4 µM end concentration), and 7.5 µl nuclease-free water to a total volume of 25 µl in PCR Tubes. PCR amplification was performed with a cycling program (2.7) using the SimpliAmp Thermal Cycler, and the resulting products were loaded onto a 2% agarose gel with a 100 bp ladder (Thermo Fisher Scientific) and electrophoresed at 100V for 45 minutes as described in section 2.2.2.

Table 2.5: Primer sequences for *CFTR* Exon 11.

Primer Exon 11	Sequence	TA
CFTR_11F	AATATACACTTCTGCTTAGGATGATAATTG	59.7°C
CFTR_11R	AGAGGAAACATAAATATATGTAGACTAACCG	59.9°C

Target Region: chr7:117559372+117559769 (398bp)

Table 2.6: Primers sequences for *CFTR* Exon 12.

Primer Exon 12	Sequence	TA
CFTR_12F	TTCAACTGTGGTTAAAGCAATAGTGT	60.4°C
CFTR_12R	GCACAGATTCTGAGTAACCATAATCTC	60.7°C

Target Region: chr7:117587621+117588047 (427bp)

Table 2.7: PCR cycle protocol for targeting *CFTR* gene exons 11 and 12.

Temperature (°C)	Time	Cycles
95	2 min	1
95	30 sec	
60	30 sec	32
72	45 sec	
72	5 min	1

2.3.4 PCR for Sanger sequencing (seqPCR) of cff-DNA: DreamTaq Green PCR or LA Takara with GC buffer I master mix

For Sanger sequencing of cff-DNA, the LA Takara with GC buffer I or DreamTaq Green PCR MM (2X) was used based on the GC content of the target sequences. The PCR reaction mixture comprised 0.5 µl of cff-DNA (diluted to 5 ng/µl) and 12 µl of the selected PCR master mix that had been mixed according to 2.8. The thermal cycling conditions were described in Table 2.9 and were run in the SimpliAmp Thermal Cycler (Applied Biosystems).

Table 2.8: Master mix reactions for Sanger sequencing based on GC content.

DreamTaq Green PCR Master Mix		**Takara with GC buffer I**	
Volume	**Component**	**Volume**	**Component**
6.25 µl	DreamTaq Green PCR MM (2X)	2 µl	dNTPs mixture (2.5 mM each)
4.5 µl	H2O	2.65 µl	H2O
0.625 µl	Fwd Primer (0.5 µM final conc.)	0.625 µl	Fwd Primer (0.5 µM final conc.)
0.625 µl	Rev Primer (0.5 µM final conc.)	0.625 µl	Rev Primer (0.5 µM final conc.)
		0.1 µl	LA Taq DNA Pol. (5 units/µl)
		6.25 µl	2X GC Buffer I
+ 0.5 µl cff-DNA (5 ng/µl)			

Table 2.9: PCR Cycling program for Sanger sequencing of cell-free fetal DNA.

Temperature (°C)	**Time**	**Cycles**
95	15 min	1
95 60/63 72	30 sec 30 sec 1 min	35x
72	10 min	1
10	-	∞

2.3.5 Sequencing PCR (SeqPCR) for Sanger sequencing of cff-DNA

To perform Sanger sequencing, the PCR products underwent sequencing PCR (SeqPCR) with fluorescently labelled dideoxynucleotides (ddNTPs) and gene-specific primers. To prepare the master mix, 1 µl of BigDye Polymerase (Applied Biosystems), 1 µl of 5x BigDye sequencing buffer

(Applied Biosystems), and 6.5 µl of nuclease-free water were combined to create a reaction volume of 8.5 µl, which was then added to a 96-well plate. To the master mix, 1 µl of DNA was added and mixed with 0.5 µl of primer (10 µM; 0.5 µM end concentration). Separate reactions were set up for the forward and reverse primers. The sequencing PCR was performed using the thermal program specified in Table 2.10.

Table 2.10: PCR cycling program for SeqPCR in Sanger sequencing.

Temperature (°C)	Time	Cycles
96	4 min	1
96 50	10 sec 5 sec	25x
60	2 min 15 sec	1
4	∞	

2.4 Methodology used for evaluating the best cff-DNA extraction kit and its parameters for cell-free fetal DNA from amniotic fluid supernatant

This section outlines the methodology for finding the best cff-DNA extraction kit and its parameters for cell-free fetal DNA from amniotic fluid supernatant. Six DNA extraction kits were selected and evaluated based on conflicting literature recommendations, cost, input volume, elution volume, and other relevant parameters for routine diagnostics. The extraction process involved binding cell-free DNA to magnetic beads or columns, washing the bound DNA to remove impurities, and eluting the purified DNA in a 30 l elution buffer provided by the respective kit manufacturer. DNA extraction kits are listed in Table 2.11.

Table 2.11: List of extraction kits tested for isolating cell-free fetal DNA from amniotic fluid.

Name of the Kit	Manufacturer
QIAamp Circulating Nucleic Acid Kit	Qiagen
QIAamp ccfDNA/RNA Kit	Qiagen
MagMAX Cell-Free DNA Isolation Kit	Applied Biosystem
cfPure V2 Cell-Free DNA Extraction Kit	Biochain
Plasma Serum Cell-Free Circulating DNA Purification Midi Kit	Norgen
XCF COMPLETE Exosome and cfDNA Isolation Kit	SBI

2.4.1 Experimental design 1: Narrowing down the selected 6 DNA extraction kits

In order to narrow down the selection of extraction kits for cff-DNA, an initial experiment was conducted. The experiment evaluated the performance of six pre-selected DNA extraction kits on four patient samples of amniotic fluid supernatant, each from a different gestational week (Table 2.12). Before cff-DNA extraction, the samples were centrifuged at 10,000 xg for 10 minutes (Centrifuge 5804, Eppendorf). To account for varying cff-DNA concentrations, 2 ml aliquots of the supernatant were prepared for each kit. The extracted cff-DNA samples were subjected to quantitative analysis and quality assessment using the Qubit Fluorometer (Thermo Scientific) as described in section 2.2.1 and quantitative polymerase chain reaction (qPCR) using the CFX96 Connect Real-Time PCR Detection System (Bio-Rad) as described in section 2.3.1, as well as fragment size analysis and purity via TapeStation system (Agilent) described in 2.2.3.

Table 2.12: Gestational age of amniotic fluid supernatant samples used for evaluating six DNA extraction kits.

Case Number	Gestational Age
1	20+1
2	21+6
3	24+2
4	25+1

2.4.2 Experimental design 2: finding the best extraction kit for cff-DNA isolated from amniotic fluid

After evaluating the extraction kits' performance on cff-DNA isolated from amniotic fluid, a follow-up experiment was conducted with the kits listed in Table 2.13. 15 patient samples were selected, representing different gestational weeks, as shown in Table 2.14. cff-DNA was handled and extracted as in the first experimental design. However, additionally, to evaluate the specificity of the extracted cff-DNA, a PCR was performed targeting two different exons of interest, Exon 11 and Exon 12 of the *CFTR* gene as described in 2.3.3. The resulting PCR products and a 100 bp ladder (Invitrogen) were loaded onto a 2% agarose gel and subjected to electrophoresis for 45 minutes at 100V based on the method described in 2.2.2. The Qubit Fluorometer (Thermo Scientific), quantitative polymerase chain reaction (qPCR) using the CFX96 Connect Real-Time PCR Detection System (Bio-Rad), and Tapestation were used to evaluate the best cff-DNA yield of the extraction kits according to the methods described in sections 2.2.1, 2.3.1, and 2.2.3, respectively.

Table 2.13: Selection of 4 DNA extraction kits for further assessment.

Name of the Kit	Manufacturer
QIAamp Circulating Nucleic Acid Kit	Qiagen
QIAamp ccfDNA/RNA Kit	Qiagen
cfPure V2 Cell-Free DNA Extraction Kit	Biochain
Plasma Serum Cell-Free Circulating DNA Purification Midi Kit	Norgen

Table 2.14: Gestational age of patient samples selected for cff-DNA extraction kit evaluation from amniotic fluid.

Gestational Age	Case Number
15+3	1
16+4	2
17+3	3
18+4	4
19+4	5
20+5	6
21+2	7
22+2	8
23+4	9
24+6	10
25+1	11
26+1	12
27+2	13
29+1	14
30+4	15

2.4.3 Experimental design 3: Evaluating the best parameters to use the QIAamp Circulating Nucleic Acid Kit

After an evaluation and comparative analysis, the QIAamp Circulating Nucleic Acid (QIAamp CNA kit) Kit was chosen as the standard for isolating cff-DNA. Two experiments were conducted to investigate further the potential of the Qiagen CNA kit concerning silica membrane capacity and different protocols (Serum/Plasma vs Urine). All amniotic fluid samples were centrifuged for 10 minutes at a specific speed using the Centrifuge 5804 (Eppendorf). For the first experiment, the efficiency of the silica membrane in the QIAamp kit was evaluated to assess its ability to process increased sample volumes of up to 8 ml amniotic fluid beyond the maximum of 5 ml, as stated by Qiagen. Therefore, both samples were aliquoted into two 4 ml and one 8 ml aliquot. The standard procedure described in the manufacturer's protocol was followed, with necessary reagents scaled up to 8 ml. For the second experiment, two different protocols, serum and urine, were compared for cff-DNA extraction from amniotic fluid supernatant using the QIAamp kit. Therefore, both samples were aliquoted into two 4 ml aliquots and extracted with the serum protocol provided by the Qiagen CNA kit. In both experiments, cff-DNA was eluted in 30 l of Qiagen Buffer AVE. The concentration of cff-DNA in each sample was measured using the

Qubit Flex fluorometer (Thermo Scientific) according to section 2.2.1 and qPCR with the CFX96 Connect Real-Time PCR Detection System (Bio-Rad) according to section 2.3.1.

2.5 Established standard cff-DNA extraction protocol

In Section 2.4, the Cell-Free DNA (cff-DNA) extraction kit and its parameters were evaluated, and the QIAamp Circulating Nucleic Acid (CNA) (Qiagen) kit was selected based on the standard protocol established in this method. The frozen amniotic supernatant (centrifuged as described in Section 2.1.2.1) was thawed and extracted using the QIAamp Circulating Nucleic Acid Kit (Qiagen) following the plasma and serum protocol for a 5 ml sample volume. For amniotic fluid supernatant samples exceeding 5 ml, they were separated into individual 50 ml centrifuge tubes before proceeding with the extraction process. Each aliquot was extracted and purified separately using the QIAamp CNA Kit.

The extraction procedure was initiated by transferring 5 ml of amniotic fluid supernatant or the appropriate aliquot to a 50 ml centrifuge tube. If the sample volume was less than 5 ml, it was supplemented with PBS (phosphate-buffered saline) to reach the required volume. Then, 500 µl of Proteinase K was added to the sample. Buffer ACL was prepared by mixing it with carrier RNA per the manufacturer's manual, according to the sample size and volume. Next, 4 ml of the Buffer ACL/Carrier RNA mixture was added to the sample. The mixture was thoroughly vortexed and incubated at 60°C for 30-40 minutes using the Professional 3500 Incubating Orbital Shaker (VWR) at 300 RPM. Afterwards, 9 ml of Buffer ACB (binding buffer) was added to the mixture, vortexed, and incubated on ice for 5 minutes. The QIAvac 24 Plus vacuum pump (Qiagen) was assembled with VacConnectors, VacValves, QIAamp Mini Columns, and tube extenders. After a five-minute wait, the sample mix was transferred to the tube extender. The cff-DNA was then bound to the silica membrane of the mini-column using the vacuum manifold and a vacuum pressure of -800 to -900 mbar as the lysate was drawn through by vacuum pressure. The VacValves allowed for processing multiple samples since they could be individually closed. The tube extenders were removed after the lysate was drawn through the mini-column. Following this, the mini-column was washed with 600 µl of wash Buffer ACW1, then 750 µl of Buffer ACW2, and finally, 750 µl of ethanol (96-100%) was added to the Mini

column. The VacValves were closed between each washing step to ensure a stable vacuum on all samples. After completing all three wash steps, the vacuum pump was switched off, releasing the pressure to 0 mbar. The Mini column lid was closed and removed from the vacuum manifold, discarding the VacConnector. The Mini column was then placed in a clean 2ml collection tube and centrifuged at full speed (21,300 x g) for 3 minutes using a Centrifuge 5804 (Eppendorf). After centrifugation, the Mini column was placed into another clean 2ml collection tube and incubated with the lid open at 56°C for 10 minutes in a Thermo. Shaker (Grant-bio) to dry the membrane and remove any residual ethanol. Then, the Mini column was placed in a clean 1.5 ml DNA LoBind Tube (Eppendorf), discarding the previous collection tube. The elution volume was kept low initially to yield higher concentrations to comply with specific molecular methods' requirements since low concentrations of cff-DNA were expected. However, it was adjusted based on the molecular methods to be conducted and their specific requirements. Nuclease-free water or elution buffer AVE was added to the Mini column in an appropriate volume considering the downstream applications. The standard elution volume was 30 µl, except if otherwise stated. The elution step was carried out by incubating the Mini column at room temperature for 3 minutes and centrifuging at full speed (21,300 x g) for 1 minute (Centrifuge 5804, Eppendorf). After conducting the pre-tests, the elution buffer AVE was permanently switched to nuclease-free water because the elution buffer AVE caused problems in downstream applications after vacuum concentration via SpeedVac (Thermo Scientific) due to increased salt concentrations. This change was implemented for all cff-DNA extractions. After cff-DNA extraction, the sample aliquots were pooled together, and their concentration was measured via Qubit Flex fluorometer (Thermo Scientific), as indicated in section 2.2.1.

2.6 Screening for maternal cell contamination in cff-DNA via STR Markers analysis

The process of prenatal diagnosis through amniocentesis can pose some difficulties due to the potential contamination of maternal cells in the amniotic fluid. While the risk of maternal cell contamination is low, it can still affect fetal analysis and lead to a misdiagnosis. Thus, it is

crucial to validate the extracted DNA. STR-marker analysis is utilised to compare the mother and fetus's DNA, and it is also essential to work with cff-DNA for practical implementation in prenatal diagnostics. A study was conducted using the AmpFlSTR Identifiler Plus PCR kit (Thermo Scientific) on 132 amniotic fluids, with 41 visibly tinged with blood. The study tested the hypothesis that maternal cells can be significantly removed by centrifuging the amniotic fluid.

2.6.1 Sample preparation and dilution of cff-DNA

91 visibly inconspicuous and 35 amniotic fluid samples visibly tinged with blood (after centrifugation) were collected and centrifuged for ten minutes at 3234 xg (Centrifuge 5804, Eppendorf) according to section 2.1.2.1. 6 samples were macroscopically heavily tinged with erythrocytes even before centrifugation. For these samples, amniotic fluid was centrifuged twice for ten minutes at 3234 xg, and the supernatant was transferred into a new centrifuge tube (Greiner) in between. cff-DNA was extracted from the supernatant according to the standard cff extraction protocol (2.5) and eluted in 30 µl nuclease-free water. The extracted cff-DNA was diluted to a 1 ng/µl concentration using TE buffer, pH 8.0, RNase-free (Invitrogen) in a final volume of 5 µl. To assess the reliability of the screening protocol, a non-template control (5 µl nuclease-free water) and positive control (DNA 9947A, Thermo Fisher Scientific) with a concentration of 1 ng/µl were included for analysis.

2.6.2 PCR amplification and fragment analysis

STR-marker analysis was performed using the AmpFlSTR Identifiler™ Plus PCR kit (Thermo Fisher Scientific). The PCR master mix was prepared by combining 5 µl of the master mix (yellow cap) with 2.5 µl of the primer mix (green cap) provided in the kit. 5 µl of diluted cff-DNA (1 ng/µl) was added to the master mix, resulting in a 12.5 µl reaction mix. Amplification was carried out in a SimpliAmp Thermal Cycler (Applied Biosystems) with the following cycling program details in Table 2.15, with a ramp temperature of 1.6°C.

Following amplification, fragment analysis was performed using either the 3500 (injection conditions: 1.2kV/15 sec) or 3500XL (injection conditions: 1.2kV/24 sec) Genetic Analyzer (Applied Biosystems) for sequencing. 1 µl PCR product was mixed in a 96-well plate with 9

Table 2.15: AmpFlSTR Identifiler Plus PCR cycling program for maternal cell contamination examination via STR Marker.

Temperature (°C)	Time	Cycles
95	11 min	1
94	1 min	
59	1 min	28x
72	1 min	
60	60 min	1
4		∞

µl master mix containing 8.5 µl Hi-Di Formamide (Applied Biosystems) and 0.5 µl GeneScan™ 500 LIZ™ dye Size Standard (Applied Biosystems). In addition to the samples, 1 µl Allelic Ladder was mixed with 9 µl master. The 96-well plate was sealed with an aluminium seal, and the samples were denatured for 5 min at 98°C in a SimpliAmp Thermal Cycler (Applied Biosystems). After denaturation and cooling for three minutes, the plate was transferred to the sequencer with the Identifiler assay settings, according to the manufacturer's protocol. The resulting data was analysed using the Gene Mapper Software (Thermo Fisher Scientific) with the AmpFLSTR_Panels_v2 (Thermo Scientific) panel.

2.7 Sanger sequencing of cff-DNA

The feasibility of using Sanger sequencing to detect gene mutations in cell-free fetal DNA (cff-DNA) extracted from amniotic fluid supernatant was evaluated. Fifteen patient samples were analysed based on the presence of previously identified gene mutations. The Sanger sequencing results of the target genes were compared with the known mutations, and the results of 19 genes of interest were compared to genomic DNA sequencing data.

The amniotic fluid samples were collected from pregnant individuals undergoing prenatal testing and were processed according to the protocol described in section 2.1.2.1. The cff-DNA was extracted using the QIAamo Circulating Nucleic Acid (CNA) kit, eluted in 30 µl nuclease-free water according to the established protocol in section 2.5 and the concentration determined using the Qubit Flex fluorometer as described in section 2.2.1. Furthermore, one sample was vacuum-concentrated using the SpeedVac Vacuum concentrator (Thermo Fisher Scientific) while

being centrifuged for 30 min at 60°C under the setting for a vacuum for aqueous solutions (V-AQ) and resuspended in 30 µl nuclease-free water.

For most genes targeted for Sanger sequencing, the primers were already available. However, new primers were designed for six genes, as mutations were found in whole-exome sequencing (WES), and no Sanger sequencing had been performed originally. PCR was performed on cff-DNA after dilution to 5 ng/µl using either the LA Takara with GC buffer I or DreamTaq Green PCR MM (2X) (Fermentas), depending on the GC content of the target sequences and the PCR master mixes according to section 2.3.4. The gene-specific primer sequences used for PCR amplification are provided in Table 1 in the Appendix.

The PCR product was subjected to enzymatic cleanup using 2 µl ExoSAP-IT Express PCR Product Cleanup Reagent® (Thermo Fisher Scientific) containing exonuclease I for degradation of unused single-stranded primers and shrimp alkaline phosphatase (SAP) for dephosphorylation of unused nucleotides. The cleanup involved two steps using the SimpliAmp Thermal Cycler (Applied Biosystems): hydrolysing excess primers and nucleotides by incubating at 37°C for 4 minutes and inactivating the Exonuclease I at 80°C for 1 minute. Successful amplification of PCR products was confirmed by visualising them on a 2% agarose gel as described in section 2.2.2.

After PCR product purification with ExoSAP-IT Express, the samples were subjected to a second round of PCR amplification known as SeqPCR as described in section 2.3.5. Additional SeqPrimers were designed for two genes, and the sequences are listed in Table 2 in the Appendix.

The SeqPCR products were purified with precipitation using the BigDye X-Terminator Purification Kit (Applied Biosystems). A master mix was prepared according to Table 2.16, and 55 µl was added to each reaction. The plate was sealed, shaken for 30 minutes on a Digital Vortex-Genie 2 with a microplate adapter (Applied Biosystems), and vortexed in between after 15 minutes for 15 seconds and centrifuged for 2 minutes at 201 xg. Approximately 20 µl of the supernatant was transferred into a new 96-well plate. This step removed excess ddNTPs, primers, and enzymes, ensuring high-quality sequencing results.

The purified sequencing reaction products were loaded onto a 3500 or 3500XL Genetic Analyzer (Applied Biosystems) for sequencing using the POP7 polymer and Fast injection assay (1h). The SeqPilot software (JSI Medical Systems GmbH) was used to analyse the raw sequencing data

Table 2.16: Precipitation master mix added to the SeqPCR samples.

Volume	Component
45 µl	SAM solution
10 µl	X-Terminator Beads
55 µl	Reaction Mix

obtained from the sequencer. The sequencing results were compared with known changes in the target genes to detect changes or mutations and to evaluate the suitability of cff-DNA from amniotic fluid supernatant for Sanger sequencing.

2.8 Multiplex Ligation-depended Probe Amplification

The Multiplex Ligation-dependent Probe Amplification (MLPA) analysis was performed on cff-DNA according to the manufacturer's instructions provided by MRC Holland. However, the working volume was reduced by half. This method detects copy number variation (CNV), such as deletions, duplications, or amplifications in genes linked with specific diseases within targeted genomic regions.

The cff-DNA sample was diluted to 10 ng/µl with TE (pH=8). Then, 5 µl of the sample and 2.5 µl of reference DNA were transferred in 0.2 µl stripes, along with the gender-specific cff-DNA control pool and the gDNA controls (sheared to 250bp and standard) pools, which were also diluted to 25 ng/ µl. A blank sample containing only 2.5 µl of TE was also included.

The hybridisation master mix was prepared using the reagents from the SALSA MLPA Reagent Kits (MRC Holland - 2.17) as listed in Table 2.18. After DNA denaturation and resuspension, 1.5 µl of the hybridisation master mix was added to each reaction. The hybridisation was conducted for 16-20 hours as indicated in Table 2.18

Table 2.17: SALSA MLPA reagent kits (MRC Holland) used for each patient sample.

Case Number	SALSA MLPA Reagent Kits
1	P049-C2
2	P1800-B3
3	P329-B1
4	P189-C2
5	P002-D1

Table 2.18: Reagents and cycling program for MLPA hybridisation.

Reagent	Volume	Description
SALSA MLPA Buffer (yellow cap)	0.75 μl	KCl, Tris-HCl, EDTA, PEG-6000, DTT, oligonucleotides
SALSA Probemix (black cap)	0.75 μl	Contains probes that are specific to the target DNA sequence
Total volume	1.50 μl	

Step	Temperature	Time
Denature DNA	98 °C	5-10 minutes
Pause to add hybridisation mix	25 °C	10 minutes
Denature DNA	95 °C	1 minute
Hybridise	60 °C	Indefinite
Hold in Thermocycler		
	60 °C	16-20 hours

The following day, the Ligase-65 master mixture was created according to Table 2.19. The temperature was increased to 54°C and maintained for 15 minutes. While the samples were in the thermocycler at 54°C, 16 µl of Ligase-Mix was added to each reaction, mixed by resuspension, and the thermocycler program was resumed by incubating at 54°C for 15 minutes. The ligase enzyme was then inactivated by incubating at 98°C for 5 minutes, after which the temperature was cooled to 20°C indefinitely as outlined in Table 2.21.

The PCR-mastermix for amplification was prepared according to Table 2.20. Afterwards, 5 µl of the mix was added to each reaction while the thermal cycler was held at 20°C. The mix was gently pipetted up and down to ensure proper mixing, and the tubes were immediately placed in the thermocycler. The PCR reaction program described in Table 2.21 was continued.

A reaction mixture was created by mixing 9.5 µl of Applied Biosystems' Hi-Di Formamide with 0.5 µl of Applied Biosystems' Size Standard GeneScanTM 500 LIZ. This mixture was added to a 96-well plate at 10 µl for each reaction, followed by 2 µl of the MLPA-PCR product. The injection plate was sealed, heated for 3 minutes at 86°C and cooled for 2 minutes at

Table 2.19: MLPA Ligase-65 master mix.

Reagent	Volume	Description
ddH2O	12.5 μl	Purified water.
SALSA Ligase Buffer A (transparent cap)	01.5 μl	Coenzyme NAD (bacterial origin).
SALSA Ligase Buffer B (white cap)	01.5 μl	Tris-HCl, MgCl2, non-ionic detergent.
SALSA Ligase-65 (green cap)	00.5 μl	Glycerol, EDTA, DTT, KCl, Tris-HCl, non-ionic detergent, Ligase-65 enzyme (bacterial origin).
Total	16.00 μl	

Table 2.20: MLPA polymerase master mix.

Reagent	Volume
ddH2O	3.75 μl
SALSA PCR Primer Mix (brown cap)	1.00 μl
SALSA Polymerase (orange cap)	0.25 μl

Table 2.21: MLPA cycling conditions.

DNA denaturation	
98°C	5 minutes
25°C	1 minute
Hybridisation reaction	
95°C	1 minute
60°C	16-20 hours
Ligation reaction	
54°C	pause
54°C	15 minutes
98°C	5 minutes
20°C	pause
PCR reaction	
	35 cycles:
95°C	30 seconds
60°C	30 seconds
72°C	60 seconds
72°C	20 minutes
15°C	pause

4°C. After that, the reaction mixture was loaded into the designated plate, and the fragment analysis was initiated using either the 3500 (injection conditions: 1.2kV/15 sec) or 3500XL (injection conditions: 1.2kV/24 sec) Genetic Analyzer (Applied Biosystems) for sequencing and Performance-Optimized Polymer 7 (POP-7, Applied Biosystems). The SeqPilot software (JSI medical systems) was used for data analysis. Quality control fragments are included in SALSA MLPA probe mixes to identify any issues that may impact the MLPA results. It is crucial to evaluate the quality of the MLPA reaction, including the quality control fragments. Most SALSA MLPA probe mixes contain 9 control fragments, including four Q-fragments (64, 70, 76, and 82 nt). These Q-fragments provide control for sufficient DNA addition and successful ligation, but they do not need to hybridise to the DNA or be ligated to be amplified during PCR. The Q-fragment height decreases as more sample DNA is included in a reaction. Two D-fragments (88 & 96 nt) are included in each probe mix to detect sequences in exceptionally strong CpG islands, which have a high GC content and are challenging to denature. A low level of the 88 and 96 nt D-fragments (less than 50% of the 92 nt benchmark fragment) indicates that the sample DNA needed more adequately denatured. Poor denaturation may be due to over 40 mM salt in a DNA sample. Incomplete sample DNA denaturation can result in false results. When using POP7 polymer (Applied Biosystems), a non-specific fragment of 80-90 nt is usually present that may coincide with the control fragments. Control samples were assessed first, followed by patient samples. By comparing the peak heights in patient samples with those in technically validated control samples, any anomalies within patient results can be identified.

2.9 Array-CGH analysis of cell-free Fetal DNA (cff-DNA)

To overcome the challenge of obtaining a sufficient amount of cell-free fetal DNA (cff-DNA) required for Array-CGH, a workflow was established for extracting cff-DNA from amniotic fluid samples using the QIAamp Circulating Nucleic Acid Kit (Qiagen). The process utilised the standard protocol for cff-DNA extraction as described in Section 2.5. The available amniotic fluid supernatant was extracted in 5 ml aliquots and eluted in 30 µl nuclease-free water. The aliquots of one sample were then pooled together, and the concentration of cff-DNA was measured in duplicate using the Qubit 1x dsDNA HS Assay Kit (Invitrogen) as described in section 2.2.1. If

the cff-DNA concentration was below 10 ng/µl, the sample was concentrated using the SpeedVac Vacuum concentrator (Thermo Fisher Scientific) while being centrifuged for 30 min at 60°C under the setting for vacuum for aqueous solutions (V-AQ) and resuspended in 30 µl nuclease-free water. The concentration of the concentrated cff-DNA was then quantified in duplicate using the Qubit 1x dsDNA HS Assay Kit (Invitrogen) again. If the new concentration was 10 ng/µl or higher, the Array-CGH was done using a Backing Slide for 4x180K (G2534-60011, Agilent). The results were compared to the routine diagnostics gf-DNA results.

The SureTag Complete DNA Labeling Kit (Agilent) was used to prepare the samples and their corresponding male or female Human Reference DNA (Agilent). The Human Reference DNA was then diluted to a concentration of 50 ng/µl, resulting in a total concentration of 1300 ng used for an Array-CGH reaction. For sample preparation, 2 µl of barcodes were added to 24 µl of cff-DNA with a concentration of at least 10 ng/µl, as described in the Agilent application note "Use of Spike-ins for Sample Tracking in Agilent Array CG". Samples and controls were prepared in an 8-tube PCR stripe, with 26 µl of each sample (sample + barcode) or control per reaction. To each 26 µl sample or control, 5 µl of random primers were added. The reagents were mixed and briefly spun before use. Samples and controls were denatured at 98°C for 3 minutes in a SimpliAmp Thermal Cycler (Applied Biosystems), as described in Table 2.22.

Table 2.22: Array-CGH denaturation program for the thermal cycler.

Step	Temperature and Time
1.	98°C for 10 Minutes
2.	4°C Indefinitely (Hold)

After denaturation, samples were spun down, and a labelling master mix was prepared with either Cy3 for the sample or Cy5 for the reference, as specified in Table 2.23. The mix was added to each reaction tube to bring the volume up to 50 µl, then pipetted up and down. The labelling program in Table 2.23 was used to run the thermal cycler.

Table 2.23: Labelling master mix components and volumes for Array-CGH.

Component	Sample (Cy3)	Reference (Cy5)
5x Reaction Buffer	10 μl	10 μl
10x dNTPs	5 μl	5 μl
Cyanine 3-dUTP	3 μl	———
Cyanine 5-dUTP	———	3 μl
Exo (-) Klenow	1 μl	1 μl
Final Volume of Labelling Mastermix		19 μl

Table 2.24: Thermal cycling conditions for labelling Array-CGH samples with cyanine dyes.

Temperature	Time
37 °C	2 hours
65 °C	10 minutes
4 °C	∞

Five minutes prior to the completion of the labelling process, the purification columns provided with the SureTag Complete DNA Labeling Kit (#5190-4240, Agilent) were assembled and prepared. Subsequently, 430 µl of TE buffer (pH=8) was added to the purification columns. After labelling, the samples (50 µl) were transferred into the purification columns with TE buffer. The columns were then centrifuged at 16000 xg for 10 minutes. The flow-through was discarded, and 480 µl TE buffer (pH=8) was added to the column and centrifuged for another 10 minutes at 16000 xg. The column was then transferred to a new 2 ml collection tube upside down and centrifuged for 1 minute at 1000 xg, after which the column was removed. The sample was then purified and concentrated with the SpeedVac Vacuum concentrator (Thermo Fisher Scientific) while being centrifuged for 30 minutes at 60°C under the setting for vacuum for Aqueous solutions (V-AQ). The remaining pellet was resuspended in 39 µl of nuclease-free water and briefly spun down. Subsequently, the 39 µl reference sample was added to the corresponding patient sample and thoroughly mixed. The hybridisation master mix was then prepared according to Table 2.25.

71 µl of hybridisation master mix was added to a 200 µl thin-wall tube PCR stripe. Next, 39 µl of the patient sample and 39 µl of the reference sample were combined and mixed thoroughly. The resulting mixture was then added to the hybridisation master mix and briefly centrifuged. Finally, the tube was preheated to 37°C for hybridisation in the SimpliAmp Thermal Cycler

Table 2.25: Components for the Array-CGH hybridisation master mix.

Component	Volume
Cot-1 DNA (1mg/ml)*	5 μL
10x aCGH Blocking Agent†	11 μL
2x HI-RPN Hybridisation Buffer†	55 μL
Final Volume of Hybridisation Master Mix	71 μL

* Human Cot-1 DNA (Agilent)

† Included in the Oligo aCGH Hybridisation Kit (Agilent)

(Applied Biosystems), following the instructions provided in 2.26.

Table 2.26: Pre-hybridisation thermal cycling program.

Temperature	Time
98°C	3 minutes
37°C	30 minutes
37°C	∞

The samples were briefly spun down after pre-hybridisation in the thermal cycler. Next, the microarray chambers were assembled, and the gasket slide was inserted, with approximately 100 µl of the combined sample pipetted into the gasket. The microarray slide was then placed on top of it, following the Agilent protocols and User Guides (Oligonucleotide Array-Based CGH for Genomic DNA Analysis - Enzymatic Labeling, Agilent Microarray Hybridization Chamber User Guide (publication G2534-90004). The assembled chamber was placed in the hybridisation oven (G2545A, Agilent, Böblingen) for 20 hours for hybridisation. The Agilent Oligo aCGH Wash Buffer 2 was pre-warmed in a slide-staining dish with a slide rack and magnetic stir bar overnight at 37°C in the INCU-Line IL23 incubator (VWR) for the following day.

The next day, the slide was washed following a specific procedure and handling instructions from the Agilent protocols and user guides. The washing and scanning process was managed in the NoZone Workspace (AlphaMetrix Biotech) with Ozonfilter (Sci Gene). First, a clean slide-staining dish was filled with Agilent Oligo aCGH Wash Buffer 1 at room temperature. Next, a slide rack was placed in a separate slide-staining dish, and a magnetic stir bar was added. The dish was filled with enough Agilent Oligo aCGH Wash Buffer 1 to cover the slide rack. The hybridisation chamber was removed from the oven and disassembled in the NoZone Workspace. As per the Agilent protocol, tweezers carefully separated the slide from the gasket in the first slide-staining dish. Then, the slide was transferred to the second slide-staining dish on the magnetic stirrer, placed in the rack and incubated at room temperature with light protection at 350 rpm for 5 minutes. The slide was transferred to a pre-warmed slide-staining dish at 37°C, containing wash buffer 2. It was then incubated on a magnetic stirrer for 1 minute under light protection. The slide was carefully removed to ensure no droplets were left on it and immediately transferred to the Microarray Scanner (InnoScan 910AL, InnoScan 710, Innopsys).

After the microarray scanning is finished, the following steps involve feature extraction and analysis, which are a vital part of our experimental process. Feature extraction takes data from

scanned microarray image files in tif format and transforms it into logarithmic ratios. This transformation allows us to identify anomalies or aberrations in our research samples. The feature extraction and analysis was done using the CytoGenomics 5.1.2.1 software (Agilent).

Quality control (QC) metrics are essential when analysing high-quality DNA samples with Agilent CGH microarrays. These metrics are included in the Feature Extraction QC report generated by the CytoGenomics 5.1.2.1 software (Agilent). QC metrics should be examined to evaluate the quality of the data produced, particularly the DLRSpread (spread of the log ratio differences), which should be below 0.3. The QC value helps to assess the hybridisation quality and the strength of the noise between samples on a chromosome.

The CNV dataset generated by Cytogenomics 5.1.2.1 (Agilent) is used for each patient, and an annotated tabular list of those called CNVs is generated using our in-house pipeline. This table contains information from common databases (DGV, dbvar, Decipher, OMIM) as well as from our internal patient collective ("intern frequency"). The information summarised in the annotated table is presented in Synopsis with the representation of the probe distribution in the Agilent software as the basis for interpretation. For analysis, all samples from the same run are opened in the "Triage View" of the program, and the probe distribution is displayed within the Called areas visually with the corresponding probes of the samples compared.

2.10 Whole Exome Sequencing

The Twist NGS workflow (Twist Bioscience) was used to conduct all steps of Whole Exome Sequencing (WES), and the NGS-Star pipette robot (Hamilton) was used to automate some of the processes. Routine diagnostics personnel conducted the standard WES procedure. The Twist Exome panel 2.0 (Twist Bioscience) was used for all samples except for WES 2, where the older Twist Exome panel 1.0 (Twist Bioscience) was used for the genomic fetal DNA (gf-DNA) run. Cell-free fetal DNA (cff-DNA) was extracted from amniotic fluid samples using the QIAamp Circulating Nucleic Acid Kit (Qiagen). The extracted cff-DNA was eluted in 30 µl of nuclease-free water, as described in section 2.5. cff-DNA with a concentration below 15 ng/µl was concentrated using the SpeedVac Vacuum concentrator (Thermo Fisher Scientific) while being centrifuged for 30 min at 60°C under the setting for a vacuum for aqueous solutions (V-AQ). In the case of WES

10, there was not enough volume left for vacuum concentration, as the rest was used for another experiment. The Qubit 1x dsDNA HS Assay Kit (Invitrogen) was used to measure cff-DNA concentration in duplicate, as described in section 2.2.1. Indexed cff-DNA libraries for target enrichment were generated using the Enzymatic Fragmentation and Twist Universal Adapter System protocol (Twist Bioscience). The cff-DNA was enzymatically fragmented with a proprietary enzyme, and the Twist universal adapters (adapter + index) were ligated and amplified using Unique Dual Index (UDI). The samples were pooled together for a multiplex hybridisation reaction, and target enrichment was conducted following the steps of Twist Target Enrichment Standard Hybridisation v2 Protocol (Twist Bioscience). The hybridisation of probes with the indexed DNA fragments was done overnight for 16 hours. Subsequently, hybridised targets were captured using streptavidin beads, followed by post-capture amplification and purification. The Qubit 1x dsDNA High Sensitivity Quantification Assay (Invitrogen) was used to quantify the final concentrations of the enriched library. Fragment lengths were measured using the D1000 Kit on the Tapestation (Agilent Technologies). Molar concentrations were calculated to prepare library pools of 2 nM for equimolar loading for sequencing, using the equation 2.1.

$$\frac{\text{Mass concentration (ng/µl)}}{(660\ \text{g/mol} \times \text{Average library size (bp)})} \times 10^6 = \text{Molar concentration (nM)} \qquad (2.1)$$

The NextSeq 2000 platform (Illumina) was used to prepare the enriched library for sequencing, according to the NextSeq 1000/2000 protocol (Illumina). A 2 nM DNA library pool was diluted with RSB and Tween 20 to 650 pM in 24 µl. A 20 µl spike-in of PhiX control was included at the same concentration as the library. The quality of all samples was determined via BaseSpace or with "Illumina Sequencing Analysis Viewer." The quality of the sequences of the individual samples was checked using the automatic downstream NGS pipeline, which utilises FastQC and Picard tools. The Burrows-Wheeler Aligner (BWA) was used to map the resulting FASTQ files to a reference genome (HG38). The Genome Analysis Toolkit (GATK) was used for variant calling, and VarSeq (Golden Helix) was used for functional annotation, visualisation, and clinical evaluation. A comparison was made between the data from cff-DNA and the original genomic fetal DNA (gf-DNA) WES run to check if the variants found in the gf-DNA WES could also be identified after sequencing from cff-DNA.

Results

3.1 Evaluating a suitable cff-DNA extraction kit for routine prenatal diagnostics and its parameters for cell-free fetal DNA from amniotic fluid supernatant

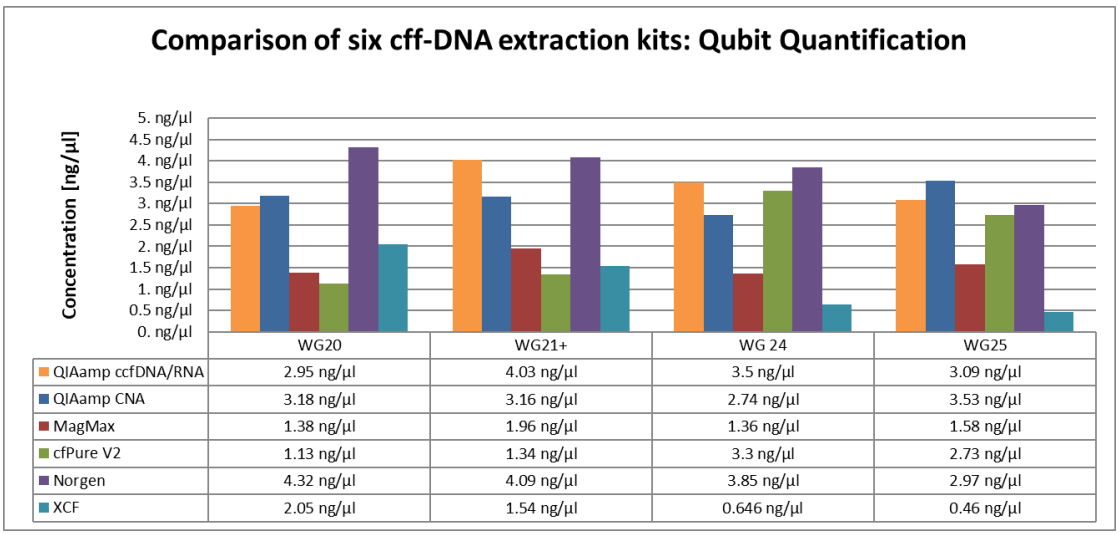
A comparative analysis of different extraction methods was performed to determine a suitable cell-free fetal DNA extraction kit (cff-DNA) for genetic prenatal diagnostics.

3.1.1 Identifying a suitable extraction kit for cff-DNA from amniotic fluid supernatant

This study aimed to find an efficient extraction kit for fetal cell-free DNA (cff-DNA) from amniotic fluid supernatant that can be used for all gestational ages in prenatal clinical samples. Since only one kit can be used for routine diagnostics, a kit that provides the best performance regarding sample purity, fragment length, cff-DNA yield, convenience, and time in cff-DNA extraction is needed. A literature review revealed several DNA extraction methods with contrasting findings regarding the optimal extraction method used. For routine diagnostics, six extraction kits were pre-selected based on cost, input, and elution volume.

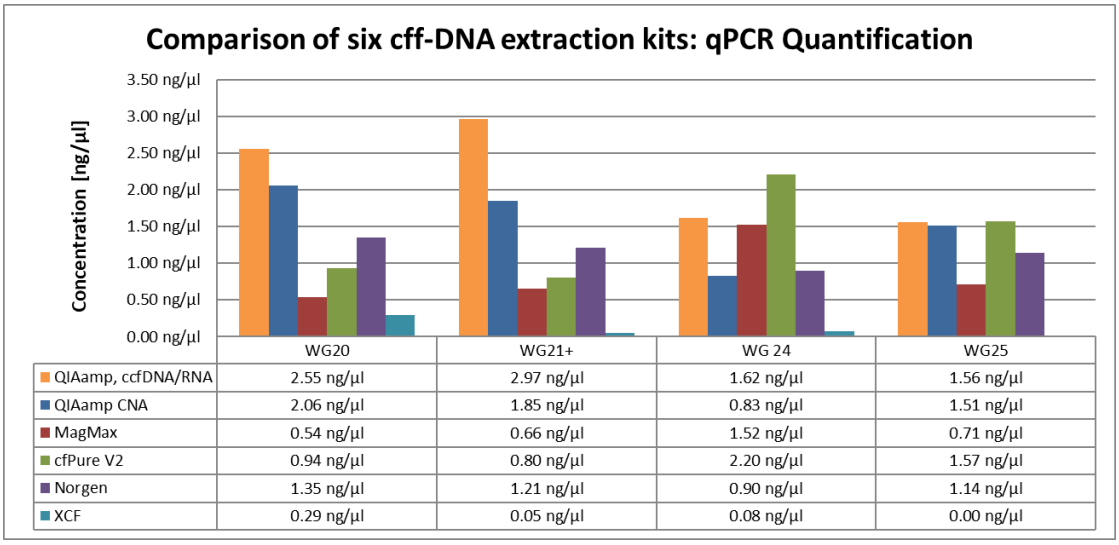
The initial experiment involved four samples of the amniotic supernatant representing different gestational ages. The extracted DNA samples were analysed for cff-DNA concentration and quality. The Qubit Flex fluorometer was used to measure the concentration of cff-DNA, and the results showed that the Norgen kit, the QIAamp ccfDNA / RNA kit, and the QIAamp CNA kit had higher overall yields, with the Norgen kit producing the most cff-DNA. The cfPure V2 kit had a higher concentration only in samples from later weeks of gestation (Figure 3.1) Similarly, the qPCR analysis showed that the QIAamp ccfDNA / RNA kit produced the highest concentration in samples from earlier gestational weeks. In contrast, the cfPure V2 kit yielded more in later weeks. The QIAamp CNA kit performed well consistently across different gestational weeks in both analyses (Figure 3.2). The TapeStation system was used to assess the fragment size of the extracted DNA samples. The results revealed that the fragment size of cff-DNA ranged from 50 bp to >1000 bp, which is longer than expected, making it challenging to evaluate fragment

size.



	WG20	WG21+	WG 24	WG25
QIAamp ccfDNA/RNA	2.95 ng/µl	4.03 ng/µl	3.5 ng/µl	3.09 ng/µl
QIAamp CNA	3.18 ng/µl	3.16 ng/µl	2.74 ng/µl	3.53 ng/µl
MagMax	1.38 ng/µl	1.96 ng/µl	1.36 ng/µl	1.58 ng/µl
cfPure V2	1.13 ng/µl	1.34 ng/µl	3.3 ng/µl	2.73 ng/µl
Norgen	4.32 ng/µl	4.09 ng/µl	3.85 ng/µl	2.97 ng/µl
XCF	2.05 ng/µl	1.54 ng/µl	0.646 ng/µl	0.46 ng/µl

Figure 3.1: Evaluation of cff-DNA yields using six extraction kits and Qubit quantification.
Norgen, QIAamp ccfDNA/RNA, and QIAamp CNA kits show higher overall yields, with Norgen providing the most cff-DNA. Notably, cfPure V2 demonstrates higher concentrations in samples from later gestational weeks.

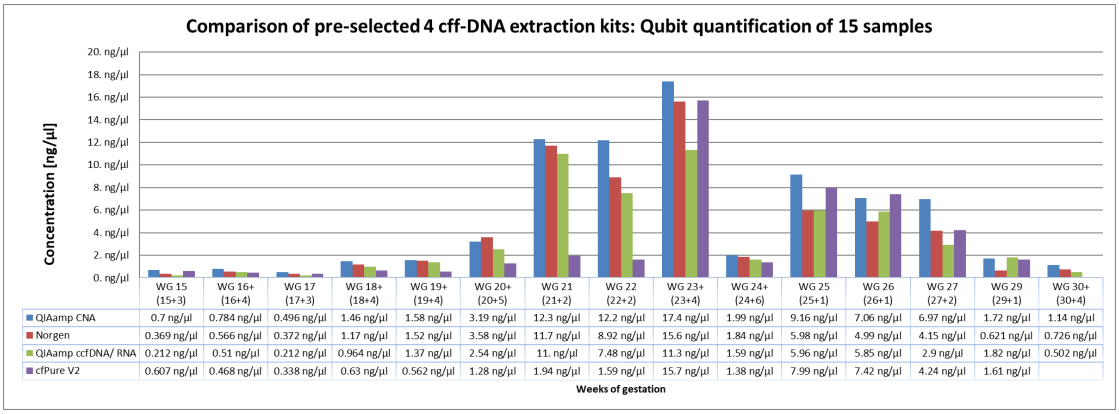


	WG20	WG21+	WG 24	WG25
QIAamp, ccfDNA/RNA	2.55 ng/µl	2.97 ng/µl	1.62 ng/µl	1.56 ng/µl
QIAamp CNA	2.06 ng/µl	1.85 ng/µl	0.83 ng/µl	1.51 ng/µl
MagMax	0.54 ng/µl	0.66 ng/µl	1.52 ng/µl	0.71 ng/µl
cfPure V2	0.94 ng/µl	0.80 ng/µl	2.20 ng/µl	1.57 ng/µl
Norgen	1.35 ng/µl	1.21 ng/µl	0.90 ng/µl	1.14 ng/µl
XCF	0.29 ng/µl	0.05 ng/µl	0.08 ng/µl	0.00 ng/µl

Figure 3.2: Evaluation of cff-DNA yields using six extraction kits and qPCR quantification.
The QIAamp ccfDNA/RNA kit yields the highest concentration of cff-DNA in samples from earlier gestational weeks, while the cfPure V2 kit shows increased yield in later weeks. The QIAamp CNA kit consistently performs well across different gestational weeks.

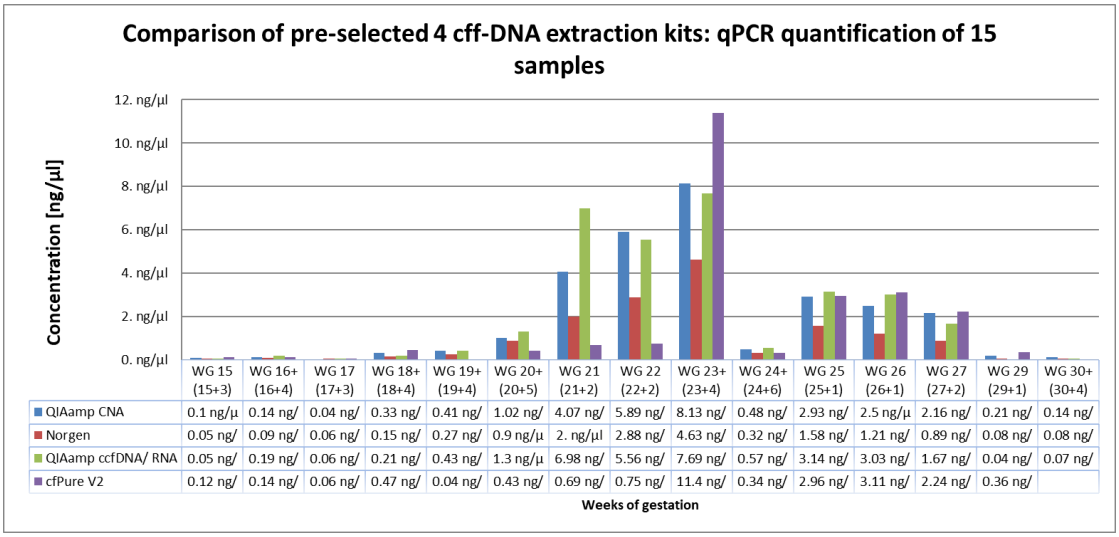
A follow-up comparison was conducted using 15 samples ranging from weeks 15 to 30 after the

initial experiment to identify a single kit that performed well throughout all weeks 15 to 30. The QIAamp CNA kit consistently produced the highest concentrations of cff-DNA, although there were specific weeks where it yielded slightly less. The Norgen kit and the QIAamp ccfDNA/RNA kit performed similarly, while the cfPure V2 kit performed better in later gestational weeks. The qPCR quantification chart shown in Figure 3.4 displays a similar view with slight changes. From GA 15 to GA 21, the QIAamp ccfDNA/RNA kit yielded slightly more cff-DNA than the QIAamp CNA kit. The Norgen extraction kit yielded the least out of the three. As observed before, the cfPure V2 extraction kit performed worse in the early weeks of gestation but showed improvement starting from WG 23.



	WG 15 (15+3)	WG 16+ (16+4)	WG 17 (17+3)	WG 18+ (18+4)	WG 19+ (19+4)	WG 20+ (20+5)	WG 21 (21+2)	WG 22 (22+2)	WG 23+ (23+4)	WG 24+ (24+6)	WG 25 (25+1)	WG 26 (26+1)	WG 27 (27+2)	WG 29 (29+1)	WG 30+ (30+4)
QIAamp CNA	0.7 ng/µl	0.784 ng/µl	0.496 ng/µl	1.46 ng/µl	1.58 ng/µl	3.19 ng/µl	12.3 ng/µl	12.2 ng/µl	17.4 ng/µl	1.99 ng/µl	9.16 ng/µl	7.06 ng/µl	6.97 ng/µl	1.72 ng/µl	1.14 ng/µl
Norgen	0.369 ng/µl	0.566 ng/µl	0.372 ng/µl	1.17 ng/µl	1.52 ng/µl	3.58 ng/µl	11.7 ng/µl	8.92 ng/µl	15.6 ng/µl	1.84 ng/µl	5.98 ng/µl	4.99 ng/µl	4.15 ng/µl	0.621 ng/µl	0.726 ng/µl
QIAamp ccfDNA/ RNA	0.212 ng/µl	0.51 ng/µl	0.212 ng/µl	0.964 ng/µl	1.37 ng/µl	2.54 ng/µl	11. ng/µl	7.48 ng/µl	11.3 ng/µl	1.59 ng/µl	5.96 ng/µl	5.85 ng/µl	2.9 ng/µl	1.82 ng/µl	0.502 ng/µl
cfPure V2	0.607 ng/µl	0.468 ng/µl	0.338 ng/µl	0.63 ng/µl	0.562 ng/µl	1.28 ng/µl	1.94 ng/µl	1.59 ng/µl	15.7 ng/µl	1.38 ng/µl	7.99 ng/µl	7.42 ng/µl	4.24 ng/µl	1.61 ng/µl	

Figure 3.3: Comparison of cff-DNA yields from 4 pre-selected kits using Qubit quantification.
Evaluation of the four top-performing kits across 15 samples spanning weeks 15 to 30 of gestation. The QIAamp CNA kit consistently demonstrates the highest concentration throughout, with occasional exceptions. The Norgen and QIAamp ccfDNA/RNA kit exhibit lower yields, while the cfPure V2 kit shows weaker performance in early gestation but a significant increase in later weeks.



	WG 15 (15+3)	WG 16+ (16+4)	WG 17 (17+3)	WG 18+ (18+4)	WG 19+ (19+4)	WG 20+ (20+5)	WG 21 (21+2)	WG 22 (22+2)	WG 23+ (23+4)	WG 24+ (24+6)	WG 25 (25+1)	WG 26 (26+1)	WG 27 (27+2)	WG 29 (29+1)	WG 30+ (30+4)
QIAamp CNA	0.1 ng/µ	0.14 ng/	0.04 ng/	0.33 ng/	0.41 ng/	1.02 ng/	4.07 ng/	5.89 ng/	8.13 ng/	0.48 ng/	2.93 ng/	2.5 ng/µ	2.16 ng/	0.21 ng/	0.14 ng/
Norgen	0.05 ng/	0.09 ng/	0.06 ng/	0.15 ng/	0.27 ng/	0.9 ng/µ	2. ng/µl	2.88 ng/	4.63 ng/	0.32 ng/	1.58 ng/	1.21 ng/	0.89 ng/	0.08 ng/	0.08 ng/
QIAamp ccfDNA/ RNA	0.05 ng/	0.19 ng/	0.06 ng/	0.21 ng/	0.43 ng/	1.3 ng/µ	6.98 ng/	5.56 ng/	7.69 ng/	0.57 ng/	3.14 ng/	3.03 ng/	1.67 ng/	0.04 ng/	0.07 ng/
cfPure V2	0.12 ng/	0.14 ng/	0.06 ng/	0.47 ng/	0.04 ng/	0.43 ng/	0.69 ng/	0.75 ng/	11.4 ng/	0.34 ng/	2.96 ng/	3.11 ng/	2.24 ng/	0.36 ng/	

Figure 3.4: Comparison of cff-DNA yields from 4 pre-selected kits using qPCR quantification.
Over the gestational weeks 15 to 21, the QIAamp ccfDNA/RNA kit slightly outperforms the QIAamp CNA kit in cff-DNA yield, while the Norgen kit exhibits lower yields. Starting from week 23, the cfPure V2 kit shows a substantial increase in yield, often surpassing the other kits.

A PCR analysis targeting Exons 11 and 12 of the CFTR gene was performed to evaluate the product specificity of the extracted cff-DNA. The QIAamp CNA and QIAamp ccfDNA/RNA kits consistently displayed specific product amplification, indicating high specificity for both exons. In contrast, the cfPure V2 and Norgen kits displayed unspecific or missing products. Based on the comparative analysis, the QIAamp CNA kit was chosen as the best extraction kit for isolating cff-DNA from amniotic fluid supernatant. This decision was primarily driven by the kit's consistently high cff-DNA yields across different gestational weeks. Additionally, the QIAamp CNA Kit offered more effortless and faster-handling advantages than the QIAamp ccfDNA/RNA Kit, making it more suitable for routine genetic prenatal diagnostics.

3.1.2 Optimisation of the QIAamp Circulating Nucleic Acid kit

The study evaluated the performance of the silica membrane in the QIAamp Circulating Nucleic Acid Kit for cff-DNA extraction. Increased sample volumes were used to optimise the procedure for routine diagnostics. Results showed that increasing the input volume from 4 to 8 ml of amniotic fluid supernatant significantly decreased the concentration of cff-DNA in the sample with the highest initial concentration but increased it in the sample with the lowest initial

concentration (Figure 3.5). The serum and urine protocols of the QIAamp Circulating Nucleic Acid Kit were compared for extracting cff-DNA from amniotic fluid supernatant. The study found that the cff-DNA yielded via the urine protocol had a slightly higher concentration than the cff-DNA yielded by the serum protocol. However, no statement about the significance can be made from the observations (Figure 3.6)

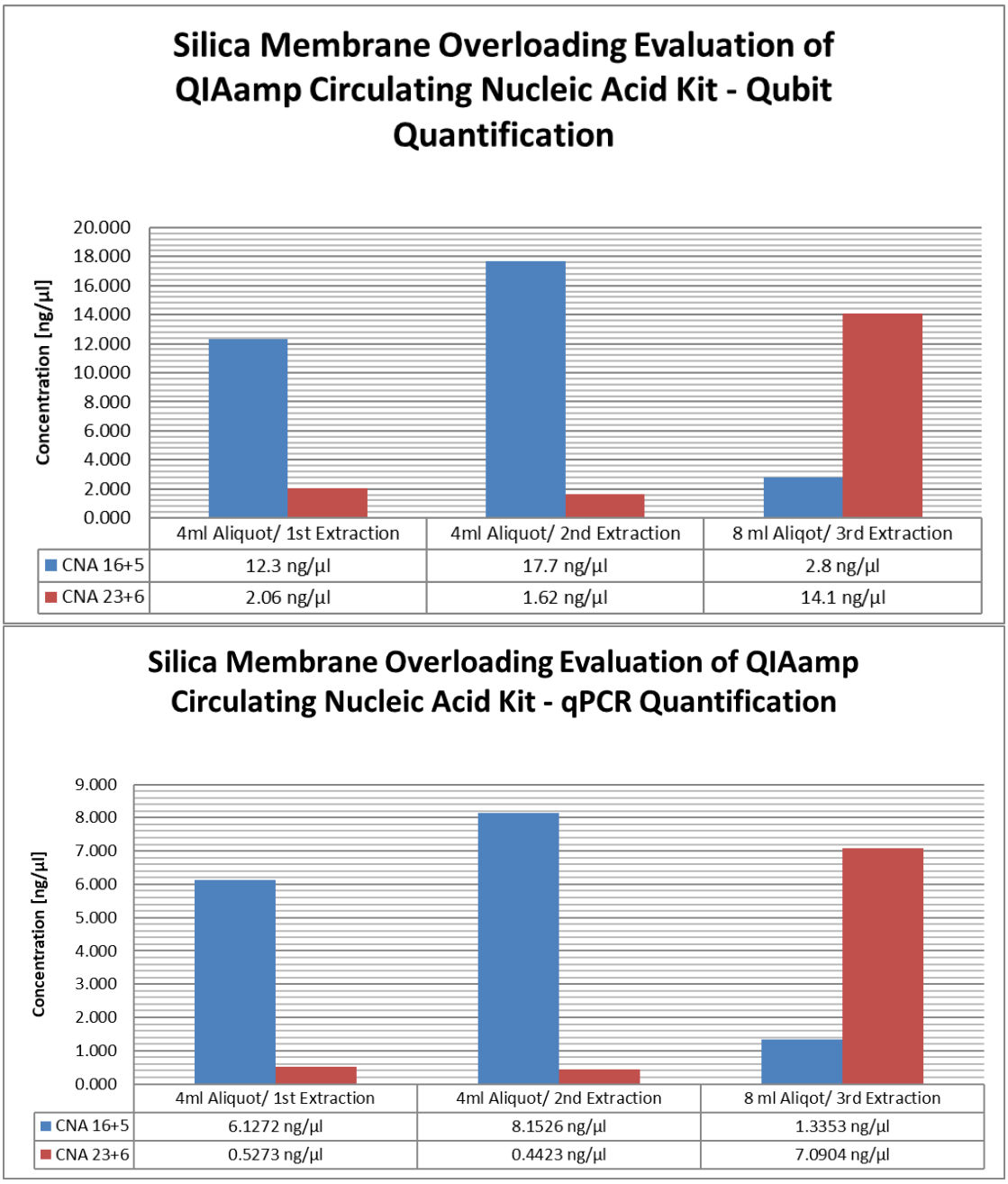


Figure 3.5: Overloading the silica membrane of the QIAamp CNA Spin column.
cff-DNA was extracted and quantified after increasing the sample input volume from 4 ml to 8 ml to compare it. cff-DNA quantified with both methods displays the same behaviour. The cff-DNA yield was reduced in the sample with a higher initial concentration but increased in the sample with a lower initial concentration.

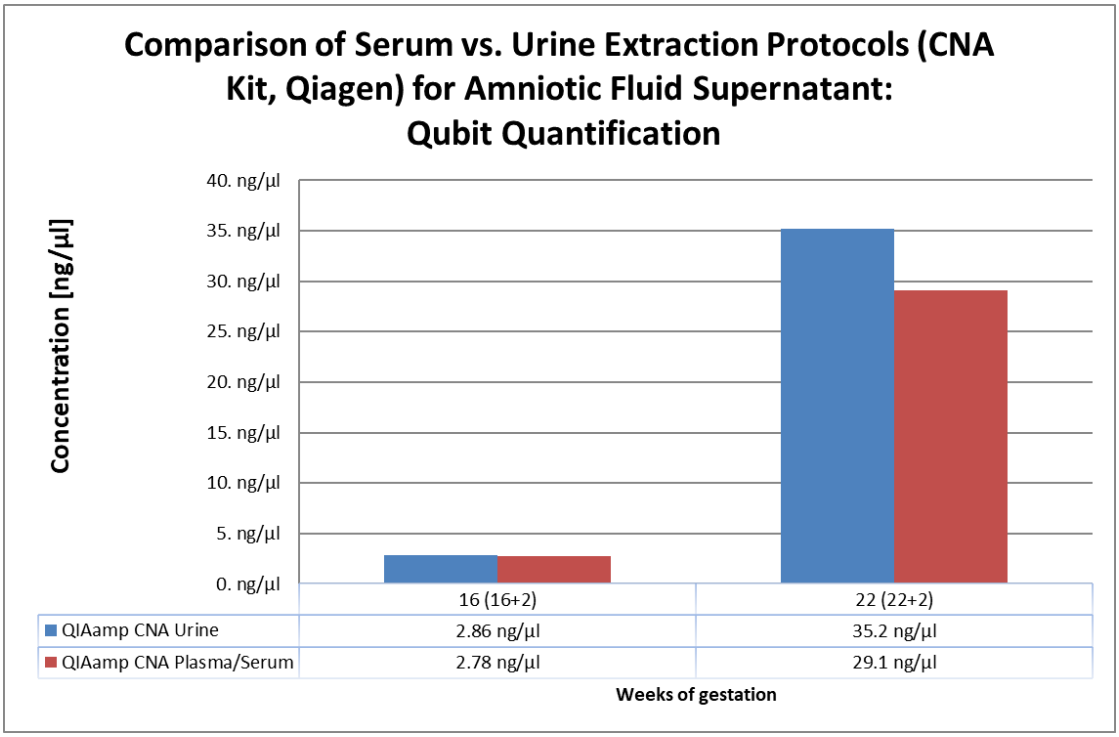


Figure 3.6: Comparison of cff-DNA yield using the serum vs. the urine extraction protocol. cff-DNA extracted via the urine protocol exhibits a slightly increased DNA yield. Due to the sample size, no testimony about the significance can be made.

3.2 Fragment size of cff-DNA extracted from amniotic fluid

The size of cell-free fetal DNA (cff-DNA) fragments was determined using the 4150 TapeStation System (Agilent) (Section 2.2.3). Initially, the Cell-free DNA ScreenTape was used to analyse fragments up to 800 bp, based on the expectation of fragment sizes corresponding to a mononucleosome and its multimers. However, it was observed that fragments beyond 800 bp were visible. Therefore, the ScreenTape D5000 was employed to analyse fragment sizes up to 5000 bp. For analysis with the D5000 ScreenTape, 10 ng/µl cff-DNA was loaded per sample. Figure 3.7 shows the distribution of fragment sizes in cff-DNA for different gestational ages. The image represents the observed fragment distribution for all samples. In Figure 3.7.A, the electrohperogram of an early gestational age (GA) (15+5) shows a prominent peak around 200 bp, with fewer longer fragments. The sampling intensity decreased towards a more extended fragment size. In Figure

3.7.B (GA 22+1), it can be observed that longer fragments were visible in higher quantities as gestational age increased. In Figure 3.7.C, a late gestational age (30+6) is analysed. No prominent peak around 200 bps is visible. Instead, longer fragments up to 1500 bp were visible in high quantity, with signal intensity similar to the peak of 200 bp in early gestational weeks. Additionally, even larger fragment sizes than 1500 bps can be observed, but they decrease in signal intensity.

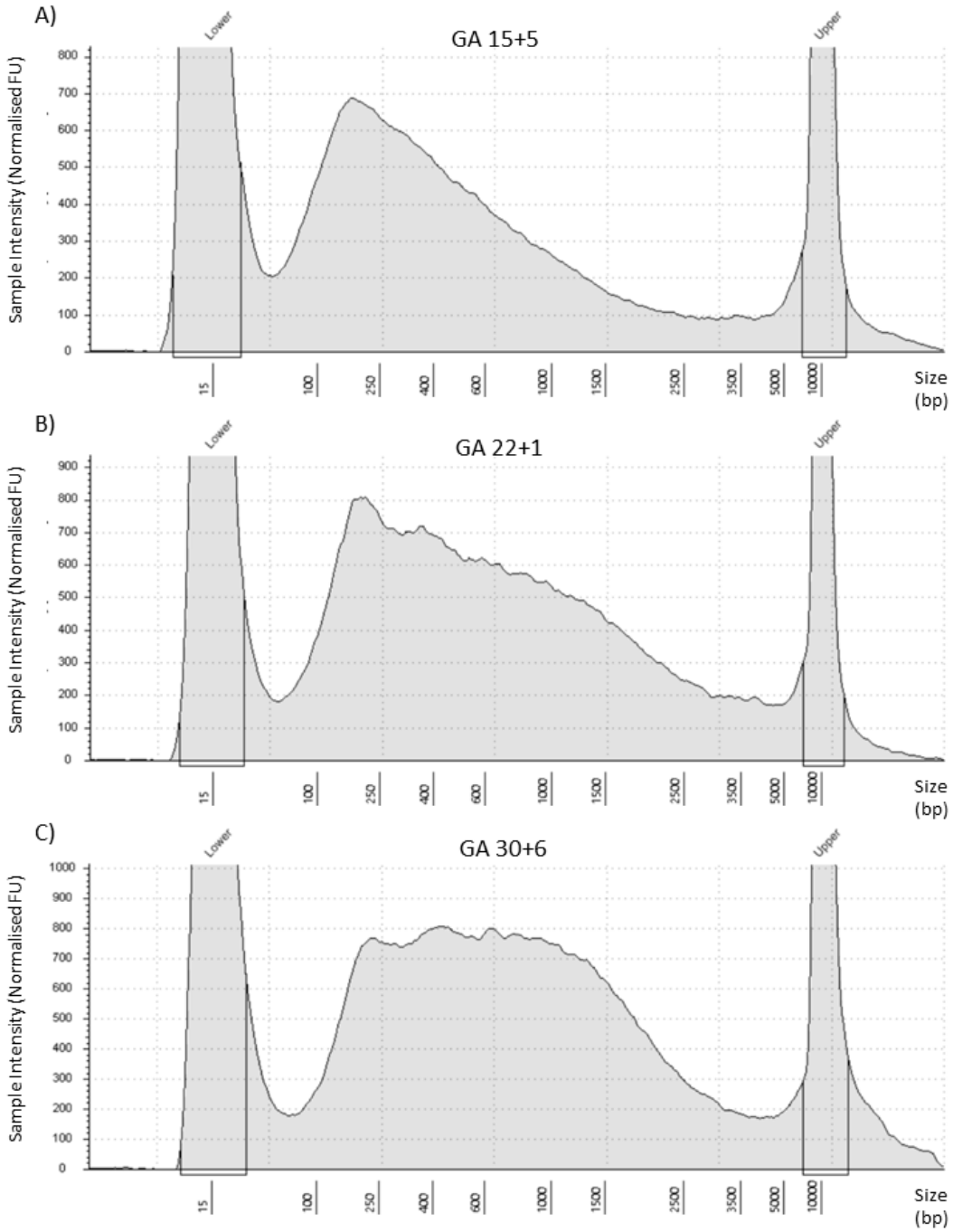


Figure 3.7: Fragment size analysis of cff-DNA using the 4150 TapeStation System.
Analysis of cff-DNA fragment size reveals a distinct pattern of size distribution associated with gestational age. **A)** At 15+5 weeks of gestation, a prominent peak around 200 bp is observed, with a gradual decrease in signal intensity for longer fragments. **B)** At 22+1 weeks, an increase in the abundance of longer fragments is evident. **C)** At 30+6 weeks, the characteristic 200 bp peak is absent, replaced by a broad distribution of fragments ranging up to 1500 bp. Even longer fragments are visible.

3.3 Normal distribution of cff-DNA concentration in euploid fetuses across gestational ages

This research sought to examine the amount of cell-free fetal DNA (cff-DNA) at different pregnancy stages and determine if there is a relationship between gestational age and the concentration of cff-DNA. Quantitative polymerase chain reaction (qPCR) was used to measure the concentration of cff-DNA. Primers for the *GAPDH* gene were used for qPCR, and the unknown sample concentration was compared to male and female human reference DNA standards. The results showed a clear pattern in cff-DNA concentration throughout gestation resembling a Gaussian curve. The qPCR concentrations in ng/µl eluate indicated that during the early stages of pregnancy, the concentration of cff-DNA was relatively low with minimal standard variability, with the concentration increasing as gestational age progressed. As the gestational period advanced towards late gestational age, a slight decrease in concentration was observed, which appears to follow a Gaussian distribution. However, due to the limited sample size in the later weeks, it is not possible to make any definite conclusions regarding the significance of this observation. Figure 3.9 displays the concentration curve recalculated to ng/ml amniotic fluid.

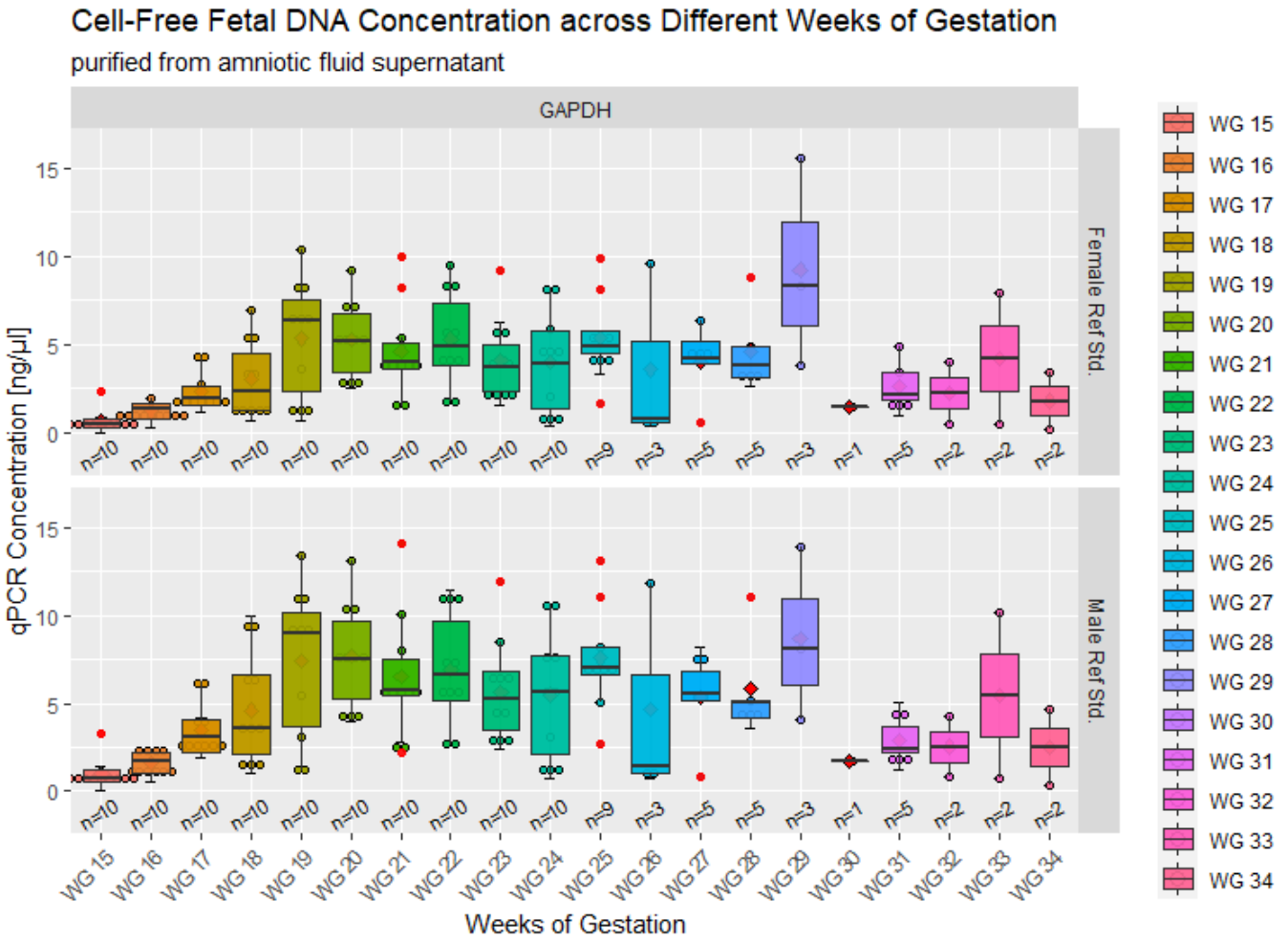


Figure 3.8: Distribution of cff-DNA Concentration (ng/ml amniotic fluid) in euploid fetuses across gestational ages.
The figure illustrates the normal distribution of cff-DNA concentrations across gestational weeks 15 to 34. Concentrations are initially low in early gestation, gradually increasing over weeks. A subtle decline is observed in later stages (Sample numbers: WG 15-24 = 10; WG 25 = 9; Sample numbers of Week 27-34 = too low for conclusive statements; outliers are depicted as red dots).

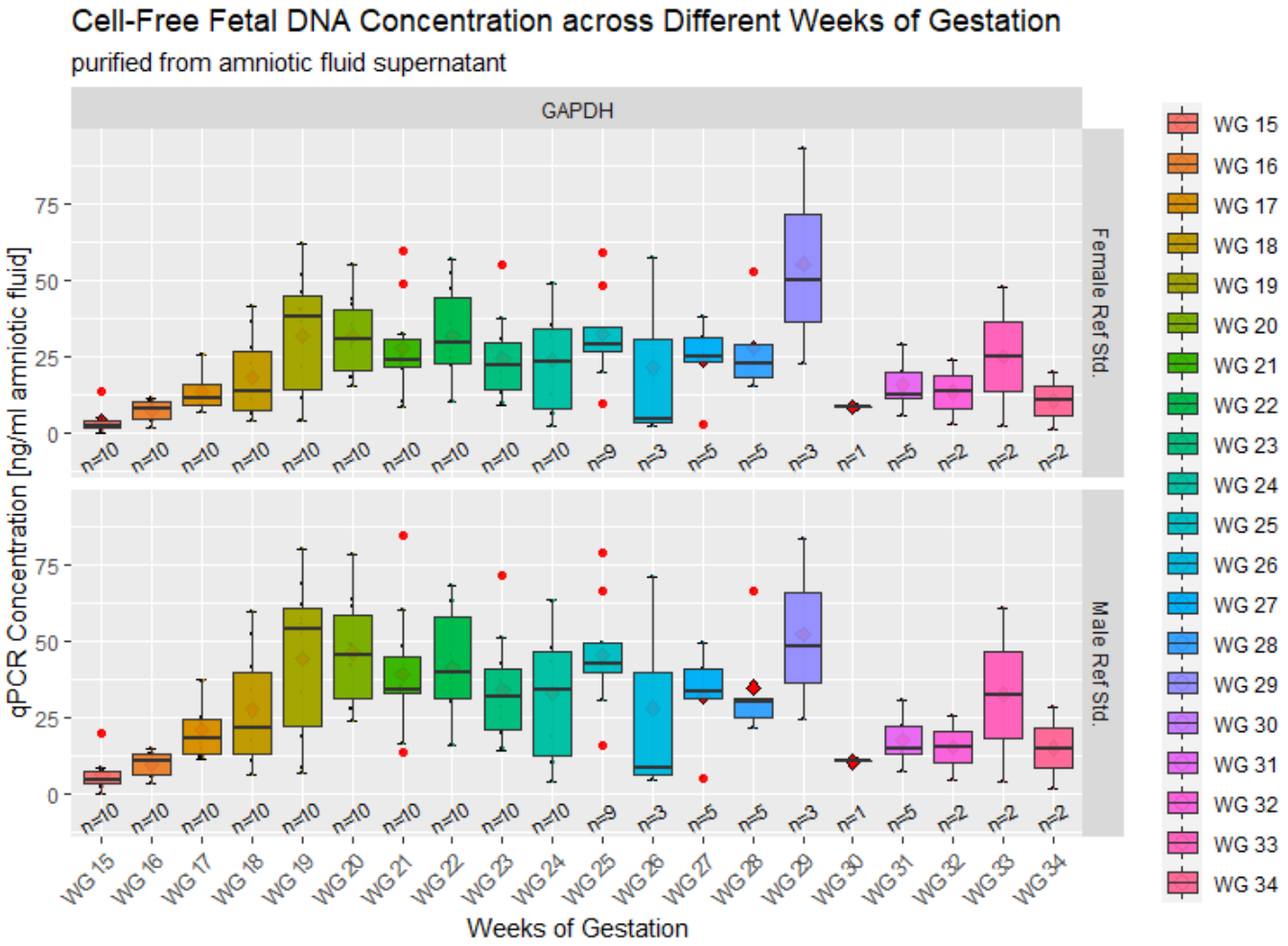


Figure 3.9: Distribution of cff-DNA Concentration (ng/ml amniotic fluid) in Euploid Fetuses across Gestational Ages
The figure illustrates the normal distribution of cff-DNA concentrations across gestational weeks 15 to 34. Concentrations are initially low in early gestation, gradually increasing over weeks. A subtle decline is observed in later stages (Sample numbers: WG 15-24 = 10; WG 25 = 9; Sample numbers of Week 27-34 = too low for conclusive statements; outliers are depicted as red dots).

3.4 Screening for maternal cell contamination in cff-DNA via STR-marker

Maternal cell contamination (MCC) can be challenging when conducting accurate fetal genetic testing on amniotic fluid samples. Prenatal diagnosis through amniocentesis can pose difficulties due to the potential contamination of maternal cells in the amniotic fluid. The AmpFlSTR™ Identifiler Plus PCR kit (Thermo Scientific) for STR-marker analysis on cff-DNA extracted from 132 amniotic fluid samples, 41 of which had visible blood tinges. Out of the 41 blood-tinged amniotic samples, 6 samples were heavily tinged with blood that was even visible before centrifugation. Figure 3.10 displays an example of a heavily blood-tinged amniotic fluid. The study found that STR-marker analysis on cff-DNA extracted from 132 amniotic patient samples was 100% concordant with STR-marker analysis from gf-DNA extracted directly from amniotic fluid or cell culture. All samples were negative or below significance (<5%) for maternal cell contamination, regardless of visible blood tinges. Centrifugation effectively separated the cellular components from the supernatant, minimising the risk of maternal cell contamination.

Figure 3.10: Heavily blood-tinged amniotic fluid in a syringe.

3.4.1 The accuracy of Sanger sequencing in detecting mutations in target genes using cff-DNA

This study aimed to evaluate the effectiveness of Sanger sequencing in detecting mutations in target genes from cell-free fetal DNA (cff-DNA) extracted from amniotic fluid. The traditional gf-DNA Sanger sequencing technique was compared with this method in detecting mutations. Sanger sequencing is a valuable tool in prenatal diagnostics for single-gene and segregation analysis, as it can detect parental mosaic patterns and help in inheritance studies. It is the most accurate form of DNA sequencing and complements NGS by verifying variants and filling in gaps in NGS data. The study focused mainly on gene variants detected via WES, regardless of whether they were responsible for the clinical phenotype, to determine whether cff-DNA could reliably detect all variants. The amplified genes' fragment sizes ranged from 363 to 1203 bp.

The effectiveness of cff-DNA was evaluated by extracting and sequencing 15 samples from different fetuses. The sequencing targeted 19 genes with 23 different approaches, as listed in Table 3.1. The largest fragment sequenced was about 1203 base pairs long. Interestingly, all 23 sequencing approaches between gf-DNA and cff-DNA were 100% consistent and detected the same variants, as shown in 3.2. A total of 4 heterozygous (het) mutations were identified in which the mother was a heterozygous carrier, 4 were inherited paternally, and for 3 cff-DNA samples, no parents were sequenced. Six had a de-novo mutation, 1 had a maternally inherited hemizygous mutation, and 6 sequencing approaches did not detect mutation, as shown in 3.3.

Table 3.1: Overview of patient samples for Sanger sequencing with cff-DNA.
Clinical indication and gestational age of 15 patients analysed via Sanger Sequencing

Case No	GA	Clinical Indication	Sequenced Genes
1	27+2	Microcephaly, cerebellar hypoplasia, abnormal cerebral cortex morphology	*COL4A1* *COL4A2*
2	24+2	Cleft palate, cleft lip, absent gallbladder, thymus hyperplasia	*DHCR7* *SOX17*
3	21+5	Ventricular septal defect, dilated bowel loops, bilateral ventriculomegaly	*ANKLE2*
4	24+2	Polyhydramnios, abnormal foot morphology, clubbing	*CAMK2B* *LRRK1* *ANO5*
5	22+1	abnormality of the diaphragm	*G6PD* *FOXI3*
6	16+5	Beckwith-Wiedemann Syndrome	*CDKN1C*
7	21+4	Meningoencephalocele, abnormality of brain morphology	*TNFRSF1A*
8	19+5	Agenesis Of corpus callosum	*CC2D2A* *PIGN* *EARS2*
9	20+0	Segregation analysis: 1st child deceased: het. mutation c.68A>G in *MEF2C* gene	*MEF2C*
10	22+1	Ventriculomegaly, aqueductal Stenosis, fetal skin edema	*L1CAM*
11	16+0	Holt-Oram-Syndrome	*TBX5*
12	27+2	periventricular cysts	*ALDH6A1*
13	19+6	Complex renal malformation, vitium cordis, suspected esophageal atresia	*ALDH6A1*
14	18+2	Double outlet right ventricle, transposition of the great arteries	*ALDH6A1*
15	15+5	NT >95 percentile	*ALDH6A1*

Table 3.2: Comparison of Sanger sequencing results.
100% concordance was achieved in identified variants between gf-DNA and cff-DNA.

Case No	Sequencing Approach	Gene	Mutation Detection in gf-DNA	Mutation Detection in cff-DNA
1	1	COL4A1	c.1970C>G	c.1970C>G
	2	COL4A2	c.4214G>A	c.4214G>A
2	3	DHCR7	c.452G>A	c.452G>A
	4	SOX17	c.499_520del	c.499_520del
3	5	ANKLE2	no mutation detected	no mutation detected
4	6	CAMK2B	c.1990C>T	c.1990C>T
	7	LRRK1	c.4699dupA	c.4699dupA
	8	ANO5	c.1520delT	c.1520delT
5	9	G6PD	c.637G>A,	c.637G>A,
	10	FOXI3	c.343_351dup GCGCCCGCC	c.343_351dup GCGCCCGCC
6	11	CDKN1C	c.555T>C	c.555T>C
7	12	TNFRSF1A	no mutation detected	no mutation detected
8	13	CC2D2A	c.4553G>A,	c.4553G>A,
	14	PIGN	c.2371-1G>T	c.2371-1G>T
	15	EARS2	c.322C>T	c.322C>T
9	16	MEF2C	no mutation detected	no mutation detected
10	17	L1CAM	c.1704-75G>T	c.1704-75G>T
11	18	TBX5	c.663+36G>T	c.663+36G>T
	19	TBX5	c.755+94C>A	c.755+94C>A
12	20	ALDH6A1	c.319C>T	c.319C>T
13	21	ALDH6A1	no mutation detected	no mutation detected
14	22	ALDH6A1	no mutation detected	no mutation detected
15	23	ALDH6A1	no mutation detected	no mutation detected

Table 3.3: Detailed sequencing results and their associated disease inheritance.
Genetic Variants, inheritance and clinical significance. All variants could be detected with gf-DNA and with cff-DNA.

Seq. Approach	Gene	Mutation	Zygosity	Associated Disease (Inheritance)	Clinical Significance
1	*COL4A1*	c.1970C>G	het (mat)	Brain small vessel disease with or without ocular anomalies (AD); Angiopathy hereditary with nephropathy aneurysms and muscle cramps (AD); Microangiopathy and leukoencephalopathy (AD)	VUS
2	*COL4A2*	c.4214G>A	het (mat)	Brain small vessel disease 2 (AD)	VUS
3	*DHCR7*	c.452G>A	het (pat)	Smith-Lemli-Opitz Syndrome (AR)	pathogenic
4	*SOX17*	c.499_520del	het (de-novo)	Vesicoureteral reflux 3 (AD)	Likely pathogenic
5	*ANKLE2*	no mutation	-	-	-
6	*CAMK2B*	c.1990C>T	het (de novo)	Intellectual developmental disorder (AD)	VUS
7	*LRRK1*	c.4699dupA	het (de novo)	Osteosclerotic metaphyseal Dysplasia (AR)	Likely pathogenic
8	*ANO5*	c.1520delT	het (mat)	Miyoshi muscular dystrophy 3, Muscular dystrophy limb-girdle (AR)	Likely pathogenic
9	*G6PD*	c.637G>A	het (de novo)	Hemolytic anemia G6PD deficient (XLD)	Likely pathogenic
10	*FOXI3*	c.343_351dup GCGCCCGCC	het (de novo)	-	VUS
11	*CDKN1C*	c.555T>C	het	-	Benign/Likely benign
12	*TNFRSF1A*	no mutation	-	-	
13	*CC2D2A*	c.4553G>A	het (pat)	COACH syndrome 2, Joubert syndrome 9, Meckel syndrome 6, Retinitis pigmentosa 93 (AR)	pathogenic
14	*PIGN*	c.2371-1G>T	het (mat)	Multiple congenital anomalies-hypotonia-seizures syndrome 1 (AR)	pathogenic
15	*EARS2*	c.322C>T	het (pat)	Combined oxidative phosphorylation deficiency 12 (AR)	pathogenic
16	*MEF2C*	no mutation	-	-	-
17	*L1CAM*	c.1704-75G>T	hemi (mat)	Hydrocephalus due to aqueductal stenosis; Hydrocephalus with congenital idiopathic intestinal pseudoobstruction; Hydrocephalus with Hirschsprung disease; Corpus callosum, partial agenesis of; CRASH syndrome; MASA syndrome (all XLR)	VUS
18	*TBX5*	c.663+36G>T	het	Holt-Oram-Syndrome (AD)	Benign
19	*TBX5*	c.755+94C>A	het	Holt-Oram-Syndrome (AD)	Benign
20	*ALDH6A1*	c.319C>T	het (de-novo)	Methylmalonate semialdehyde dehydrogenase deficiency (AR)	VUS
21	*ALDH6A1*	no mutation	-	-	-
22	*ALDH6A1*	no mutation	-	-	-
23	*ALDH6A1*	no mutation	-	-	-

Note: het = heterozygous, hemi = hemizygous, mat = maternal inheritance, pat = paternal inheritance; VUS = variant of uncertain significance.

3.5 Validating MLPA CNV results in cff-DNA using

Five fetal samples underwent analysis using Multiplex Ligation-Dependent Probe Amplification (MLPA) with cell-free fetal DNA (cff-DNA) to detect Copy Number Variation (CNV) changes related to their clinical indication or to validate an Array-CGH or Whole Exome Sequencing (WES) result using an independent method. The results were compared to those obtained using genomic fetal DNA (gf-DNA). MLPA requires reference samples in the same run to detect relative differences. The MLPA reaction was performed using 50 ng of total cff-DNA. A decrease in peak height indicates a deletion of one or more target sequences, while an increase in relative peak height reflects a copy number increase. Incorrect controls for cff-DNA samples can lead to false-positive duplications or deletions. Table 3.4 lists the clinical indications and targeted gene regions with MLPA. Table 3.5 presents the detailed results of the five samples analysed via MLPA that all displayed consistent results when the correct reference samples were used for cff-DNA.

An example of a heterozygous deletion is shown in case number 5 in Figure 3.11, where the MLPA was conducted to verify the deletion found via WES. The deletion in the *BRCA1* gene was identified up to exon 16 without any false positives while using cff-DNA controls. However, when gDNA was used as a reference, false positive deletions and duplications were observed in other exons.

Figure 3.12 shows an example of a MLPA result that does not indicate any clinical abnormalities. The results of routine diagnostic MLPA of gf-DNA were consistent with those obtained

Table 3.4: Clinical indications and targeted genes for MLPA analysis of cff-DNA.

Case No	GA	Karyotype	Clinical Indication	Targeted Gene Regions for MLPA
1	22+1	46,XY	Ventriculomegaly	*L1CAM*
2	16+0	46,XY	Holt-Oram Syndrome	*TBX5*
3	17+3	46,XY	Nuchal Translucency > 95%	*CRLF2, CSF2RA*
4	20+0	46,XY	Nuchal Translucency > 3mm	*CDKL5*
5	22+3	46,XX	Dysplastic Corpus Callosum, Cerebellar Hypoplasia, Vitium Cordis, Echogenic Kidneys	*BRCA1*

Note: GA = Gestational Age, MLPA = Multiplex Ligation-dependent Probe Amplification, cff-DNA = cell-free fetal DNA, gf-DNA = genomic fetal DNA, CNV = Copy Number Variation.

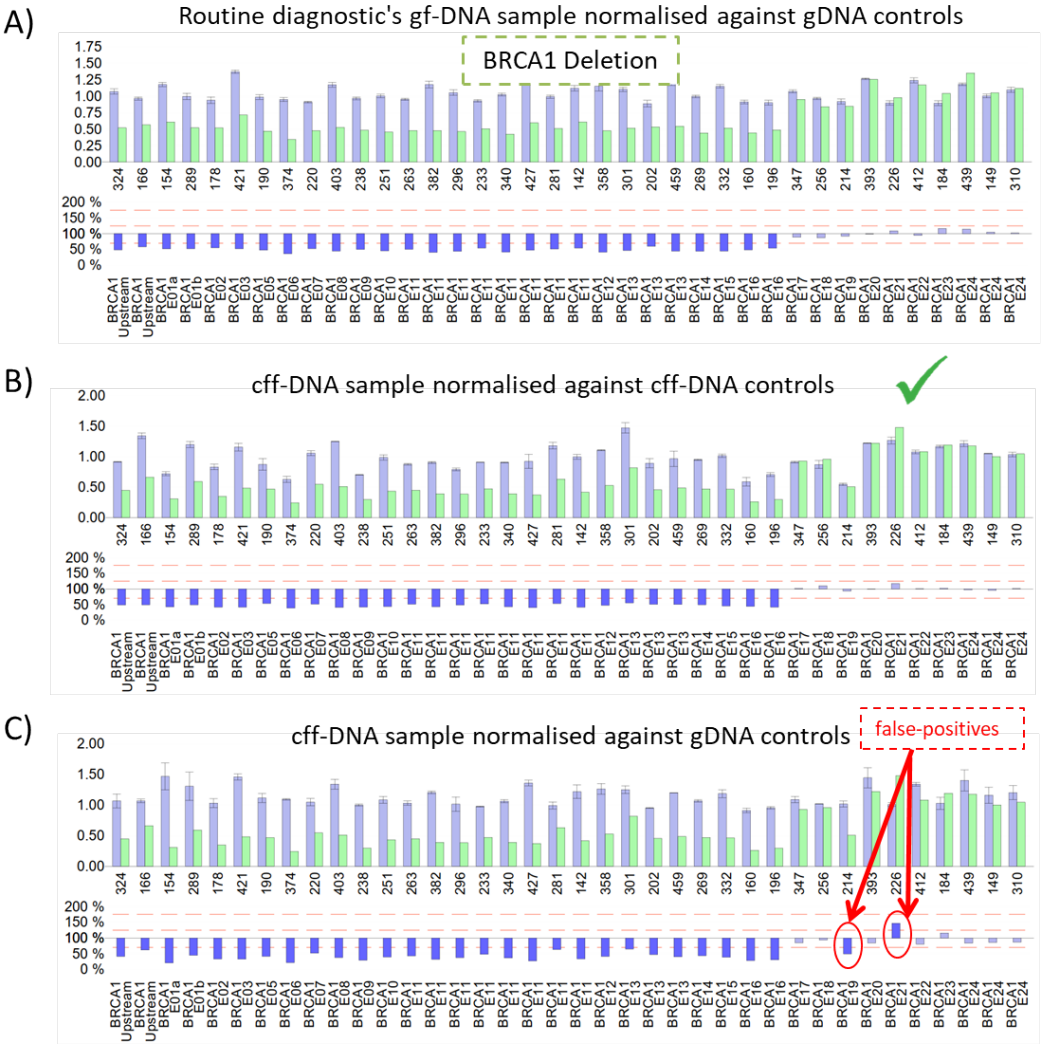


Figure 3.11: Comparison of MLPA results for cff-DNA and gf-DNA in Case 5.
MLPA analysis of case number 5 revealed consistent results between gf-DNA and cff-DNA when using cff-DNA references. Both methods detected a deletion in the *BRCA1* gene up to exon 16. However, when using gDNA as a reference for cff-DNA samples, false positive deletions and duplications were observed in other exons, indicating the importance of using appropriate reference material for cff-DNA analysis.

Table 3.5: MLPA analysis results using cff-DNA.
MLPA analysis confirms consistent copy number variations (CNVs) between genomic fetal DNA (gf-DNA) and cell-free fetal DNA (cff-DNA), demonstrating the reliability of cff-DNA in CNV detection.

Case No	CNV Detection in gf-DNA	CNV Detection in cff-DNA	MLPA Results
1	No CNV detected	No CNV detected	Concordant
2	No CNV detected	No CNV detected	Concordant
3	*CRLF2*/ partial *CSF2RA* duplication	*CRLF2*/ partial *CSF2RA* duplication	Concordant
4	No CNV detected	No CNV detected	Concordant
5	*BRCA1* deletion	*BRCA1* deletion	Concordant

Note: MLPA = Multiplex Ligation-dependent Probe Amplification, cff-DNA = cell-free fetal DNA, gf-DNA = genomic fetal DNA, CNV = Copy Number Variation.

from cff-DNA using cff-DNA reference DNAs. None of the exons of the kit showed deletions or duplications. However, false positive results were obtained using gDNA (regular or sheared) as a reference. False positives were observed in all 5 cases using gDNA reference DNA. Case number 3, where duplications of *CRLF2* and partial duplications of *CSF2RA* were detected using gf-DNA, was a peculiar case regarding reference DNA experiments. False positives were observed when using gDNA reference DNA, but sheared gDNA did not lead to false positives in this case. However, a higher probe spread was observed in comparison to when using cff-DNA as the reference DNA.

In conclusion, the results of the MLPA analysis of cff-DNA were 100% concordant in all 5 samples when using the correct controls for cff-DNA that were treated the same way as the samples.

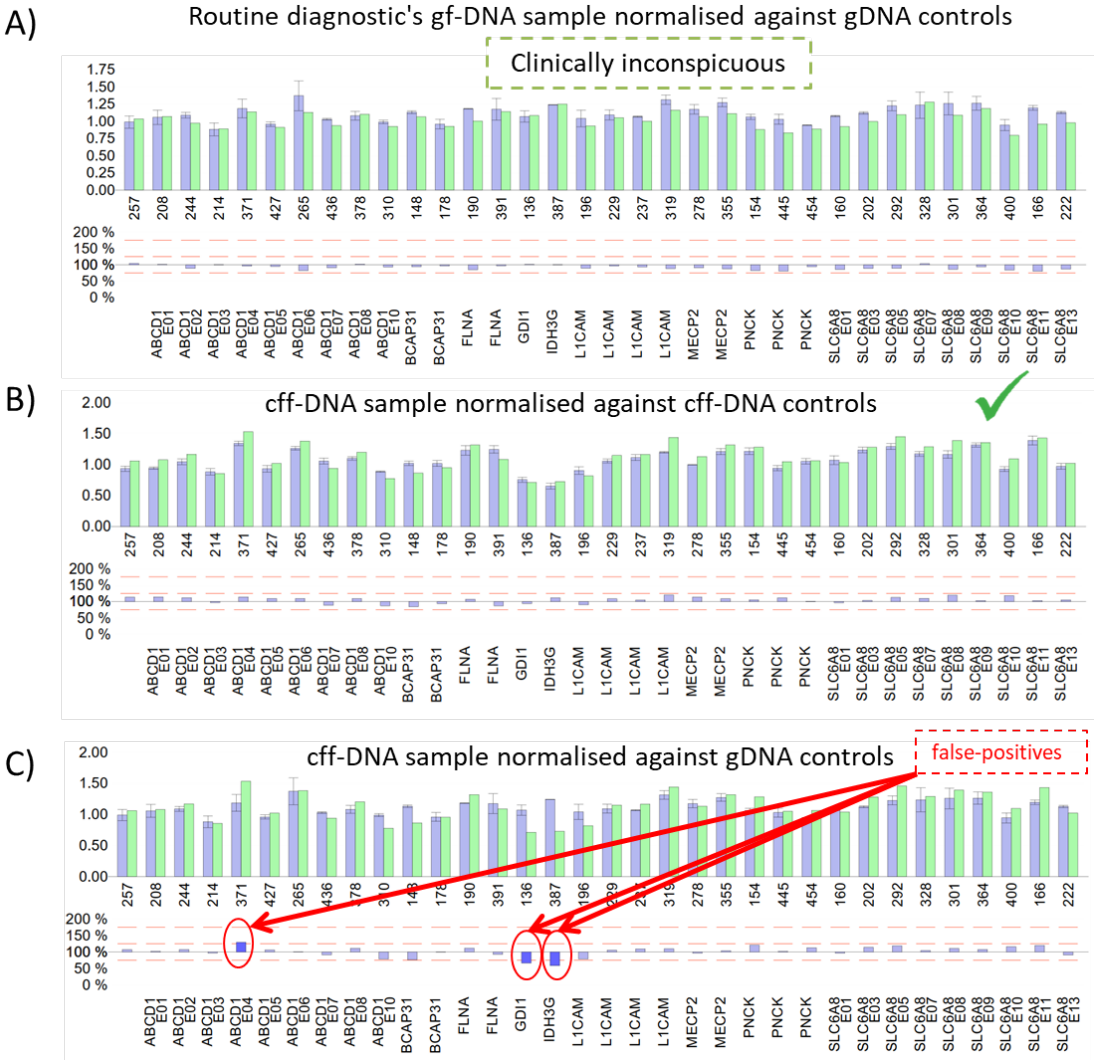


Figure 3.12: Comparison of MLPA results for cff-DNA and gf-DNA in case 1.
MLPA analysis of Case number 1 demonstrated consistent results between cff-DNA and gf-DNA, both methods detecting no copy number variations (CNV) when using the appropriate reference DNA. Using gDNA or sheared gDNA as a reference for cff-DNA samples resulted in false positives.

3.6 100% detection success rate of CNVs using cff-DNA in aCGH analysis

This section presents an Array-CGH (aCGH) analysis conducted with cell-free fetal DNA (cff-DNA) in amniotic fluid supernatant. The study included 25 patients, and the goal was to detect targeted deletions and duplications (Del/Dup) within cff-DNA samples, as had been identified in routine diagnostic runs using gf-DNA. The use of cff-DNA was also evaluated in cases of suspected maternal cell contamination samples that would have to wait for culture and later gf-DNA extraction, as well as for early pregnancy weeks (GA $<$ 20 weeks) when direct gf-DNA extraction is impossible.

Before the primary study, preliminary experiments were conducted to determine the required concentration of cff-DNA to a detection limit of 10 ng/µl, corresponding to 240 ng of total cff-DNA per reaction. Data were collected from all 25 patients, and a 100% success rate was achieved in detecting copy number variants (CNVs) that were either primary or additional findings in the clinical report of the aCGH analysis conducted with gf-DNA. Fifteen samples were concentrated before aCGH analysis, while the other ten had a sufficient cff-DNA concentration for direct processing. Furthermore, a subgroup analysis was conducted on six samples from pregnancies in the early stages (gestational age <20 weeks). Three of these samples were taken from amniotic fluid, which revealed a clear presence of maternal erythrocyte contamination, suggesting potential maternal cell contamination that could result in maternal DNA being blended with fetal DNA during the usual extraction. As seen in Table 3.6, all reported CNVs could be successfully detected for all samples.

Table 3.6: Array-CGH CNV analysis results.
Array-CGH analysis of cell-free fetal DNA (cff-DNA) accurately identifies all copy number variations (CNVs) compared to gf-DNA in routine diagnostic tests.

Case No	GA	Karyotype	Qubit Conc. (ng/µl)	cff-DNA Vacuum Conc.?	aCGH result	Copy Number Variants (validated and reported)	Cytoband
1	19+1	46,XX	35.8	no	arr[GRCh38] 1q41(216086637_216143568)x1	57 kbp heterozygous deletion	1q41
2	23+5	46,XX	27.60	no	arr[GRCh38] 13q12.3(31173042_31313267)x3, 22q11.22(21959009_22202339)x1	a) 140 kbp heterozygous duplication b) 243 kbp heterogeneous deletion	a) 13q12.3 b) 22q11.22
3	24+1	46,XX	35.60	no	arr[GRCh38] 4q13.1(63036755_63409632)x3	373 kbp heterozygous duplication	4q13.1
4	27+2	46,XY	48.00	no	clinically inconspicuous	clinically inconspicuous	-
5	21+3	46,XY	15.60	no	arr[GRCh38] 11q11q12.1(54667246_56129373)x3	1.46 Mbp heterozygous duplication	11q11q12.1
6	24+2	46,XY	16.60	no	arr[GRCh38] 9p13.1p11.2(38662490_41614886)x3, 21p11.2(8632506_10068169)x3, Xq11.1(62839246_63590485)x2	a) 2.95 Mbp heterozygous duplication b) 1.44 Mbp heterozygous duplication c) 751.24 kbp hemizygous duplication	a) 9p13.1p11.2 b) 21p11.2 c) Xq11.1
7	25+0	46,XY	18.10	no	arr[GRCh38] 15q26.2(96079510_96240348)x1	160.84 kbp heterozygous deletion	15q26.2
8	21+0	46,XY	9.31	yes	clinically inconspicuous	clinically inconspicuous	-
9	21+3	46,XY	25.90	yes	arr[GRCh38] 11q11q12.1(54667246_56129373)x3	1.46 Mbp heterozygous duplication	11q11q12.1
10	17+6	46,XY	10.00	yes	arr[GRCh38] 2p14(65365163_67424875)x1	2.06 Mbp, heterozygous deletion	2p14
11	26+6	46,XY	24.50	yes	arr[GRCh38] 5q23.1(117109109_118989611)x3	1.88 Mbp, heterozygous duplication	5q23.1
12	22+4	46,XX	92.60	yes	arr[GRCh38] 7q36.3(157348614_157619299)x3	271 kbp heterozygous duplication	7q36.3
13	22+2	46,XX	42.40	yes	arr[GRCh38] 22q11.21(18965499_21151128)x1	2.18 Mbp heterozygous deletion; region critical for DiGeorge syndrome.	22q11.21
14	16+0	46,XY	23.50	yes	clinically inconspicuous	clinically inconspicuous	-
15	21+0	46,XY	13.30	yes	arr[GRCh38] 7q34(143307663_143342026)x3	34.36 kbp heterozygous duplication	7q34
16	20+6	46,XY	9.88	yes	arr[GRCh38] 11q13.2q13.3(68481380_68777703)x3	296.32 kbp heterozygous duplication	11q13.2q13.3
17	24+6	46,XY	32.10	yes	arr[GRCh38] 1.13(81486739_81709268)x3, 9p23(12046864_12405079)x1, 16q23.1(77811253_78258887)x3	a) duplication b) deletion c) duplication	a) 8q21.13 b) 9 p23 c) 16q23.1
18	17+0	46,XX	9.08	yes	clinically inconspicuous	clinically inconspicuous	-
19	19+6	46,XY	15.95	yes	arr[GRCh38] 2p21(44303991_44390279)x1	86 kbp heterozygous deletion	2p21
20	21+5	46,XX	11.70	yes	arr[GRCh38] 1q21.3q22(155020146_155443013)x3	423 kbp heterozygous duplication	1q21.3q22
21	25+5	46,XY	11.72	yes	arr[GRCh38] 17q12(36110957_37829126)x1	1.7 Mbp heterozygous deletion	17q12
22	26+0	46,XX	14.15	yes	arr[GRCh38] 7p12.3(47759533_48508753)x1, Xp11.23(48561980_49130452)x3	a) 749 kbp heterozygous deletion b) 568 kbp heterozygous duplication	a) 7p12.3 b) Xp11.23
23	22+6	46,XX	22.00	yes	arr[GRCh38] 22q11.22q11.23(22916059_23312035)x1	396 kbp heterozygous deletion	22q11.22q11.23
24	23+5	46,XY	23.40	yes	arr[GRCh38] 1q31.1(186040959_186338412)x3	297 kbp heterozygous duplication	1q31.1
25	18+1	47,XX,+21 (free T21)	11.75	yes	array canceled because Trisomy 21 identified in karyogram		Trisomy 21 visible in aCGH

Note: GA = gestational age in weeks, aCGH = array comparative genomic hybridization, CNV = copy number variant

The analysis of the cff-DNA samples' quality control measurements is shown in Table 3.7, which summarises the results of the passed and failed QC metrics. The CytoGenomics Software (Agilent) provides 26 metrics, out of which 12 are considered particularly important and are generally displayed in the QC report. Samples with cff-DNA concentrations above 15 ng/µl (360 ng total cff-DNA/reaction) usually pass the QC report, with most of them failing only one additional QC metric, the Standard Deviation of Log Ratio, which indicates a higher degree of variability. Samples with cff-DNA concentrations higher than 20 ng/µl typically pass all 26 QC metrics, except for case 14. However, when the concentration drops towards 10 ng/µl, additional QC values such as Derivative Log Ratio spread and Log Ratio Imbalance fail the QC range, and when it falls even further below, noise increases, making analysis impossible.

Figures 3.13 and 3.14 compare the aCGH results of cases 19 and 24. Both cases show a clear CNV with low overall noise, and one artefact excluded. In contrast, Figure 3.15 displays higher noise and more artefacts, particularly in regions where Agilent probes are known to generate higher noise signals. The log (2) (sample / reference) expresses the copy number variations (CNV) between the patient sample and the reference DNA. The blue lines indicate a heterozygous duplication (0.58), and the red line represents a heterozygous deletion (-1), but the actual data is slightly compressed from ideal values. The green rectangle marks the CNVs reported in the routine prenatal report, while the yellow rectangle highlights the identified artefacts. In gestational age 19+6, the expected CNV for case 19 is a putative 86 kbp heterozygous deletion, visible in Figure 3.13.A. The concentration of cff-DNA was moderate with 15.95 ng/µl, and the total cff-DNA input was 382.8 ng (Table 3.6). The additional QC value Standard Deviation of Log Ratio failed. However, the artefact was not visible in the gf-DNA results in Figure 3.13.B and could be excluded by the standard workflow. Figure 3.13.C shows a close-up gene view comparison of the probes of the reported heterozygous deletion, which reveals a similar probe distribution between cff-DNA and gf-DNA.

Case 24, with a concentration higher than 20 ng/µl and no failed QC values, exhibits a similar pattern, as demonstrated in Figure 3.14. In Figure 3.14.A, a single artefact is visible that is composed of only three probes and can be easily excluded from the analysis when compared to the gf-DNA results in Figure 3.14.B. Figure 3.14.C again displays a similar number and distribution of probes between cff-DNA and gf-DNA. The 297 kbp heterozygous duplication was

Table 3.7: Quality control metrics for Array-CGH analysis of cff-DNA.

Case No	GA	Conc. Qubit (ng/µl)	cff-DNA Vacuum Conc.?	QC Report "selected important values"	All QC values	Failed QC values
1	19+1	35.8	no	pass	pass	-
2	23+5	27.6	no	pass	pass	-
3	24+1	35.6	no	pass	pass	-
4	27+2	48.0	no	pass	pass	-
5	21+3	15.6	no	pass	1/26	StdDevLR
6	24+2	16.6	no	pass	1/26	StdDevLR
7	25+0	18.1	no	pass	1/26	StdDevLR
8	21+0	9.31	yes	pass	1/26	StdDevLR
9	21+3	25.9	yes	pass	pass	-
10	17+6	10.1	yes	pass	1/26	StdDevLR
11	26+6	24.5	yes	pass	pass	-
12	22+4	92.6	yes	pass	pass	-
13	22+2	42.4	yes	pass	Pass	-
14	16+0	23.5	yes	Pass	1/26	StdDevLR
15	21+0	13.3	yes	1 value	2/26	StdDevLR DerivativeLR spread
16	20+6	9.9	yes	2 values	4/26	StdDevLR DerivativeLR spread DerivativeLR spread norm
17	24+6	32.1	yes	pass	pass	- LogRatioImbalance
18	17+0	9.08	yes	2 values	4/26	StdDevLR DerivativeLR spread DerivativeLR spread norm
19	19+6	16	yes	pass	pass	- LogRatioImbalance
20	21+5	11.7	yes	1 value	3/26	StdDevLR DerivativeLR spread DerivativeLR spread norm
21	25+5	11.7	yes	pass	pass	-
22	26+0	14.2	yes	pass	1/26	StdDevLR
23	22+6	22.0	yes	pass	pass	-
24	23+5	23.4	yes	pass	pass	-
25	18+1	11.8	yes	pass	1/26	StdDevLR

Note: GA = gestational age in weeks, aCGH = array comparative genomic hybridization, StdDevLR = Standard Deviation of Log Ratio, DerivativeLR spread = Derivative Log Ratio (LR) spread, DerivativeLR spread norm = Derivative Log Ratio spread normalized.

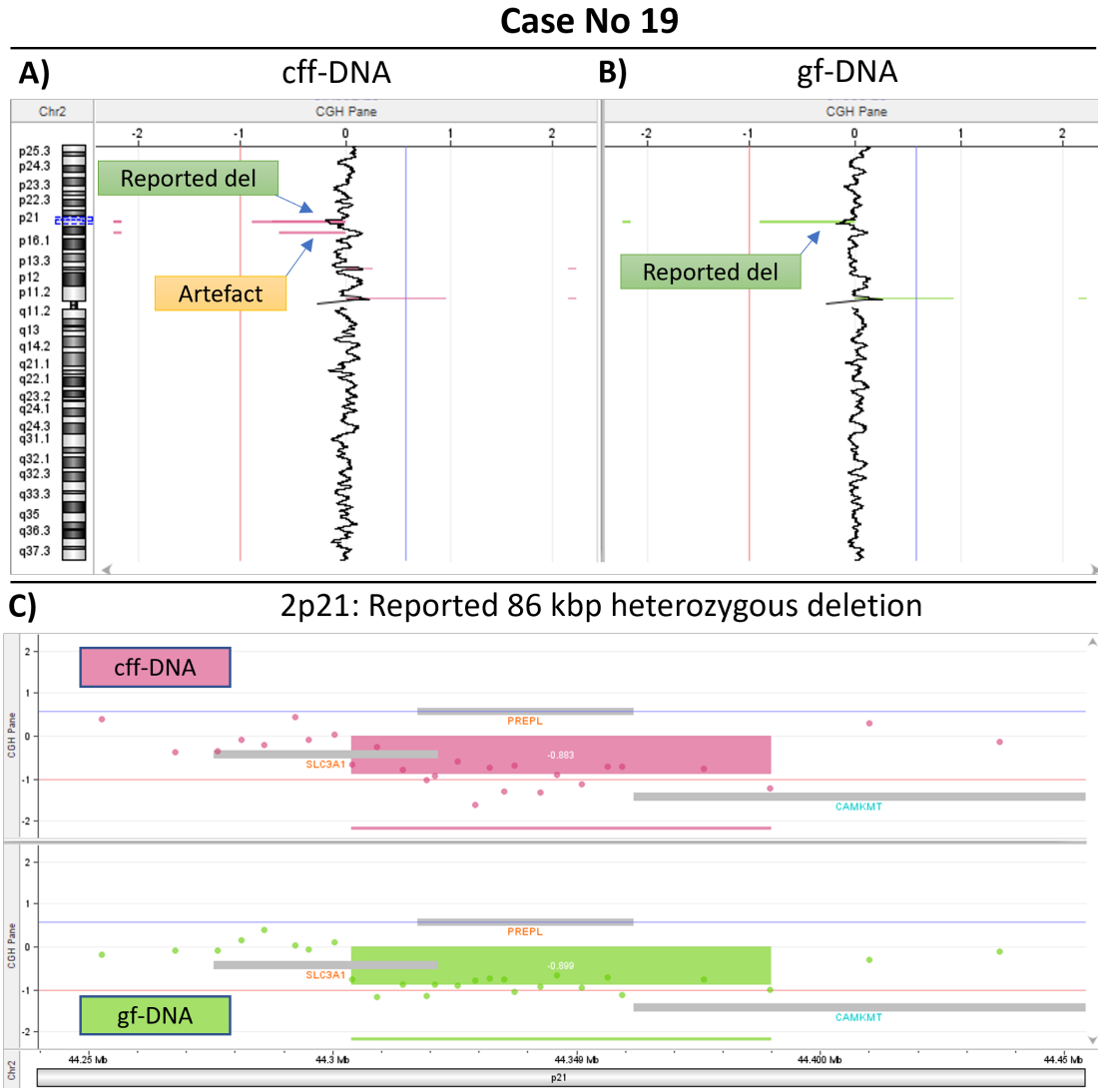


Figure 3.13: Array-CGH analysis of cff-DNA gf-DNA identifies concordant 86-kb heterozygous deletion in chromosome 2p21 at gestational age 19+6.
The aCGH analysis on cff-DNA, with a concentration of 15.95 ng/µl (total cff-DNA of 382.8 ng/reaction), presents Copy Number Variation (CNV) data expressed as log2 (Sample/reference). Duplications are represented by a blue line (0.58), and heterozygous deletions by a red line (-1). Green rectangles highlight CNVs reported in the prenatal analysis, while yellow rectangles denote identified artefacts. **A)** Chromosome 2 view of cff-DNA aCGH reveals an 86-kilobase (kbp) heterozygous deletion in the p21 region, with one excluded artefact. **B)** Corresponding aCGH results for gf-DNA. **C)** Comparative gene-level view of probes associated with the reported heterozygous deletion, demonstrating similar probe distribution between cff-DNA and gf-DNA.

accurately identified in cff-DNA.

Case 20 had a concentration of 11.7 ng/µl, a total cff-DNA input of 280.8 ng, and an increased Derivative Log Ratio spread normalised. In this case, more artefacts were called than with gf-DNA. The artefacts can be observed in Figure 3.15.B. However, it should be noted that not all chromosomes exhibited the same amount of noise as depicted in the image. Furthermore, the probe distribution of the reported 423 kbp heterozygous duplication on chromosome 1 in the region of q21.3q22 was similar in number and distribution to that of gf-DNA, as seen in Figure 3.15.C.

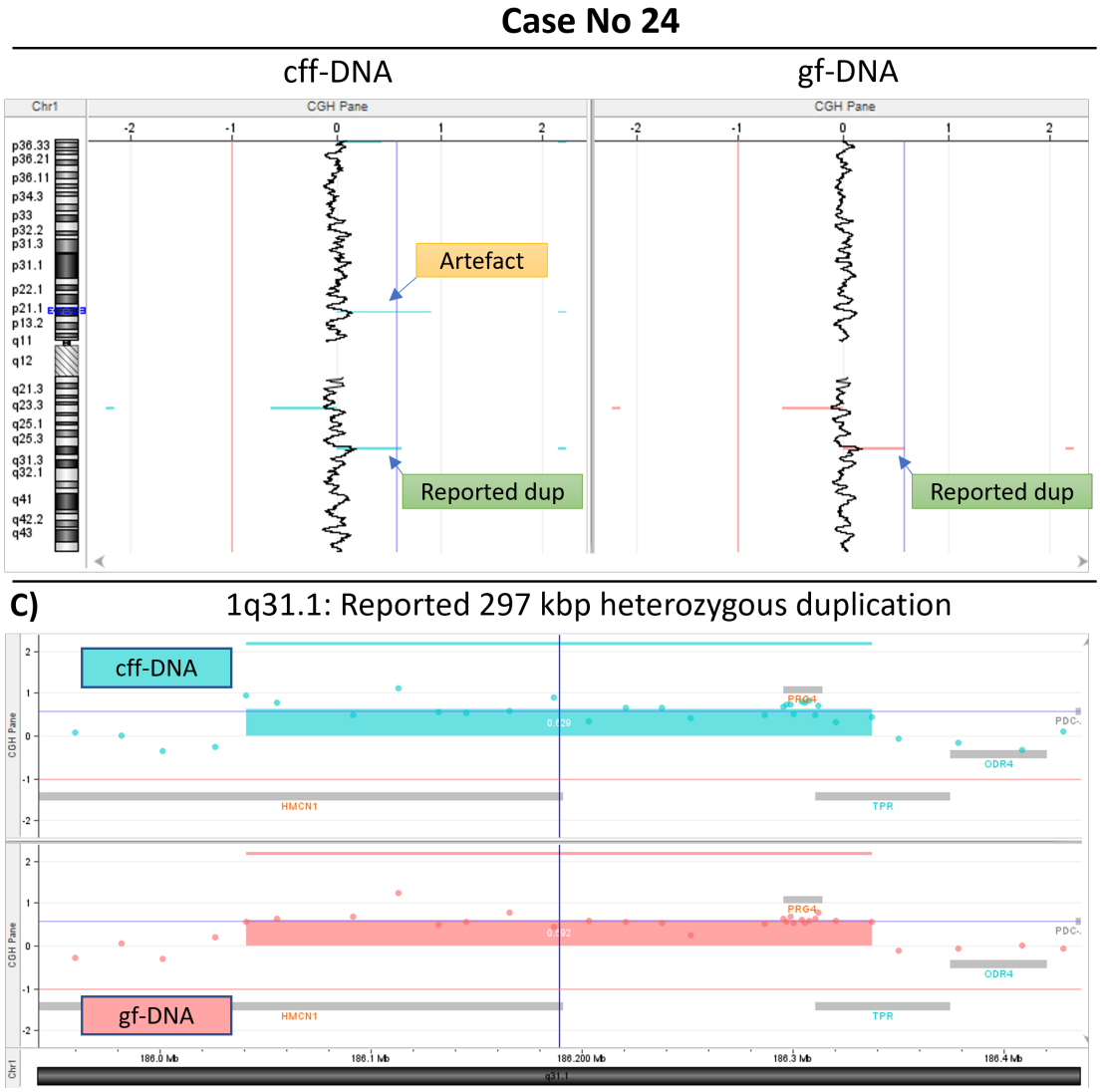


Figure 3.14: Array-CGH analysis with cff-DNA accurately detects a 297 kbp heterozygous duplication in chromosome 1q31.1
cff-DNA (23.4 ng/µl, total 561.6 ng/reaction) underwent log2 CNV analysis. Blue line: duplication (0.58), red line: heterozygous deletion (-1). Green rectangles highlight CNVs, yellow rectangles mark artefacts. **A)** Chromosome 1 aCGH shows a 297-kbp heterozygous duplication in q31.1, with one excluded artefact. **B)** Corresponding gf-DNA results. **C)** Gene-level view displays similar probe distribution between cff-DNA and gf-DNA

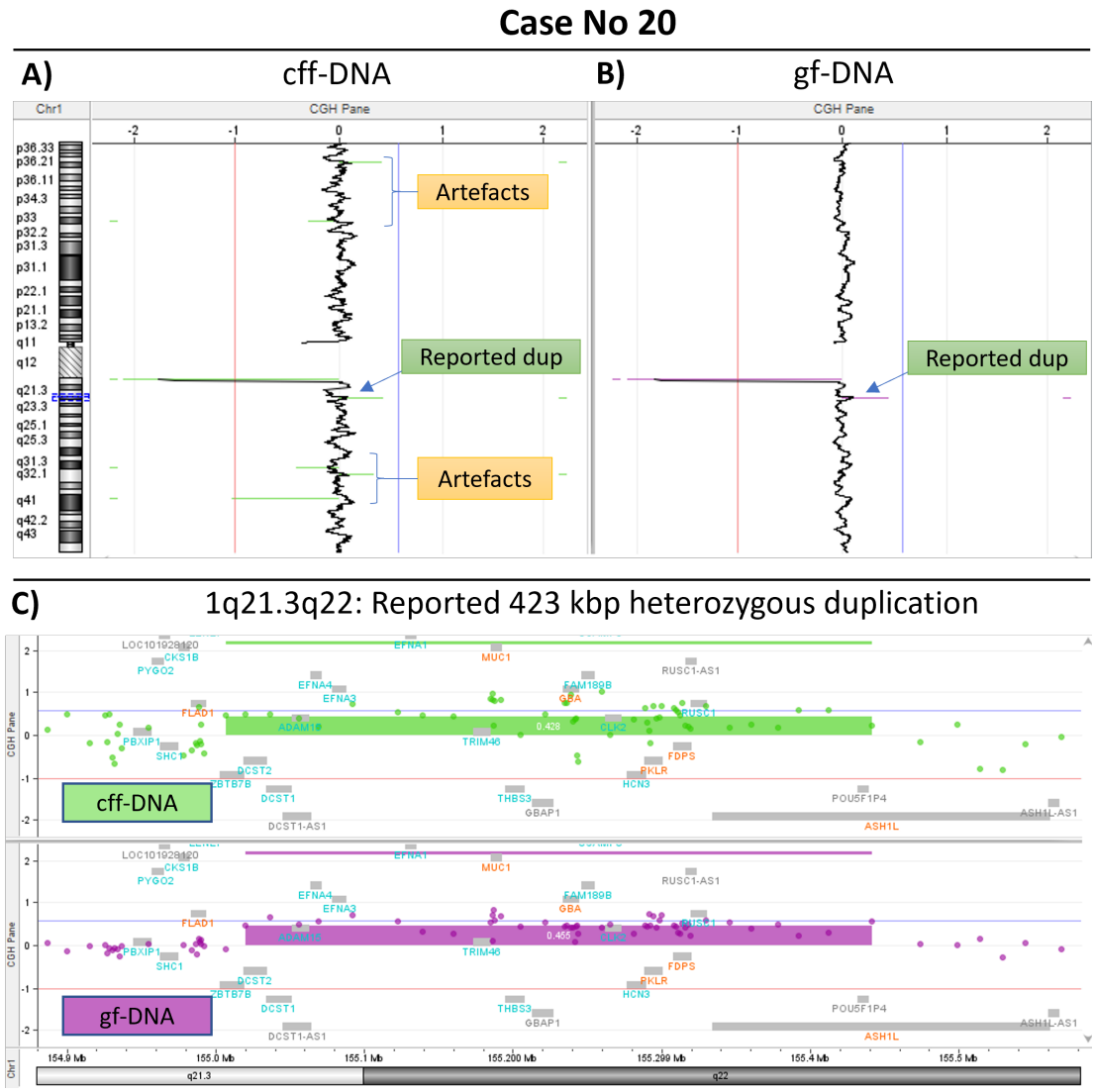


Figure 3.15: Array-CGH analysis with cff-DNA detects a 423-kbp heterozygous duplication in chromosome 1q21.3q22.
The cff-DNA with a concentration of 11.7 ng/µl (total cff-DNA of 280.8 ng/ reaction) underwent copy number variation (CNV) analysis expressed as log(2) (Sample/reference). The blue line denotes a heterozygous duplication (0.58), while the red line represents a heterozygous deletion (-1). CNVs included in the routine prenatal report are marked with a green rectangle, while artefacts are marked with a yellow rectangle. **A)** The aCGH chromosome view for chromosome 1 shows a 423-kilobase (kbp) heterozygous duplication in the q21.3q22 region. Various artefacts are identified and excluded. **B)** The aCGH results for the corresponding gf-DNA. **C)** A close-up gene view comparison of the probes of the reported heterozygous duplication reveals that the probe distribution is similar between cff-DNA and gf-DNA.

3.7 Whole Exome Sequencing

The study aimed to evaluate the effectiveness of using cell-free fetal DNA (cff-DNA) from amniotic fluid for whole-exome sequencing (WES) in prenatal diagnostics. The research investigated whether cff-DNA could detect the same variants as genomic fetal DNA (gf-DNA) extracted directly from amniotic fluid or cell culture. The impact of vacuum concentration on the WES analysis was also studied, particularly for early gestational age. Additionally, the study tested whether cff-DNA could be used in case of possible maternal cell contamination that often interferes with proper diagnosis if the mother is analysed instead of the fetus. For this purpose, one visibly contaminated sample (WES 1) was analysed to check if maternal DNA was mixed with fetal DNA. The study found no significant contamination of maternal DNA in the cff-DNA WES samples.

The cff-DNA was collected and extracted using a standard protocol, and the samples were vacuum-concentrated if the cff-DNA concentration was below 15 ng/µl. The library concentration of the samples before and after target enrichment was measured using Qubit. The study observed that all samples with a high starting concentration led to sufficient target enrichment, regardless of whether they were vacuum-concentrated. However, one sample had a low starting concentration and a significantly low library concentration, making it unsuitable for sequencing and was excluded from further analysis. The Twist Exome panel 2.0 was used for all cff-DNA runs, except for one sample where the older Twist Exome panel 1.0 was used for the original gf-DNA run.

Table 3.8: cff-DNA concentrations before and after library preparation for Whole Exome Sequencing. Low concentrations are highlighted in red *.

Case No	GA	Concentration before VC	Concentration after VC (ng/µl)	Concentration after Library Prep/ Target Enrichment (ng/µl)
WES 1	20+2	15.15	-	94.20
WES 2	19+6	8.30	22.70	56.50
WES 3 Fet I	18+6	6.40	14.70	79.00
WES 4 Fet II	18+6	5.84	19.60	85.80
WES 5	16+4	3.65	13.55	86.50
WES 6*	**15+3**	**0.39**	**3.6**	**2.13**
WES 7	21+3	15.90	-	113.50
WES 8	19+5	18.35	-	108.00
Wes 9	23+1	5.99	17.50	107.00
WES 10	18+2	6.78	-	96.60
Control	-	15.80	-	58.00

Note: GA = gestational age in weeks, VC = vacuum concentration.

3.7.1 Sequencing and Mapping Quality Statistics

According to established NGS practice guidelines and internal recommendations, Table 3.9 presents essential quality control (QC) metrics to ensure accurate and reliable data analysis. The results of the cff-DNA and gf-DNA samples show high Q30 scores, indicating dependable data for further analysis. However, using different reference genomes (hg19 for gf-DNA WES runs and hg38 for cff-DNA) has led to genome size variations, affecting some specific metrics. Median insert size, alternate allele frequency, zero coverage targets, and coverage rate are all crucial QC metrics. Median target coverage is an essential metric for assessing the depth of coverage across target regions, and it falls within an acceptable range for reliable variant calling. However, it fluctuates in all WES runs. It is important to note that some gf-DNA WES samples did not undergo prior QC and are marked with an X in the table. Additionally, one of the runs used Twist Exome Panel 1.0 instead of 2.0 for the library prep workflow. Despite noticeable differences when comparing cff-DNA and gf-DNA samples within each WES run, a balance was struck to minimise duplicate reads and ensure adequate coverage. All samples obtained good quality scores, regardless of gestational age and vacuum concentration. After filtering for high-quality pass-filter (PF) reads, the total number of reads may vary across different WES runs due to library preparation and sequencing efficiency inherent to the sequencing process.

Table 3.9: WES quality control metrics.

		Q30 (%)	Genome Size	Total Reads (PF)	Duplicate Rate	Median Target Coverage
WES 1	cff-DNA	93.40	3,215,250,450	137,642,108	10.67	113
	gf-DNA	93.90	3,215,250,450	129,815,120	21.12	87
WES 2	cff-DNA	93.30	3,215,250,450	100,899,072	10.57	83
	gf-DNA		3,217,346,917	137,307,224	4.39	87
WES 3 Fet I	cff-DNA	93.40	3,215,250,450	79,884,310	9.22	61
	gf-DNA		3,215,250,450	112,818,722	14.76	74
WES 4 Fet II	cff-DNA	93.30	3,215,250,450	102,492,082	9.79	86
	gf-DNA		3,217,346,917	117,128,184	5.74	73
WES 5	cff-DNA	93.20	3,215,250,450	97,513,276	9.89	78
	gf-DNA		x	x	x	x
WES 6	not sequenced					
WES 7	cff-DNA	93.40	3,215,250,450	82,383,588	8.17	71
	gf-DNA		x	x	x	x
WES 8	cff-DNA	93.50	3,215,250,450	111,105,254	9.28	93
	gf-DNA		3,217,346,917	138,352,878	25.46	88
Wes 9	cff-DNA	93.30	3,215,250,450	100,536,842	9.58	84
	gf-DNA		3,217,346,917	116,985,154	20.62	79
WES 10	cff-DNA	93.30	3,215,250,450	91,701,116	8.65	72
	gf-DNA	92.40	3,217,346,917	123,768,494	5.18	77
Control		93.50	3,215,250,450	72,788,456	8.08	61

		Uniformity of Coverage - Fold 80 Base Penalty	Median Insert Size	Alternate Allele Frequency (< 30)	Zero Coverage Targets	Coverage Rate (> 20X)
WES 1	cff-DNA	1.15	1.44	3.04	97.94%	163
	gf-DNA	1.00	1.37	2.40	97.99%	218
WES 2	cff-DNA	1.39	1.54	2.91	97.43%	144
	gf-DNA	4.01	1.67	2.04	94.15%	217
WES 3 Fet I	cff-DNA	1.32	1.46	2.97	97.09%	154
	gf-DNA	1.04	1.34	2.20	97.95%	289
WES 4 Fet II	cff-DNA	1.32	1.38	2.76	97.73%	163
	gf-DNA	4.11	1.41	2.38	94.23%	206
WES 5	cff-DNA	1.41	1.43	3.28	97.43%	141
	gf-DNA	x	x	x	x	x
WES 6	not sequenced					
WES 7	cff-DNA	1.23	1.42	2.68	97.39%	163
	gf-DNA	x	x	x	x	x
WES 8	cff-DNA	1.16	1.44	2.95	97.79%	159
	gf-DNA	3.81	1.38	1.73	94.29%	208
Wes 9	cff-DNA	1.22	1.45	2.63	97.66%	158
	gf-DNA	3.71	1.38	2.06	94.25%	241
WES 10	cff-DNA	1.24	1.42	5.07	97.63%	176
	gf-DNA	4.10	1.41	2.26	94.33%	201
Control		1.22	1.34	3.08	97.67%	241

3.7.2 Variant detection and comparison

After performing sequencing and quality control, BWA mapped the resulting FASTQ files to the reference genome (HG38). Variant calling was performed with GATK, and functional annotation, visualisation, and clinical evaluation were performed using commercial software VarSeq, which employed phenotype-driven approaches with Human Phenotype Ontology (HPO) terms and genotype-driven approaches.

Whole-exome sequencing (WES) was performed on cff-DNA from 10 patient samples, of which 9 were sequenced, and the results were compared with the corresponding gf-DNA analysis. Assoc. Prof. Priv. Doz. Dr. med. Franco Laccone conducted the initial evaluation using the same filter setting as the original gf-DNA run.

A discrepancy was observed in one patient's sample (WES 5), where gf-DNA was extracted from cell culture. The gf-DNA analysis initially reported two variants, but the cff-DNA analysis identified only one. This discrepancy prompted further investigation, and it was discovered that the missing variant might have been an artefact arising from cell culture. Among the 9 sequenced samples, WES detected all actual variants, showing a high level of agreement between cff-DNA and gf-DNA analyses regardless of whether the cff-DNA was vacuum concentrated due to low starting concentration or early gestational age. The detected variants are comprehensively summarised in Table 3.10. It should be noted that no CNV analysis has been conducted yet.

Table 3.10: Comparison of genetic variants detected during Whole Exome Sequencing (WES) in 10 prenatal samples using cell-free DNA (cff-DNA) and genomic fetal DNA (gf-DNA). Samples were collected at different weeks of gestation, and gf-DNA was extracted directly or via culture. The table presents potentially clinically relevant gene variants detected in each sample using gf-DNA, along with a comparison to cff-DNA. All variants, except one (LUZP1 c.1771_1773delGAT in WES 5), were concordant between cff-DNA and gf-DNA WES results.

Sample ID	Week of Gestation	Extraction Material for Genomic Fetal DNA	Expected Gene Variants Detected	Comparison of cff-DNA vs gf-DNA Variants
WES 1	20+2	AC	*KIF4A* c.2603A>G, *FOXF1* c.710C>T	Consistent
WES 2	19+6	AC	*GLB1L3* c.433G>A, *SMARCAL1* c.2542G>T	Consistent
WES 3 Fet I	18+6	AC-culture	*TNNT2* c.40G>T, *DMWD* c.86G>T de-novo, *PLEC* c.5236C>T, *PLEC* c.1130T>G, *TRIT1* c.334delC	Consistent
WES 4 Fet II	18+6	AC-culture	*TNNT2* c.40G>T, *DMWD* c.86G>T de-novo, *PLEC* c.5236C>T, *PLEC* c.1130T>G, *TRIT1* c.334delC	Consistent
WES 5	16+4	AC-culture	*OR8J3-2* c.682A>T *, *LUZP1* c.1771_1773delGAT	**OR8J3-2 c.682A>T identified as artefact LUZP1 in cff-DNA*
WES 6	15+3	AC-culture	*NPHP3* c.3824_3826delGAG, *NPHP3* c.9_10insT, *RECQL4* 1 573delT (mat)	Not sequenced
WES 7	21+3	AC	Clinically inconspicuous	Consistent
WES 8	19+5	AC	*CC2D2A* c.4553G>A, *EARS2* c.322C>T, *PIGN* c.2371-1G>T	Consistent
WES 9	23+1	AC-culture	*KANSL1* c.611delG (de-novo), *FOXQ1* c.1109C>G, *TSEN54* c.1039A>T	Consistent
WES 10	18+2	AC-culture	Clinically inconspicuous	Consistent

Discussion

Timely decision-making and appropriate pregnancy management depend on fast turnaround times in prenatal diagnostics. In cases where fetal abnormalities are detected, quick diagnoses can help parents and healthcare providers make informed decisions about the next steps, such as whether to continue the pregnancy or consider termination. Furthermore, a fast turnaround time can alleviate the anxiety and stress associated with waiting for test results, which can significantly impact the mental health and well-being of the parents [3], [4]. The Medical University of Vienna employs various molecular methods, including Sanger sequencing, Multiplex Ligation-Dependent Probe Amplification, Array Comparative Genomic Hybridization, and Whole Exome Sequencing, in addition to conventional Karyotyping. However, these molecular methods require high-quality DNA and, for some methods, high quantity. If possible, gf-DNA is obtained from non-cultivated cells from amniotic fluid or cultivated cells from amniotic fluid. However, if cell culture is required, waiting time can be prolonged to at least two weeks and, in most cases, 4-6 weeks. In the worst case, there could also be a cell-culture failure, leading to the need for a possible re-puncture. The waiting time can be excruciatingly long and painful for the expectant parents. Reasons for not directly extracting gf-DNA include early gestational age < 20 weeks, all amniocytes being used for cell culture since only a small amount is available, or maternal cell contamination. Bianchi et al. (2001) were the first to show that amniotic fluid supernatant samples contain a large amount of cff-DNA, which is 100-200 times more than the maternal plasma per millilitre of amniotic fluid [50]. Since then, the use of cff-DNA in amniotic fluid as an alternative source of fetal genetic material has been neglected mainly due to the emergence of cell-free fetal DNA in maternal plasma, enabling the development of a non-invasive prenatal diagnostic test (NIPT). Despite its advantages, the potential of AF has yet to be fully explored for applications beyond prenatal diagnosis of aneuploidy. Unlike cell-free fetal DNA from maternal plasma, AF is a relatively pure fetal sample not contaminated by maternal- and trophoblast-derived nucleic acids. The placental origin of circulating cell-free fetal DNA in plasma can lead to false negatives and positives in NIPT results in cases of discordance between placental and fetal tissues [51]–[53]. The study aimed to investigate the properties of cff-DNA from amniotic fluid supernatant and its suitability as a reliable source of genetic information. The goal was to establish its use in a clinical prenatal diagnostic setting when traditional gf-DNA extraction faces challenges and establish a reliable and robust workflow that can be transferred to routine diagnostics.

4.1 Fragmentation pattern and correlation of cff-DNA concentration to gestational age in euploid fetuses

Investigations into the biophysical properties of cff-DNA have been conducted. Analysis of the fragment pattern using the TapeStation System by Agilent revealed a correlation between fragment size and gestational age [54]. It was found that the cff-DNA fragments were longer than expected. Initially, the fragment sizes were thought to be similar to circulating cell-free DNA, which consists of a mononucleosome and its multimers. While the length of circulating cell-free DNA varies based on the tissue, it typically ranges from 100-300 bp, with a peak at 167 bp. This length corresponds to the DNA wrapped around a single nucleosome plus a short stretch of 20 bp (linker DNA) bound to a histone H1 [54]. Fetal-specific end sites were mainly located at the border or within the nucleosome core, leading to the trimming of approximately 20 bp linkers. On the other hand, maternal-specific end sites were primarily located in the linker region [55]. Both maternal and fetal cell-free DNA (cfDNA) showed a series of 10-bp periodicities in the smaller cfDNA molecules, with the fetal cfDNA having increased amplitudes across the periodicities. This periodicity suggests the DNA helix interacting with the nucleosome core at 10 bp per turn. It indicates that the fragmentation of cfDNA occurs in association with nucleosome structures related to the tissue of origin [56]–[58]. The small fragment size was a limitation in diagnosing genome-wide single gene disorders or SNPs, being limited to one gene or complex repeat expansion like Fragile X syndrome. However, recent reports have found large circulating cell-free DNA fragments increasing in size as gestational age advances in maternal plasma, possibly due to increased degradation stability of the placenta through sequencing with Nanopore (ONT) [59].

Even though long fragments of cell-free fetal DNA (cff-DNA) have been observed in amniotic fluid even at early gestational ages, they display a prominent peak between 150-180 base pairs (bps), as expected. As the gestational age increases, the fragment size increases in signal intensity and thus predominance. No distinct peak for small fragment size is seen anymore, but more of a plateau up to 1500 bp, with even larger fragment sizes visible.

The fragmentation pattern of cell-free fetal DNA (cff-DNA) in amniotic fluid is distinct

from that in maternal plasma and serum, possibly due to the different sources of the cff-DNA. Maternal plasma contains cff-DNA, which originates from the placenta, whereas cff-DNA in amniotic fluid originates directly from fetal cells. The syncytiotrophoblast is the primary source of circulating cff-DNA in maternal plasma, which is continuously released through apoptosis with minimal contribution from the fetal hematopoietic system. The stability of circulating cff-DNA is attributed to their association with placenta-derived microparticles, which protect them from nuclease degradation. The source of genetical material for circulating cell-free fetal DNA in plasma is not only a drawback in fragment size for diagnosis but also due to the source of the material [51], [60]. Confined placental mosaicism is a condition that can cause false-positive results when using NIPT. This is because the cell-free fetal DNA found in the mother's blood plasma comes from the trophoblast of the blastocyst, which is different from the inner cell mass that (epiblast) forms the fetus. Chromosomal mosaicism can occur due to post-zygotic mitotic division errors, leading to discordance. This condition is found in around 1% to 2% of cases, with monosomy X and trisomy 13 being more common than trisomy 21 or 18. False-negative results can also occur when there is true fetal mosaicism, where the fetus has a chromosomal abnormality, but the results of the cfDNA test indicate otherwise. Vanishing twins can also contribute to discordant cases, leading to false-positive reports. Since chromosomal abnormalities are a leading cause of miscarriages, trisomies could be the reason for vanishing twins, which could lead to a potentially high number of false-positive results if the analysed cff-DNA is from the vanishing twin [59], [61].

cff-DNA can be found in amniotic fluid through various mechanisms. The fetal kidneys can excrete it or come from local degradation or permeation through non-keratinised fetal skin. As the fetus develops its organs, the immature fetal kidney can release longer fragments of cff-DNA into the amniotic fluid. Other potential sources of DNA include blood cells, direct degradation of the placenta, membranes, skin, and organs undergoing apoptosis with direct contact with the amniotic fluid. For example, during the second trimester, fetal lung fluid is in direct contact with the amniotic fluid as the lung is actively developing and remodelling [50]–[52].

The distinct fragmentation pattern and source of cff-DNA in amniotic fluid, characterised by larger fragment sizes even in earlier gestational age, is a significant advantage for prenatal diagnostics compared to circulating cell-free DNA in plasma. The longer fragments make cff-DNA

suitable for genome-wide detection of point mutations and CNVs, such as NGS-based WES and Sanger sequencing. Results are not biased by the discordance of the source material. It is also suitable for most PCR-based methods, provided the fragment size is considered. MLPA reactions result in PCR amplicons ranging from 64-500 nt in length, and hybridisation methods like Array-CGG should not use fragment sizes above 1000 bp for efficient hybridisation. Therefore, single-gene conditions, including X-linked, recessive, and dominant conditions, should be diagnosable in principle, and CNV analysis for microdeletion/microduplication syndromes should be achievable in most cases. However, optical genome mapping such as Bionano, which detects genome-wide structural variants, is impossible as it requires ultra-high molecular weight (UHMW) DNA. It is impossible to make assumptions about repeat expansion diseases as they have yet to be tested. However, long-read sequencing such as Nanopore (ONT), which allows for both short and long-read sequencing and calling methylation without the need for PCR, could be tested, for example, for Fragile X syndrome. Alternatively, Nanopore could be utilised further to explore fragment patterns of cff-DNA in amniotic fluid, identifying the limit of fragment size or SNP calling.

The correlation between gestational age in euploid fetuses and cff-DNA concentration was investigated via quantitative Polymerase Chain Reaction (qPCR) in 137 fetal cff-DNA samples. Amplification primer targeted the *GAPDH* locus with a target region of 291 bp, lying in a fragmented range even available during early gestational age according to the TapeStation results, thereby making sure amplification through several weeks of gestation is not biased due to fragment size. We observed a correlation between gestational age and cff-DNA concentration in amniotic fluid, similar to a Gaussian curve. However, the limited sample size in later weeks (GA 27-34) requires cautious interpretation, which requires further research to elucidate the significance of this observation. Due to the significant attention given to circulating cell-free DNA in plasma about gestational age, obesity, and disease [62]–[65], no research has been done to explore the correlation between cff-DNA in amniotic fluid and gestational age across all gestation weeks. Lapaire et al. (2007a) reported a correlation between gestational age in fresh amniotic samples, but not in frozen samples stored at −80 °C, and cff-DNA when they used qPCR targeting *GAPDH* [66] in 39 euploid and 4 aneuploid pregnancies, thus reinforcing our findings.

Furthermore, the group of Lapaire et al. (2007a) in their preliminary study investigating 39 euploid and 4 aneuploid pregnancies, followed by their follow-up study by Peter et al. (2008)

of 36 euploid and 29 aneuploid fetuses, archived frozen euploid AF samples gradually lose long cff-DNA fragments and that this loss accurately distinguishes them from the fresh samples [66], [67]. In this experiment, cff-DNA was extracted from 5 ml amniotic fluid supernatant taken on consecutive days by the same operator, followed by qPCR one week later to reduce technical bias. The amniotic fluid was quickly spun at 300 xg for 10 minutes upon arrival and then stored at 20°C for up to one year. The second centrifugation step at 3234 xg for 10 minutes was done before cff-DNA extraction. Although abnormalities were seen in preliminary tests where old stored at -20°C amniotic fluid was used without the first centrifugation step to remove cells from the amniotic fluid upon arrival, no significant change in the correlation was observed in the preliminary experiment when frozen amniotic fluid supernatant was used. However, further studies focusing on cff-DNA concentration and fragmentation about storage with a statistical analysis need to be conducted in the future. Due to observed low concentration during early gestational age below 20 weeks, we suggest that all available amniotic fluid supernatant should be extracted routinely in nuclease water to avoid salt residues in order to obtain a higher concentration of cff-DNA for molecular methods such as Array-CGH and WES. This concentration step is likely to be necessary.

4.2 Screening for maternal cell contamination in cff-DNA via STR-markers and mitigating maternal cell contamination

Prenatal diagnosis currently involves testing material obtained through amniocentesis sampling since the procedure may lead to contamination of the sample with maternal cells, which can cause misdiagnosis. It is thus essential to identify the presence and extent of maternal cell contamination (MCC) in a prenatal sample, as this may affect sample processing and result interpretation. MCC of amniotic fluid (AF) is usually caused by the presence of maternal blood cells that lyse during DNA extraction, introducing maternal gDNA into the sample. MCC is usually indicated by blood-staining of the AF and was associated with an increased risk for MCC $> 5\%$ [68]. Maternal lymphocytes in uncultured AF can cause significant issues but are rarely a problem for cultured amniocytes, as maternal lymphocytes are lost during the culture

process. Therefore, blood-stained samples are routinely excluded from direct gf-DNA extraction, and analysis needs to wait for cell culture. Suppose MCC is detected in uncultured AF without blood staining or in cultured amniocytes. In that case, it is likely due to the presence of small fragments of maternal tissue removed by the sampling needle during the amniocentesis. It is important to note that cells from maternal tissue fragments may proliferate during the culture process. To ensure accurate prenatal genetic testing, stringent measures must be taken to prevent MCC [69], [70]. Our research has demonstrated the effectiveness of using cell-free fetal DNA (cff-DNA) extracted from amniotic fluid for STR marker analysis in 132 patient samples. We have also found that centrifugation in the cff-DNA extraction process effectively reduces maternal cell contamination (MCC) in blood-tinged samples. Of the 132 samples, 41 had visible blood tinges, and STR-marker analysis showed no or minimal (<5%) MCC contamination. This is a novel approach, and cff-DNA presents an alternative approach in scenarios where maternal lymphocytes pose challenges to direct gf-DNA extraction, allowing for a timely diagnosis without waiting for cell culture and advancing patient outcomes. We further separated two cases of blood-stained AF samples: a) erythrocytes visible after centrifugation of an aliquot for FISH analysis in a 1.5 ml Eppendorf Tube, and b) heavily blood-tinged AF already visible in the syringe. However, further investigation is needed for efficient MCC reduction in blood-stained syringes, as only 6 samples were available for analysis. To determine if there was maternal lymphocyte contamination, future research involves extracting gf-DNA from the cell pellet and comparing it to the STR-Marker analysis of cff-DNA. Additionally, the influence of processing time needs to be further explored, as it was observed in preliminary experiments that freezing the potentially contaminated AF before removing all cell residues or extended storage time in the fridge (> 1 week) can affect the results likely due to lysis of the maternal cells.

4.3 Implementing and validating cff-DNA for prenatal diagnostic methods

We successfully implemented reliability in the use of cff-DNA in various prenatal analysis methods, including Sanger sequencing, Multiplex Ligation-Dependent Probe Amplification (MLPA),

Array Comparative Genomic Hybridization (Array-CGH), and Whole Exome Sequencing (WES), for comprehensive prenatal genetic analysis using of-DNA. Using cff-DNA extracted from amniotic fluid allows for timely diagnosis, particularly when traditional cff-DNA extraction faces challenges, such as early weeks of gestation or blood-tinged amniotic fluid indicating maternal cell contamination or cell culture challenges.

4.3.1 Sanger sequencing with cff-DNA

Sanger sequencing, or the chain termination method, is a reliable way to validate variants identified through Next-Generation Sequencing (NGS) and can fill in any gaps in genomic regions that need to be better covered by NGS [71]. Developed by Frederick Sanger and colleagues in 1977, it is limited in throughput, processing 96-384 samples per run in 1-2 hours, and is expensive per base pair [71]. Over the past decade, there have been few improvements to Sanger sequencing, with the majority of the focus being on advancing NGS in research, resulting in few inventions to support or enhance Sanger sequencing [72], [73]. Despite these drawbacks, Sanger sequencing is still a valuable tool in prenatal diagnostics due to its accuracy and established effectiveness [71]. Despite newer technologies, Sanger sequencing is still valuable for prenatal diagnostics due to its precision and proven track record [71]. Even though Sanger sequencing was mainly used in this study to verify WES results, we have demonstrated that cff-DNA can be successfully employed to identify autosomal-dominant diseases and reliably used to detect hetero and hemizygous mutations and its use in segregation analysis with 100% concordance with genomic fetal DNA diagnosis. Fragment size was fine for Sanger sequencing, as the maximum sequence size is limited to 1000 base pairs. In cases where the gene can be directly amplified, no issues with cff-DNA were encountered. However, in some cases, a different approach is necessary for diagnosis. For example, the polycystic kidney is caused by biallelic hypomorphic variants in the *PKD1* gene, which is associated with adult-onset autosomal dominant polycystic kidney disease (ADPKD). The *PKD1* gene is approximately 14 kbs in length and requires long-range PCR amplification followed by nested PCR to amplify individual exons, followed by Sanger sequencing[74], [75]. In this scenario, a long cff-DNA fragment size would be required if the whole gene needs to be amplified. Even though we have observed longer pieces of cff-DNA and an increase in their

length as gestational age increases, it is uncertain if this size can be achieved and requires further exploration. Recently, we have already utilised cff-DNA for diagnosis in *PKD1* of a blood-tinged and early gestational age patient sample without amplifying the whole gene, as we had knowledge of the family history and the father was a carrier of a known pathogenic heterozygous mutation c.12691C>T (p.Gln4231T) associated with polycystic kidney disease (AD). With a known family history, cff-DNA still functions as an addition for early detection or indication in these cases.

4.3.2 Multiplex Ligation-depended Probe Amplification using cell-free fetal DNA from amniotic fluid

MLPA is a technique that can detect the relative copy number of up to 60 different DNA sequences (40 in 2022) in a single reaction. This method is useful in prenatal diagnosis for detecting aneuploidies, familial single-gene disorders, common microdeletion/duplication syndromes, sub-telomeric alterations, and imprinting disorders (MS-MLPA) in fetuses. Compared to other diagnostic methods such as FISH, karyotyping, and Array-CGH, MLPA is relatively low-cost and high-throughput, making it a preferred method in clinical settings. It is necessary to confirm copy number changes detected by only one probe using another method since a single probe can give misleading results. To avoid false positives, it is necessary to analyse the probe target sequence to establish whether mutations or polymorphisms are present. The Medical University of Vienna uses the MLPA technique to diagnose diseases caused by CNVs that affect entire exons for genes found appropriate for the test. For instance, in case number 3, Array-CGH identified a heterozygous duplication from the PAR1. This duplication involves a portion of the *CSF2RA* gene associated with autosomal recessive inherited alveolar proteinosis. The gene's partial duplication was confirmed using the MLPA SALSA Kit P329-B1 (MRC-Holland) and identified using cell-free DNA (cff-DNA). In case number 5, the MLPA data confirmed the presence of a heterozygous deletion in the fetus found via WES. MS-MLPA can detect abnormalities in genomic imprinting that cause imprinting disorders (ImpDis), including syndromes like Beckwith-Wiedemann (BWS), Prader-Willi (PWS), and Angelman Syndrome (AS). MS-MLPA employs HhaI endonuclease to digest unmethylated CpGs after hybridising probes. The target sequences of MLPA probes are typically 50-100 nucleotides long, making MS-MLPA capable of

detecting methylation in highly fragmented DNA samples and suitable according to the observed fragment size of cff-DNA. MS-MLPA with cff-DNA is still uncertain, primarily due to limited sample collection and its susceptibility to contaminants. We are currently collecting cff-DNA samples for future analysis using MS-MLPA. Our experiments have demonstrated that MLPA with cff-DNA extracted from amniotic fluid supernatant is a dependable technique for detecting CNVs in target genes, provided that the correct reference DNA is used. Therefore, we suggest using cff-DNA as a reference DNA, treated and extracted like the test samples. In upcoming tests, we plan to extract cff-DNA from plasma to investigate whether bias is due to differences in the extraction kit or physical properties between cff-DNA and gDNA. Utilising cff-DNA as reference DNA may raise ethical issues; if another suitable reference DNA could be used, these issues would be avoided. We also intend to conduct MS-MLPA testing and evaluate the lower detection limit to eliminate the need for early gestational age vacuum concentration since the concentration in early gestation age is still a limitation, especially for MS-MLPA where double the amount of cff-DNA is needed due to the restriction process. Otsuji et al. (2023) have already demonstrated that cell-free DNA in MLPA can be drastically lowered if the fragment size is above 100 bp and still gives reliable results. Even though cell-free DNA from cerebrospinal fluid with diffuse glioma was studied, the main fragment size and limited DNA concentrations are similar to early gestational age fragment size in cff-DNA in amniotic fluid.

4.3.3 Array-CGH with cff-DNA

Using high-resolution oligonucleotide and single nucleotide polymorphism arrays has revolutionised prenatal diagnosis. These arrays are more effective than traditional karyotyping, which has a resolution of 5-10 Mb, in detecting chromosomal abnormalities and have a higher resolution than BAC Array-CGH [76]. This allows for rapid genome-wide screening for aneuploidies or submicroscopic copy number imbalances [77]–[79]. Array-CGH is an accurate method for identifying conditions such as DiGeorge syndrome and other microdeletion or microduplication syndromes. In this genetic testing method, the patient's DNA and a reference sample are labelled with different fluorescent dyes and then hybridised into a microarray chip. This chip contains thousands of probes, which are specific to different genes and regions of DNA. Analysing the

data generated makes it possible to detect any gains or losses of DNA in the patient's sample.

However, Array-CGH is not as effective in identifying balanced translocations and structural rearrangements, making classical techniques such as karyotyping and FISH more suitable for these cases [80]–[83].

Array-CGH (aCGH) is a critical component of prenatal diagnosis, which can be used to comprehensively evaluate copy number variations (CNVs). However, it is essential to be aware of the potential for aCGH to detect CNVs of uncertain significance or incidental findings of late-onset diseases, which can raise ethical issues [84]. Additionally, aCGH has reduced sensitivity in detecting conditions such as triploidy and low-rate mosaicism and misidentifying chromosomal markers when located in highly condensed heterochromatin [85]. Small deletions or duplications cannot be examined with this analysis; this clarification is done with the help of Sanger sequencing or fragment analysis. At the Medical University of Vienna's Medical Genetics department, uncertain gains or losses can be verified using MLPA, qPCR, or WES (if requested). Generally, deletions must have at least ten consecutive probes or duplications larger than 200 kbp with at least ten consecutive probes to be considered. Exceptions may occur. Variants listed in the Database of Genomic Variants (DGV) or dbVar, or those that appear multiple times in our patients and are thus considered normal variants, are not mentioned in the report.

In 2004, Larrabee et al. demonstrated the potential of cff-DNA from amniotic fluid for hybridisation to BAC Array-CGH using GenoSensor arrays 300 (Vysis). This array contained 287 targets, spotted in triplicate, which included subtelomeric regions, microdeletions, and other loci of interest. They tested euploid and aneuploid samples containing at least 100 ng of cff-DNA. The results showed that cff-DNA exhibited similar performance in hybridisation to microarrays compared to cellular DNA but with higher clone-clone variability (noise) due to the natural degradation of cff-DNA, causing inefficient labelling. The study successfully identified fetal gender and whole chromosome gains or losses, including trisomy 21 and monosomy X, according to the strength of the hybridisation signals from chromosome-specific markers. However, the most significant issue was the cff-DNA concentration, as only 17 of 28 amniotic fluid samples yielded adequate amounts of high-quality cff-DNA (>100 ng DNA) for analysis [86].

The current literature uses cff-DNA in amniotic fluid, focusing mainly on whole chromosome changes using the BAC Array-CGH. However, our novel investigation with our extraction

procedure identifies small gains and losses on a high-resolution using oligonucleotide 4x180k Array-CGH (Agilent). We established a lower detection limit and set a lower detection threshold of 10 ng/µl (240 ng cff-DNA/ reaction). All twenty-five patients who exceeded or were close to the threshold yielded analysable data, resulting in a 100% success rate in identifying the same CNV as the corresponding gf-DNA. Array-CGH analysis of cff-DNA consistently and definitively detected all deletions and duplications, even the smallest putative, approximately 34.36 kbp heterozygous duplication from the q34 region of chromosome 7, whereas the largest detected gain was a 2.95 Mbp heterozygous duplication. We confirmed that there were no false positives that were not identifiable as artefacts through standard analytical processes, confirming the method's accuracy and its potential for prenatal diagnosis. However, the bottleneck of this procedure is the required DNA amount, which is hardly achieved in early gestational age, even with cff-DNA vacuum concentration. Several samples were unsuccessful in the pre-experiment phase because DNA concentration was lower than 10 ng/µl. QC values dropped rapidly, indicating a need for improvement in extraction techniques. The problem of low cff-DNA concentrations in Array-CGH is described by Miura et al. (2006) and Larrabee et al. (2004), who already encountered significant issues regarding cff-DNA concentration in BAC Array-CGH for whole-chromosome changes [87].

Miura et al. (2006) investigated using Array-CGH to detect larger chromosomal abnormalities in fetuses. They created a panel of BAC clones from chromosomes 13, 18, 21, X, and Y and applied it to cff-DNA from uncultured amniotic fluid of 13 fetuses with congenital anomalies. The results of the molecular karyotyping were successful in 12 of the 13 fetuses, which were later confirmed by conventional chromosome analysis of cultured amniocytes. However, the method was unable to detect balanced rearrangements and diploid/tetraploid mosaics, as was the case with the one fetus whose molecular karyotype was indicated as normal Array-CGH but was found to have a balanced translocation, 45,XY,-der(14;21)(q10;q10) [87]. Interestingly, they reported that all their cff-DNA extracts from 10 ml amniotic fluid supernatant contained at least 800-1250 ng. The study included seven fetuses in the 15-17th weeks of gestation that should also fit in that range. While the lower end of this range was also achieved in previous experiments with our standard extraction protocol, not all samples in early pregnancy weeks showed the same amount of DNA as they have achieved. Unfortunately, they only mentioned using a Qiagen kit but did

not specify which one or if they modified the protocol. Still, cff-DNA concentration issues were mentioned.

Trying to improve the concentration of cff-DNA, Lapaire et al. (2007b) extracted cff-DNA from 10 ml of residual amniotic fluid (AF) supernatant with a modified version of the QIAamp DNA MAxi Kit (Quiagen). They reported a typical yield of 150-200 ng of cff-DNA in early gestational age (week 16-20). This yield is in line with, or even lower than, the cff-DNA yield obtained with our standard protocol using the QIAamp circulating nucleic acid kit, depending on the week of gestation. However, our experience has shown that the yield can vary due to processing time, internal handling aneuploidy, and patient variety. All cases in their study were successful in the hybridisation process, which was in contrast to the results of the previous study by Larrabee et al. (2004) [86]. Lapaire et al. (2007b) focused on Array-CGH on Spectral Constitutional Chip, which was designed to contain 434 bacterial artificial chromosomes (BACs) clones associated with 43 constitutional syndromes and 41 sub-telomeric regions. They were able to detect whole chromosome aneuploidy in 8 out of 9 cases tested, including the case of trisomy 9 mosaicism. However, the case of triploidy was not detected [79].

Quality scores are another key element in guaranteeing precise results. Quality control (QC) metrics assess the accuracy of generated data, reproducibility, and signal-to-noise ratios. Despite being undervalued in current literature, these metrics are essential for maintaining outcomes' integrity and should be considered more. Table 3.7 outlines the QC values of cff-DNA samples. Generally, samples with cff-DNA concentrations higher than 15 ng/µl (360 ng total cff-DNA) passed the QC report, with most of them failing only one additional QC metric, the Standard Deviation of Log Ratio, which suggests a higher degree of variability. Samples with cff-DNA concentrations higher than 20 ng/µl usually passed all 26 QC metrics, except for case 14. According to Larrabee et al. (2004), higher noise ratios were detected at lower concentrations [86], which is to our observation that samples with 10 ng/µl in 24 µl (total DNA input of 240 ng) could still be analysed, but significantly higher noise ratios were detected (3.7), making them more challenging to analyse. As seen in Figure 3.15, when the concentration is around 10 ng/µl, there is an increase in noise, resulting in more artefacts, particularly in locations where the Agilent probes tend to hybridise poorly. When dealing with cff-DNA samples with low concentration and high noise, it is advisable not to hybridise them partially on the Array-CGH slide. This is because

it can be difficult to distinguish between the analysis results and artefacts. To make it easier to identify artefacts, they should be compared to the probe signals of other cff-DNA samples in the same run. Thus, using a whole Array-CGH slide is recommended instead of a partial one. Samples with lower concentrations showed a higher Derivative Log Ratio Spread (DLR Spread), indicating log ratio variation. Accompanied by a decrease in Log Ratio Imbalance, data analysis became more challenging. Although the conventional input for Array-CGH (Agilent) usually requires a higher quantity, it was deliberately reduced in the case of cff-DNA.

Addressing the still apparent issue of the cff-DNA concentration necessary for the Array-CGH, we recommend the following workflow to perform an Array-CGH with cff-DNA, as shown in Figure 4.1. Furthermore, even though our extraction method performs well regarding cff-DNA yield and purity, further enhancements should be investigated. First, we recommend extracting all amniotic fluid supernatants' available elutes in nuclease-free water and measuring the sample using the Qubit instrument to ensure optimal results from the Array-CGH analysis. If the concentration of the patient sample is 15 ng/µl or higher, we advise proceeding with Array-CGH analysis, with higher concentrations being preferred. If the concentration is less than 15 ng/µl, we recommend performing a vacuum concentration step followed by remeasurement. Depending on the outcome, if the concentration after the vacuum concentration step is above 10 ng/µl, Array-CGH analysis should be carried out. The feasibility of the analysis is uncertain if the concentration remains below 10 ng/µl post-concentration.

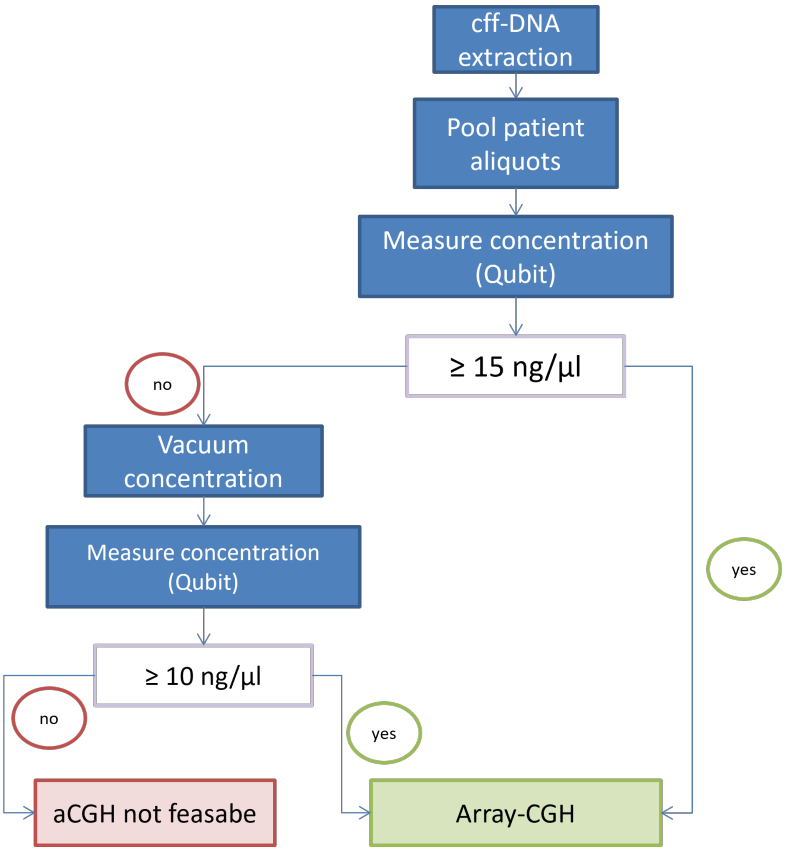


Figure 4.1: Proposed pre-Array-CGH workflow for cff-DNA

Future considerations to optimise and incorporate cff-DNA Array-CGH CNV analysis into routine diagnostics involve modifying the analysis parameters used by the Agilent Software program. The current settings are very sensitive and can detect gains/losses with only three probes and a LogRatio of 0.25, which is dependent on the expertise of the professional. To reduce the number of artefacts, it may be necessary to increase the required probes, LogRatio, or size to identify CNVs quickly and accurately. This approach was preliminarily tested by increasing the required probes from 3 to 6 and the log2ratio from 0.25 to 0.3, which yielded nearly identical results in samples above 15 ng/µl. There may be a trade-off in sensitivity and the potential to miss tiny heterozygous deletions or duplications, but accuracy would be improved.

4.3.4 Whole Exome Sequencing with cff-DNA

Whole Exome Sequencing (WES) is a genetic disorder diagnostic method that can detect gene coding region variants with a success rate of over 95% for exons and 85% for mutations causing Mendelian disorders and disease-predisposing SNPs. However, its limitations include incomplete coverage and difficulty detecting large insertions/deletions, copy number variants, repeat expansions, and structural rearrangements [88]–[91]. The WES library preparation technique requires a low amount of DNA for sequence reads, and cff-DNA is advantageous in obtaining fetal DNA in early gestational age. Previous experiments have shown positive results for the impact of cff-DNA quality on other sequencing methods, and fragment size does not pose an issue. To our knowledge, no literature on cff-DNA in amniotic fluid has been found. This study successfully performed WES on 9 cff-DNA samples. However, NGS practices must adhere to recommended and internal guidelines for sequencing sample quality to accurately detect genetic variations. Several quality parameters such as data output, coverage, insert size, PCR duplicate rate, coverage rate, and uniformity are critical in ensuring accurate detection [92]–[95].

All cff-DNA samples that underwent WES sequencing had excellent quality control parameters, ensuring precise detection of genetic variations. Even when the cff-DNA was vacuum-concentrated due to low starting concentration, WES could detect all the variants diagnosed with the corresponding gf-DNA. One amniotic fluid sample was tinged with blood, so gf-DNA was extracted from cell culture. Prolonged cell maintenance in a regulated environment can lead

to genetic alterations such as mutations, recombination, and chromosomal rearrangements. Various factors, including oxidative stress, DNA replication errors, impaired DNA repair, increased recombinase activity, and chromosomal instability, can cause these changes. An imbalance between the production and accumulation of oxygen-reactive species (ROS) in cells and tissues and the ability of a biological system to detoxify these reactive products can cause oxidative stress. This stress can result in DNA damage, mutations, and chromosomal instability. DNA replication errors can occur even under ideal conditions and become more frequent due to DNA damage and telomere shortening. Extended cell culture can also impair DNA repair mechanisms, accumulating mutations and chromosomal instability. Recombination, the exchange of genetic material between DNA molecules, can also be more frequent due to increased recombinase activity. Finally, chromosomal instability, characterised by increased chromosomal rearrangements, can arise from DNA damage, replication errors, and defects in chromosome segregation. These genetic changes can have severe consequences for the cells, potentially influencing gene expression cell function and even contributing to tumorigenesis. They can also lead to false-positive sequencing results [96], [97]. In the WES 5 run, gf-DNA extracted from the cell culture caused a false-positive variant calling, invalid with cff-DNA. Therefore, using cff-DNA for WES has the additional benefit of avoiding culture-related artefacts, providing more accurate results, and a faster turnabout time. No statement about WES CNV analysis can be made, but it will be analysed as a future prospect.

Conclusion and Prospects

Integrating cell-free fetal DNA (cff-DNA) from amniotic fluid is a promising method for expediting prenatal diagnostics, especially in the early stages of pregnancy, where direct genomic fetal DNA extraction is impossible. The use of cff-DNA can overcome the challenges posed by the traditional method, which requires all amniocytes for cell culture, leading to a long waiting period or even requiring a re-puncture in cases of cell culture failure or maternal cell contamination. Additionally, the issue of maternal cell contamination can be effectively prevented through centrifugation, ensuring reliable separation of fetal and maternal cells and the reliability of fetal genetic testing. Various molecular techniques, including Array Comparative Genomic Hybridisation, Multiplex Ligation-Dependent Probe Amplification, Short Tandem Repeat marker analysis, Sanger sequencing, and Whole Exome Sequencing, have been employed in the study and have proved to be successful when specific protocols were used. The study also found that cff-DNA concentration increases with gestational age, and a concentration step may be necessary for earlier weeks for DNA-intensive methods. The study's findings highlight the potential benefits of cff-DNA in prenatal diagnostics, including faster turnaround times and improved patient care. However, the study also emphasizes the need for an improved extraction method to achieve higher throughput. Further research is necessary to optimize extraction and concentration processes and refine analysis parameters. Exploring various prenatal genetic diagnosis methods, including Array-CGH and WES, shows high success rates and promising possibilities for revolutionizing the field. Overall, the results of this study provide valuable insights for developing more efficient and reliable prenatal diagnostic methods.

Appendices

Primers used for Sanger sequencing of cff-DNA

Table 1: Primer sequences used for Sanger sequencing of cell-free fetal DNA

Gene	Exon	Forward Primer Sequence	Reverse Primer Sequence	Product Size (hg38)
COL4A1	E27_28	GTGGTGCTGGGTAGGATGTGG	ACCAGCTTCTGTGGTGTTTTGATG	486bp
COL4A2	E44	GGCATGGGTCACATGTTGTAAAGAG	CGATCCACAAAGAGAACACAAGAGG	514bp
DHCR7	E06	AGCAGCAGAGGCCCAGAAAG	TCTCAGTGCTCAGGGCTTTACAAC	432bp
SOX17	E02	GCTTCCAGGAGACCAAAAGAAAGG	GAGACCTGCGCGTAGCTGTAGG	680bp
ANKLE2	E12	CCTTGCACACTGTTGTTTTGTGTG	CTCAGCTTGTCAGGAGCTGACC	767bp
CAMK2B	E23	CTTCGAGAACCGTGAGTGAGGAAG	CCTAATTTGACATCATTCCTGTGGC	729 bp
LRRK1	E30	CAGGATGCAACCCTGGGTG	GGCTCCTGGCATTACTTGGG	403 bp
ANO5	E15	TGCCATCTAGGGAAAGAGAGACTGAC	CCTTGAATGGTGGCTCTTGAGC	557 bp
G6PD	Exon 6-7	GAAGGTGTTGAGCCAGAGGGTC	GATAGCTCAGACACTTAGGTTTTGAACTG	765 bp
FOXI3	E01	TGCTTTTTGCCCGAAACCTG	TCTCCTGCTCCTTCCCTTTGTC	1203bp
CDKN1C	E01_2	GAAGTGGACAGCGACTCGGTG	CTGTGTAAGCATTTCCCCTTGTCC	950bp
TNFRSF1A	E2-3	TTTCACTGAGGAAGGACTTGAGCC	GTCCCACCAAAACACACACCTTC	722bp
CC2D2A	E37	CCCATCTCAACTCATATACAACCC	GCAACGTGGGTCTTTTGATTC	498 bp
PIGN	E26	CTGAGTGAGGATGACCTCTCAGG	CAGAACAAAAGGTATAGAAGTCCCAGG	363 bp
EARS2	E03	GCGGCGGGGAGGACATTG	GGGAATTTCTTACGCAGCACAAGG	410bp
MEF2C	E03	GTTCCCTGGTCACCTTCATTAAAATAC	GCAAAGACCAGATCTTACATGGTCTC	694bp
L1CAM	E14_15	AGGTTAAAGGTCAGGCAACCCTTG	CCTGAGGGTGGGGAGGGTC	660bp
TBX5	E06	CTGCTCTGTGTGCCTTGGGTAGATTCCT	TGGGGTCGAAGTTGGTGACTGCTG	518 bp
	E07	GCTTAATTTGCTTCTTTTGGTTGCCAGAG	TGAAGGTTATCAGAAAATGGGACAGAGGG	389 bp
ALDH6A1	E04	CTTTGGGACTTCAACTTATGAGCCTAG	GACTGGAGGAGATGCAGTACAAGG	471 bp

Primer sequences of SeqPrimer used for the SeqPCR, where the original amplification primers could not be used

Table 2: Primer sequences of SeqPrimer used for the SeqPCR, where the original amplification primers could not be used

Gene	Forward Seq Primer Sequence	Reverse Seq Primer Sequence
CAMK2B	CCACCAGGGCAGTCATGGTAAC	
FOXI3	AACCCCTACCTGTGGCTCAAC	ACACCATCTTCATCAGGTCCTCGC

Bibliography

[1] Z. Alfirevic, K. Navaratnam, and F. Mujezinovic, "Amniocentesis and chorionic villus sampling for prenatal diagnosis," *The Cochrane Database of Systematic Reviews*, vol. 2017, no. 9, Sep. 2017, ISSN: 1469493X. DOI: 10.1002/14651858.CD003252.PUB2. [Online]. Available: /pmc/articles/PMC6483702/%20/pmc/articles/PMC6483702/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6483702/.

[2] "Fetal sex pridiction by sex chromatin of chorionic villi cells during early pregnancy.," *Chinese medical journal*, vol. 1, no. 2, pp. 117–26, Mar. 1975, ISSN: 0366-6999. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/811431.

[3] S. J. Allison, J. Stafford, and D. O. Anumba, "The effect of stress and anxiety associated with maternal prenatal diagnosis on feto-maternal attachment," *BMC Women's Health*, vol. 11, p. 33, Jul. 2011, ISSN: 14726874. DOI: 10.1186/1472-6874-11-33. [Online]. Available: /pmc/articles/PMC3148201/%20/pmc/articles/PMC3148201/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3148201/.

[4] M. Health and D. Kabonge Kaye, "Addressing ethical issues related to prenatal diagnostic procedures," *Maternal Health, Neonatology and Perinatology 2023 9:1*, vol. 9, no. 1, pp. 1–9, Feb. 2023, ISSN: 2054-958X. DOI: 10.1186/S40748-023-00146-4. [Online]. Available: https://mhnpjournal.biomedcentral.com/articles/10.1186/s40748-023-00146-4%20http://creativecommons.org/publicdomain/zero/1.0/.

[5] B. Durmaz, H. Bolat, Z. Cengisiz, *et al.*, "20-year experience on prenatal diagnosis in a reference university medical genetics center in Turkey," *Turkish Journal of Medical Sciences*, vol. 51, no. 4, p. 1775, 2021, ISSN: 13036165. DOI: 10.3906/SAG-2006-103. [Online]. Avail-

able: /pmc/articles/PMC8569778/%20/pmc/articles/PMC8569778/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8569778/.

[6] D. M. Serr, L. Sachs, and M. Danon, "The diagnosis of sex before birth using cells from the amniotic fluid (a preliminary report).," *Bulletin of the Research Council of Israel*, vol. 5B, no. 2, pp. 137–8, Dec. 1955, ISSN: 0578-8994.

[7] L. M. Carlson and N. L. Vora, "Prenatal Diagnosis: Screening and Diagnostic Tools," DOI: 10.1016/j.ogc.2017.02.004.

[8] R. Ciortea, A. M. Malutan, C. E. Bucuri, *et al.*, "Amniocentesis—When It Is Clear That It Is Not Clear," *Journal of Clinical Medicine 2023, Vol. 12, Page 454*, vol. 12, no. 2, p. 454, Jan. 2023, ISSN: 2077-0383. DOI: 10.3390/JCM12020454. [Online]. Available: https://www.mdpi.com/2077-0383/12/2/454/htm%20https://www.mdpi.com/2077-0383/12/2/454.

[9] M. Haniffa, D. Taylor, S. Linnarsson, *et al.*, "A roadmap for the Human Developmental Cell Atlas," *Nature 2021 597:7875*, vol. 597, no. 7875, pp. 196–205, Sep. 2021, ISSN: 1476-4687. DOI: 10.1038/s41586-021-03620-1. [Online]. Available: https://www.nature.com/articles/s41586-021-03620-1.

[10] C. Kenner, L. B. Altimier, and M. V. Boykova, "Fetal Development: Environmental Influences and Critical Periods," *Comprehensive Neonatal Nursing Care*, Sep. 2019. DOI: 10.1891/9780826139146.0001.

[11] *Stages of Development of the Fetus - Women's Health Issues - MSD Manual Consumer Version*. [Online]. Available: https://www.msdmanuals.com/en-jp/home/women-s-health-issues/normal-pregnancy/stages-of-development-of-the-fetus.

[12] R. Oliver and H. Basit, "Embryology, Fertilization," *StatPearls*, Apr. 2023. [Online]. Available: https://www.ncbi.nlm.nih.gov/books/NBK542186/.

[13] Y. S. Khan and K. M. Ackerman, *Embryology, Week 1*. StatPearls Publishing, Apr. 2023. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/27194351%20http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5769129.

[14] M. Constância, E. Angiolini, I. Sandovici, *et al.*, "Adaptation of nutrient supply to fetal demand in the mouse involves interaction between the Igf2 gene and placental transporter systems," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 52, p. 19 219, Dec. 2005, ISSN: 00278424. DOI: 10.1073/PNAS.0504468103. [Online]. Available: /pmc/articles/PMC1316882/%20/pmc/articles/PMC1316882/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1316882/.

[15] J. D. Aplin, J. E. Myers, K. Timms, and M. Westwood, "Tracking placental development in health and disease," *Nature Reviews Endocrinology 2020 16:9*, vol. 16, no. 9, pp. 479–494, Jun. 2020, ISSN: 1759-5037. DOI: 10.1038/s41574-020-0372-6. [Online]. Available: https://www.nature.com/articles/s41574-020-0372-6.

[16] T. Cindrova-Davies and A. N. Sferruzzi-Perri, "Human placental development and function," *Seminars in Cell & Developmental Biology*, vol. 131, pp. 66–77, Nov. 2022, ISSN: 1084-9521. DOI: 10.1016/J.SEMCDB.2022.03.039.

[17] L. Woods, V. Perez-Garcia, and M. Hemberger, "Regulation of Placental Development and Its Impact on Fetal Growth—New Insights From Mouse Models," *Frontiers in Endocrinology*, vol. 9, p. 416 241, Sep. 2018, ISSN: 16642392. DOI: 10.3389/FENDO.2018.00570/BIBTEX.

[18] G. J. Burton and A. L. Fowden, "The placenta: A multifaceted, transient organ," *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 370, no. 1663, Mar. 2015, ISSN: 14712970. DOI: 10.1098/RSTB.2014.0066. [Online]. Available: https://my.clevelandclinic.org/health/body/22337-placenta.

[19] E. J. Herrick and B. Bordoni, "Embryology, Placenta," *StatPearls*, May 2023. [Online]. Available: https://www.ncbi.nlm.nih.gov/books/NBK551634/.

[20] M. Y. Turco and A. Moffett, "Development of the human placenta," *Development (Cambridge)*, vol. 146, no. 22, Nov. 2019, ISSN: 14779129. DOI: 10.1242/DEV.163428/223131. [Online]. Available: https://dx.doi.org/10.1242/dev.163428.

[21] J. D. Aplin and P. T. Ruane, "Embryo–epithelium interactions during implantation at a glance," *Journal of Cell Science*, vol. 130, no. 1, pp. 15–22, Jan. 2017, ISSN: 0021-9533. DOI: 10.1242/JCS.175943. [Online]. Available: https://dx.doi.org/10.1242/jcs.175943.

[22] P. T. Ruane, S. C. Berneau, R. Koeck, *et al.*, "Apposition to endometrial epithelial cells activates mouse blastocysts for implantation," *Molecular Human Reproduction*, vol. 23, no. 9, pp. 617–627, Sep. 2017, ISSN: 1360-9947. DOI: 10.1093/MOLEHR/GAX043. [Online]. Available: https://dx.doi.org/10.1093/molehr/gax043.

[23] S. Schlafke and A. C. Enders, "Cellular Basis of Interaction Between Trophoblast and Uterus at Implantation," *Biology of Reproduction*, vol. 12, no. 1, pp. 41–65, Feb. 1975, ISSN: 0006-3363. DOI: 10.1095/BIOLREPROD12.1.41. [Online]. Available: https://dx.doi.org/10.1095/biolreprod12.1.41.

[24] A. T. Hertig, J. Rock, and E. C. Adams, "A description of 34 human ova within the first 17 days of development," *American Journal of Anatomy*, vol. 98, no. 3, pp. 435–493, May 1956, ISSN: 1553-0795. DOI: 10.1002/AJA.1000980306. [Online]. Available: https://onlinelibrary.wiley.com/doi/full/10.1002/aja.1000980306%20https://onlinelibrary.wiley.com/doi/abs/10.1002/aja.1000980306%20https://onlinelibrary.wiley.com/doi/10.1002/aja.1000980306.

[25] M. N. Shahbazi, A. Jedrusik, S. Vuoristo, *et al.*, "Self-organization of the human embryo in the absence of maternal tissues," *Nature Cell Biology 2016 18:6*, vol. 18, no. 6, pp. 700–708, May 2016, ISSN: 1476-4679. DOI: 10.1038/ncb3347. [Online]. Available: https://www.nature.com/articles/ncb3347.

[26] J. D. Aplin, H. Whittaker, Y. T. Jana Lim, S. Swietlik, J. Charnock, and C. J. Jones, "Hemangioblastic foci in human first trimester placenta: Distribution and gestational profile," *Placenta*, vol. 36, no. 10, pp. 1069–1077, Oct. 2015, ISSN: 0143-4004. DOI: 10.1016/J.PLACENTA.2015.08.005.

[27] R. Vento-Tormo, M. Efremova, R. A. Botting, *et al.*, "Single-cell reconstruction of the early maternal–fetal interface in humans," *Nature 2018 563:7731*, vol. 563, no. 7731, pp. 347–353, Nov. 2018, ISSN: 1476-4687. DOI: 10.1038/s41586-018-0698-6. [Online]. Available: https://www.nature.com/articles/s41586-018-0698-6.

[28] T. Tilburgs, J. H. Evans, Â. C. Crespo, and J. L. Strominger, "The HLA-G cycle provides for both NK tolerance and immunity at the maternal-fetal interface," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 43, pp. 13 312–

13 317, Oct. 2015, ISSN: 10916490. DOI: 10.1073/PNAS.1517724112/SUPPL{_}FILE/PNAS.201517724SI.PDF. [Online]. Available: https://www.pnas.org/doi/abs/10.1073/pnas.1517724112.

[29] Z. Madeja, H. Yadi, R. Apps, *et al.*, "Paternal MHC expression on mouse trophoblast affects uterine vascularization and fetal growth," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 10, pp. 4012–4017, Mar. 2011, ISSN: 00278424. DOI: 10.1073/PNAS.1005342108/SUPPL{_}FILE/PNAS.201005342SI.PDF. [Online]. Available: https://www.pnas.org/doi/abs/10.1073/pnas.1005342108.

[30] R. Pijnenborg, G. Dixon, W. B. Robertson, and I. Brosens, "Trophoblastic invasion of human decidua from 8 to 18 weeks of pregnancy," *Placenta*, vol. 1, no. 1, pp. 3–19, Jan. 1980, ISSN: 0143-4004. DOI: 10.1016/S0143-4004(80)80012-9.

[31] E. Jauniaux and G. J. Burton, "Pathophysiology of placenta accreta spectrum disorders: A review of current findings," *Clinical Obstetrics and Gynecology*, vol. 61, no. 4, pp. 743–754, Dec. 2018, ISSN: 15325520. DOI: 10.1097/GRF.0000000000000392. [Online]. Available: https://journals.lww.com/clinicalobgyn/fulltext/2018/12000/pathophysiology_of_placenta_accreta_spectrum.12.aspx.

[32] C. M. A. Ten Broek, J. Bots, I. Varela-Lasheras, M. Bugiani, F. Galis, and S. Van Dongen, "Amniotic Fluid Deficiency and Congenital Abnormalities both Influence Fluctuating Asymmetry in Developing Limbs of Human Deceased Fetuses," *PLoS ONE*, vol. 8, no. 11, p. 81 824, Nov. 2013, ISSN: 19326203. DOI: 10.1371/JOURNAL.PONE.0081824. [Online]. Available: /pmc/articles/PMC3842303/%20/pmc/articles/PMC3842303/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3842303/.

[33] X. L. Tong, L. Wang, T. B. Gao, Y. G. Qin, Y. Q. Qi, and Y. P. Xu, "Potential function of amniotic fluid in fetal development—novel insights by comparing the composition of human amniotic fluid with umbilical cord and maternal serum at mid and late gestation," *Journal of the Chinese Medical Association : JCMA*, vol. 72, no. 7, pp. 368–373, Jul. 2009, ISSN: 1726-4901. DOI: 10.1016/S1726-4901(09)70389-2. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/19581143/.

[34] J. Suliburska, R. Kocyłowski, I. Komorowicz, M. Grzesiak, P. Bogdański, and D. Barałkiewicz, "Concentrations of Mineral in Amniotic Fluid and Their Relations to Selected Maternal and Fetal Parameters," *Biological Trace Element Research*, vol. 172, no. 1, p. 37, Jul. 2016, ISSN: 15590720. DOI: 10.1007/S12011-015-0557-3. [Online]. Available: /pmc/articles/PMC4893386/%20/pmc/articles/PMC4893386/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4893386/.

[35] E. D. Fitzsimmons and T. Bajaj, "Embryology, Amniotic Fluid," *StatPearls*, Jul. 2023. [Online]. Available: https://www.ncbi.nlm.nih.gov/books/NBK541089/.

[36] J. Calleja-Agius, S. Muttukrishna, and E. Jauniaux, "The effect of coelomic fluid on the production of cytokines by the first trimester human placenta," *Placenta*, vol. 32, no. 11, pp. 893–900, Nov. 2011, ISSN: 1532-3102. DOI: 10.1016/J.PLACENTA.2011.06.024. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/21872926/.

[37] J. A. Laudy and J. W. Wladimiroff, "The fetal lung. 1: Developmental aspects," *Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology*, vol. 16, no. 3, pp. 284–290, 2000, ISSN: 0960-7692. DOI: 10.1046/J.1469-0705.2000.00228.X. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/11169299/.

[38] M. H. Beall, J. P. van den Wijngaard, M. J. van Gemert, and M. G. Ross, "Amniotic fluid water dynamics," *Placenta*, vol. 28, no. 8-9, pp. 816–823, Aug. 2007, ISSN: 0143-4004. DOI: 10.1016/J.PLACENTA.2006.11.009. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/17254633/.

[39] B. A. Dale, K. A. Holbrook, J. R. Kimball, M. Hoff, and T. T. Sun, "Expression of epidermal keratins and filaggrin during human fetal skin development," *The Journal of Cell Biology*, vol. 101, no. 4, p. 1257, Oct. 1985, ISSN: 15408140. DOI: 10.1083/JCB.101.4.1257. [Online]. Available: /pmc/articles/PMC2113922/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2113922/.

[40] R. A. Brace, "Physiology of amniotic fluid volume regulation," *Clinical obstetrics and gynecology*, vol. 40, no. 2, pp. 280–289, 1997, ISSN: 0009-9201. DOI: 10.1097/00003081-199706000-00005. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/9199840/.

[41] R. Grassi, R. Farina, I. Floriani, F. Amodio, and S. Romano, "Assessment of fetal swallowing with gray-scale and color Doppler sonography," *AJR. American journal of roentgenology*, vol. 185, no. 5, pp. 1322–1327, 2005, ISSN: 0361-803X. DOI: 10.2214/AJR.04.1114. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/16247157/.

[42] J. CAMPBELL, N. WATHEN, M. MACINTOSH, P. CASS, T. CHARD, and R. MAINWARING-BURTON, "Biochemical composition of amniotic fluid and extraembryonic coelomic fluid in the first trimester of pregnancy," *British journal of obstetrics and gynaecology*, vol. 99, no. 7, pp. 563–565, 1992, ISSN: 0306-5456. DOI: 10.1111/J.1471-0528.1992.TB13821.X. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/1525096/.

[43] J. Kornacki, M. Adamczyk, P. Wirstlein, M. Osiński, and E. Wender-Ozegowska, "Polyhydramnios - frequency of congenital anomalies in relation to the value of the amniotic fluid index," *Ginekologia polska*, vol. 88, no. 8, pp. 442–445, 2017, ISSN: 0017-0011. DOI: 10.5603/GP.A2017.0081. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/28930371/.

[44] N. Rabie, E. Magann, S. Steelman, and S. Ounpraseuth, "Oligohydramnios in complicated and uncomplicated pregnancy: a systematic review and meta-analysis," *Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology*, vol. 49, no. 4, pp. 442–449, Apr. 2017, ISSN: 1469-0705. DOI: 10.1002/UOG.15929. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/27062200/.

[45] A. van den Heuvel, L. Chitty, E. Dormandy, *et al.*, "Will the introduction of non-invasive prenatal diagnostic testing erode informed choices? An experimental study of health care professionals," *Patient Education and Counseling*, vol. 78, no. 1, pp. 24–28, Jan. 2010, ISSN: 07383991. DOI: 10.1016/j.pec.2009.05.014. [Online]. Available: https://linkinghub.elsevier.com/retrieve/pii/S0738399109002304.

[46] A. N. Barrett, B. G. Zimmermann, D. Wang, A. Holloway, and L. S. Chitty, "Implementing Prenatal Diagnosis Based on Cell-Free Fetal DNA: Accurate Identification of Factors Affecting Fetal DNA Yield," *PLOS ONE*, vol. 6, no. 10, e25202, Oct. 2011, ISSN: 1932-6203. DOI: 10.1371/JOURNAL.PONE.0025202. [Online]. Available: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0025202.

[47] R. W. K. Chiu, Y. M. Dennis Lo, Y. M. Dennis, and L. Ka, "Cell-free fetal DNA coming in all sizes and shapes," *Prenatal Diagnosis*, vol. 41, no. 10, pp. 1193–1201, Sep. 2021, ISSN: 1097-0223. DOI: 10.1002/PD.5952. [Online]. Available: https://onlinelibrary.wiley.com/doi/full/10.1002/pd.5952%20https://onlinelibrary.wiley.com/doi/abs/10.1002/pd.5952%20https://obgyn.onlinelibrary.wiley.com/doi/10.1002/pd.5952.

[48] D. T. Miller, K. Lee, A. S. Gordon, *et al.*, "Recommendations for reporting of secondary findings in clinical exome and genome sequencing, 2021 update: a policy statement of the American College of Medical Genetics and Genomics (ACMG)," *Genetics in Medicine*, vol. 23, no. 8, pp. 1391–1398, Aug. 2021, ISSN: 15300366. DOI: 10.1038/S41436-021-01171-4.

[49] Y. Yang, D. M. Muzny, J. G. Reid, *et al.*, "Clinical Whole-Exome Sequencing for the Diagnosis of Mendelian Disorders," *New England Journal of Medicine*, vol. 369, no. 16, pp. 1502–1511, Oct. 2013, ISSN: 0028-4793. DOI: 10.1056/NEJMoa1306555.

[50] D. W. Bianchi, E. S. LeShane, and J. M. Cowan, "Large Amounts of Cell-free Fetal DNA Are Present in Amniotic Fluid," *Clinical Chemistry*, vol. 47, no. 10, pp. 1867–1869, Oct. 2001, ISSN: 0009-9147. DOI: 10.1093/CLINCHEM/47.10.1867. [Online]. Available: https://dx.doi.org/10.1093/clinchem/47.10.1867.

[51] L. Hui and D. Bianchi, "Cell-free fetal nucleic acids in amniotic fluid," *Human Reproduction Update*, vol. 17, no. 3, pp. 362–371, 2011, ISSN: 1460-2369. DOI: 10.1093/humupd/dmq049.

[52] H. J. Park, H. Y. Cho, and D. H. Cha, "The Amniotic Fluid Cell-Free Transcriptome Provides Novel Information about Fetal Development and Placental Cellular Dynamics," *International Journal of Molecular Sciences 2021, Vol. 22, Page 2612*, vol. 22, no. 5, p. 2612, Mar. 2021, ISSN: 14220067. DOI: 10.3390/IJMS22052612. [Online]. Available: https://www.mdpi.com/1422-0067/22/5/2612/htm%20https://www.mdpi.com/1422-0067/22/5/2612.

[53] Y. Xue, G. Zhao, L. Qiao, J. Lu, B. Yu, and T. Wang, "Sequencing Shorter cfDNA Fragments Decreases the False Negative Rate of Non-invasive Prenatal Testing," *Frontiers in Genetics*, vol. 11, p. 522 906, Mar. 2020, ISSN: 16648021. DOI: 10.3389/FGENE.2020.00280/BIBTEX.

[54] H. Che, K. Stanley, T. Jatsenko, B. Thienpont, and J. R. Vermeesch, "Expanded knowledge of cell-free DNA biology: potential to broaden the clinical utility," *Extracellular Vesicles and Circulating Nucleic Acids*, vol. 3, no. 3, pp. 216–234, Aug. 2022, ISSN: 27676641. DOI: 10.20517/EVCNA.2022.21. [Online]. Available: https://www.oaepublish.com/articles/evcna.2022.21.

[55] K. Hong, H. J. Park, H. Y. Jang, *et al.*, "A Novel Paradigm for Non-Invasive Prenatal Genetic Screening: Trophoblast Retrieval and Isolation from the Cervix (TRIC)," *Diagnostics*, vol. 13, no. 15, Aug. 2023, ISSN: 20754418. DOI: 10.3390/DIAGNOSTICS13152532. [Online]. Available: /pmc/articles/PMC10417081/%20/pmc/articles/PMC10417081/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10417081/.

[56] S. Holdenrieder, A. Bronkhorst, S. C. Ding, and Y. M. Dennis Lo, "Cell-Free DNA Fragmentomics in Liquid Biopsy," *Diagnostics 2022, Vol. 12, Page 978*, vol. 12, no. 4, p. 978, Apr. 2022, ISSN: 2075-4418. DOI: 10.3390/DIAGNOSTICS12040978. [Online]. Available: https://www.mdpi.com/2075-4418/12/4/978/htm%20https://www.mdpi.com/2075-4418/12/4/978.

[57] M. Alcaide, M. Cheung, J. Hillman, *et al.*, "Evaluating the quantity, quality and size distribution of cell-free DNA by multiplex droplet digital PCR," *Scientific Reports 2020 10:1*, vol. 10, no. 1, pp. 1–10, Jul. 2020, ISSN: 2045-2322. DOI: 10.1038/s41598-020-69432-x. [Online]. Available: https://www.nature.com/articles/s41598-020-69432-x.

[58] M. Grunt, T. Hillebrand, and H. Schwarzenbach, "Clinical relevance of size selection of circulating DNA," *Translational Cancer Research*, vol. 7, no. Suppl 2, S171–S184, Feb. 2018, ISSN: 2219-6803. DOI: 10.21037/TCR.2017.10.10. [Online]. Available: https://tcr.amegroups.org/article/view/17051/html%20https://tcr.amegroups.org/article/view/17051.

[59] S. C. Yu, P. Jiang, W. Peng, *et al.*, "Single-molecule sequencing reveals a large population of long cell-free DNA molecules in maternal plasma," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 118, no. 50, p. 2 114 937 118, Dec. 2021, ISSN: 10916490. DOI: 10.1073/PNAS.2114937118/-/DCSUPPLEMENTAL. [Online]. Available:

/pmc/articles/PMC8685924/%20/pmc/articles/PMC8685924/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8685924/.

[60] A. G. Edlow and D. W. Bianchi, "Tracking fetal development through molecular analysis of maternal biofluids," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1822, no. 12, pp. 1970–1980, Dec. 2012, ISSN: 0925-4439. DOI: 10.1016/J.BBADIS.2012.04.005.

[61] T. S. Hartwig, L. Ambye, S. Sørensen, and F. S. Jørgensen, "Discordant non-invasive prenatal testing (NIPT) – a systematic review," *Prenatal Diagnosis*, vol. 37, no. 6, pp. 527–539, Jun. 2017, ISSN: 1097-0223. DOI: 10.1002/PD.5049. [Online]. Available: https://onlinelibrary.wiley.com/doi/full/10.1002/pd.5049%20https://onlinelibrary.wiley.com/doi/abs/10.1002/pd.5049%20https://obgyn.onlinelibrary.wiley.com/doi/10.1002/pd.5049.

[62] Y. Hou, J. Yang, Y. Qi, *et al.*, "Factors affecting cell-free DNA fetal fraction: statistical analysis of 13,661 maternal plasmas for non-invasive prenatal screening," *Human genomics*, vol. 13, no. 1, p. 62, Dec. 2019, ISSN: 14797364. DOI: 10.1186/S40246-019-0244-0/FIGURES/3. [Online]. Available: https://humgenomics.biomedcentral.com/articles/10.1186/s40246-019-0244-0.

[63] L. Xing, T. Bai, S. Liu, *et al.*, "Maternal, neonatal, pregnancy outcome characteristics of pregnant women with high plasma cell-free DNA concentration in non-invasive prenatal screening: a retrospective analysis," *Frontiers in Pediatrics*, vol. 11, p. 1 195 818, Aug. 2023, ISSN: 22962360. DOI: 10.3389/FPED.2023.1195818/BIBTEX.

[64] F. M. Lun, R. W. Chiu, K. C. Chan, Y. L. Tak, K. L. Tze, and Y. M. Lo, "Microfluidics Digital PCR Reveals a Higher than Expected Fraction of Fetal DNA in Maternal Plasma," *Clinical Chemistry*, vol. 54, no. 10, pp. 1664–1672, Oct. 2008, ISSN: 0009-9147. DOI: 10.1373/CLINCHEM.2008.111385. [Online]. Available: https://dx.doi.org/10.1373/clinchem.2008.111385.

[65] E. Wang, A. Batey, C. Struble, T. Musci, K. Song, and A. Oliphant, "Gestational age and maternal weight effects on fetal cell-free DNA in maternal plasma," *Prenatal Diagnosis*, vol. 33, no. 7, pp. 662–666, Jul. 2013, ISSN: 01973851. DOI: 10.1002/PD.4119. [Online].

Available: https://www.researchgate.net/publication/236104637_Gestational_age_and_maternal_weight_effects_on_fetal_cfDNA_in_maternal_plasma.

[66] O. Lapaire, D. W. Bianchi, I. Peter, *et al.*, "Cell-Free Fetal DNA in Amniotic Fluid: Unique Fragmentation Signatures in Euploid and Aneuploid Fetuses," *Clinical Chemistry*, vol. 53, no. 3, pp. 405–411, Mar. 2007, ISSN: 0009-9147. DOI: 10.1373/CLINCHEM.2006.076083. [Online]. Available: https://dx.doi.org/10.1373/clinchem.2006.076083.

[67] I. Peter, H. Tighiouart, O. Lapaire, K. L. Johnson, D. W. Bianchi, and N. Terrin, "Cell-free DNA Fragmentation Patterns in Amniotic Fluid Identify Genetic Abnormalities and Changes due to Storage," *Diagnostic molecular pathology : the American journal of surgical pathology, part B*, vol. 17, no. 3, p. 185, Sep. 2008, ISSN: 10529551. DOI: 10.1097/PDM.0B013E31815BCDB6. [Online]. Available: /pmc/articles/PMC4459511/%20/pmc/articles/PMC4459511/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4459511/.

[68] J. Weida, A. S. Patil, F. P. Schubert, *et al.*, "Prevalence of maternal cell contamination in amniotic fluid samples," *The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstetricians*, vol. 30, no. 17, pp. 2133–2137, Sep. 2017, ISSN: 1476-4954. DOI: 10.1080/14767058.2016.1240162. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/27744775/.

[69] N. Nagan, N. E. Faulkner, C. Curtis, and I. Schrijver, "Laboratory Guidelines for Detection, Interpretation, and Reporting of Maternal Cell Contamination in Prenatal Analyses: A Report of the Association for Molecular Pathology," *The Journal of Molecular Diagnostics : JMD*, vol. 13, no. 1, p. 7, 2011, ISSN: 15251578. DOI: 10.1016/J.JMOLDX.2010.11.013. [Online]. Available: /pmc/articles/PMC3069929/%20/pmc/articles/PMC3069929/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3069929/.

[70] L. Volozonoka, L. Gailite, D. Perminov, *et al.*, "Reducing misdiagnosis caused by maternal cell contamination in genetic testing for early pregnancy loss," *Systems Biology in Reproductive Medicine*, vol. 66, no. 6, pp. 410–420, Nov. 2020, ISSN: 19396376. DOI:

10.1080/19396368.2020.1827081. [Online]. Available: https://www.tandfonline.com/doi/abs/10.1080/19396368.2020.1827081.

[71] I. S. Hagemann, "Overview of Technical Aspects and Chemistries of Next-Generation Sequencing," in *Clinical Genomics*, Elsevier, Jan. 2015, pp. 3–19, ISBN: 9780124051737. DOI: 10.1016/B978-0-12-404748-8.00001-0. [Online]. Available: https://linkinghub.elsevier.com/retrieve/pii/B9780124047488000010.

[72] G. A. Hawkins, "Analysis of Human Genetic Variations Using DNA Sequencing," in *Basic Science Methods for Clinical Researchers*, Elsevier, Jan. 2017, pp. 77–98, ISBN: 9780128030783. DOI: 10.1016/B978-0-12-803077-6.00005-9. [Online]. Available: https://linkinghub.elsevier.com/retrieve/pii/B9780128030776000059.

[73] "Techniques for Oral Microbiology," in *Atlas of Oral Microbiology*, Elsevier, Jan. 2015, pp. 15–40. DOI: 10.1016/B978-0-12-802234-4.00002-1. [Online]. Available: https://linkinghub.elsevier.com/retrieve/pii/B9780128022344000021.

[74] Y. C. Tan, A. Michaeel, J. Blumenfeld, *et al.*, "A Novel Long-Range PCR Sequencing Method for Genetic Analysis of the Entire PKD1 Gene," *The Journal of Molecular Diagnostics : JMD*, vol. 14, no. 4, p. 305, Jul. 2012, ISSN: 15251578. DOI: 10.1016/J.JMOLDX.2012.02.007. [Online]. Available: /pmc/articles/PMC3391417/%20/pmc/articles/PMC3391417/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3391417/.

[75] Y. Zheng, L. Wong, A. H. W. Kwan, *et al.*, "Prenatal diagnosis of polycystic kidney caused by biallelic hypomorphic variants in the PKD1 gene," *Prenatal Diagnosis*, 2023, ISSN: 1097-0223. DOI: 10.1002/PD.6419. [Online]. Available: https://onlinelibrary.wiley.com/doi/full/10.1002/pd.6419%20https://onlinelibrary.wiley.com/doi/abs/10.1002/pd.6419%20https://obgyn.onlinelibrary.wiley.com/doi/10.1002/pd.6419.

[76] E. Karampetsou, D. Morrogh, and L. Chitty, "Microarray Technology for the Diagnosis of Fetal Chromosomal Aberrations: Which Platform Should We Use?" *Journal of Clinical Medicine*, vol. 3, no. 2, p. 663, Jun. 2014. DOI: 10.3390/JCM3020663. [Online]. Available: /pmc/articles/PMC4449692/%20/pmc/articles/PMC4449692/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4449692/.

[77] B. S. Emanuel and S. C. Saitta, "From microscopes to microarrays: dissecting recurrent chromosomal rearrangements," *Nature Reviews Genetics*, vol. 8, no. 11, pp. 869–883, Nov. 2007, ISSN: 1471-0056. DOI: 10.1038/nrg2136. [Online]. Available: https://www.nature.com/articles/nrg2136.

[78] M. Freitas, J. Pinto, C. Ramalho, and S. Dória, "Prenatal diagnosis: the clinical usefulness of array comparative genomic hybridization," *Porto Biomedical Journal*, vol. 3, no. 2, e13, Oct. 2018. DOI: 10.1016/J.PBJ.0000000000000013. [Online]. Available: /pmc/articles/PMC6726309/%20/pmc/articles/PMC6726309/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6726309/.

[79] O. Lapaire, X. Y. Lu, K. L. Johnson, *et al.*, "Array-CGH analysis of cell-free fetal DNA in 10 mL of amniotic fluid supernatant," *Prenatal Diagnosis*, vol. 27, no. 7, pp. 616–621, Jul. 2007, ISSN: 01973851. DOI: 10.1002/PD.1752.

[80] A. Kallioniemi, O.-P. Kallioniemi, D. Sudar, *et al.*, "Comparative Genomic Hybridization for Molecular Cytogenetic Analysis of Solid Tumors," *Science*, vol. 258, no. 5083, pp. 818–821, Oct. 1992, ISSN: 0036-8075. DOI: 10.1126/science.1359641. [Online]. Available: https://www.science.org/doi/10.1126/science.1359641.

[81] T. Sahoo, S. W. Cheung, P. Ward, *et al.*, "Prenatal diagnosis of chromosomal abnormalities using array-based comparative genomic hybridization," *Genetics in Medicine*, vol. 8, no. 11, pp. 719–727, Nov. 2006, ISSN: 10983600. DOI: 10.1097/01.gim.0000245576.47154.63. [Online]. Available: https://linkinghub.elsevier.com/retrieve/pii/S1098360021034766.

[82] B. J. Trask, "Human cytogenetics: 46 chromosomes, 46 years and counting," *Nature Reviews Genetics*, vol. 3, no. 10, pp. 769–778, Oct. 2002, ISSN: 1471-0056. DOI: 10.1038/nrg905. [Online]. Available: https://www.nature.com/articles/nrg905.

[83] R. J. Wapner, C. L. Martin, B. Levy, *et al.*, "Chromosomal Microarray versus Karyotyping for Prenatal Diagnosis," *The New England journal of medicine*, vol. 367, no. 23, p. 2175, Dec. 2012, ISSN: 0028-4793. DOI: 10.1056/NEJMOA1203382. [Online]. Available: /pmc/articles/PMC3549418/%20/pmc/articles/PMC3549418/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3549418/.

[84] Q. Qi, S. Lu, X. Zhou, *et al.*, "Copy number variation sequencing-based prenatal diagnosis using cell-free fetal DNA in amniotic fluid," *Prenatal Diagnosis*, vol. 36, no. 6, pp. 576–583, Jun. 2016, ISSN: 10970223. DOI: 10.1002/PD.4830.

[85] E. M. Vestergaard, R. Christensen, O. B. Petersen, and I. Vogel, "Prenatal diagnosis: array comparative genomic hybridization in fetuses with abnormal sonographic findings," *Acta Obstetricia et Gynecologica Scandinavica*, vol. 92, no. 7, pp. 762–768, Jul. 2013, ISSN: 00016349. DOI: 10.1111/aogs.12146. [Online]. Available: https://onlinelibrary.wiley.com/doi/10.1111/aogs.12146.

[86] P. B. Larrabee, K. L. Johnson, E. Pestova, *et al.*, "Microarray analysis of cell-free fetal DNA in amniotic fluid: a prenatal molecular karyotype.," *American journal of human genetics*, vol. 75, no. 3, pp. 485–91, Sep. 2004, ISSN: 0002-9297. DOI: 10.1086/423288. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/15252756%20http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1182026.

[87] S. Miura, K. Miura, H. Masuzaki, *et al.*, "Microarray comparative genomic hybridization (CGH)-based prenatal diagnosis for chromosome abnormalities using cell-free fetal DNA in amniotic fluid," *Journal of Human Genetics*, vol. 51, no. 5, pp. 412–417, May 2006, ISSN: 1434-5161. DOI: 10.1007/s10038-006-0376-7. [Online]. Available: https://www.nature.com/articles/jhg200662.

[88] A. Abou Tayoun and H. Mason-Suares, *Considerations for whole exome sequencing unique to prenatal care*, Sep. 2020. DOI: 10.1007/s00439-019-02085-7.

[89] M. Vinkšel, K. Writzl, A. Maver, and B. Peterlin, "Improving diagnostics of rare genetic diseases with NGS approaches," *Journal of Community Genetics*, vol. 12, no. 2, pp. 247–256, Apr. 2021, ISSN: 18686001. DOI: 10.1007/S12687-020-00500-5.

[90] L. Lei, L. Zhou, and J.-j. Xiong, "Whole-exome sequencing increases the diagnostic rate for prenatal fetal structural anomalies," *European Journal of Medical Genetics*, vol. 64, no. 9, p. 104 288, Sep. 2021, ISSN: 17697212. DOI: 10.1016/j.ejmg.2021.104288. [Online]. Available: https://linkinghub.elsevier.com/retrieve/pii/S1769721221001543.

[91] E. Janicki, M. De Rademaeker, C. Meunier, N. Boeckx, B. Blaumeiser, and K. Janssens, "Implementation of Exome Sequencing in Prenatal Diagnostics: Chances and Challenges," *Diagnostics*, vol. 13, no. 5, Mar. 2023, ISSN: 20754418. DOI: 10.3390/DIAGNOSTICS13050860/S1. [Online]. Available: /pmc/articles/PMC10000387/%20/pmc/articles/PMC10000387/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10000387/.

[92] M. Mokry, H. Feitsma, I. J. Nijman, *et al.*, "Accurate SNP and mutation detection by targeted custom microarray-based genomic enrichment of short-fragment sequencing libraries," *Nucleic Acids Research*, vol. 38, no. 10, Feb. 2010, ISSN: 03051048. DOI: 10.1093/NAR/GKQ072.

[93] A. S. Gargis, L. Kalman, M. W. Berry, *et al.*, "Assuring the quality of next-generation sequencing in clinical laboratory practice," *Nature Biotechnology*, vol. 30, no. 11, pp. 1033–1036, Nov. 2012, ISSN: 10870156. DOI: 10.1038/NBT.2403.

[94] K. Yauy, C. Van Goethem, H. Pégeot, *et al.*, "Evaluating the Transition from Targeted to Exome Sequencing: A Guide for Clinical Laboratories," *International Journal of Molecular Sciences*, vol. 24, no. 8, Apr. 2023, ISSN: 14220067. DOI: 10.3390/IJMS24087330/S1. [Online]. Available: /pmc/articles/PMC10138641/%20/pmc/articles/PMC10138641/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10138641/.

[95] G. Matthijs, E. Souche, M. Alders, *et al.*, "Guidelines for diagnostic next-generation sequencing," *European Journal of Human Genetics 2016 24:1*, vol. 24, no. 1, pp. 2–5, Oct. 2015, ISSN: 1476-5438. DOI: 10.1038/ejhg.2015.226. [Online]. Available: https://www.nature.com/articles/ejhg2015226.

[96] G. Pizzino, N. Irrera, M. Cucinotta, *et al.*, "Oxidative Stress: Harms and Benefits for Human Health," *Oxidative Medicine and Cellular Longevity*, vol. 2017, 2017, ISSN: 19420994. DOI: 10.1155/2017/8416763. [Online]. Available: /pmc/articles/PMC5551541/%20/pmc/articles/PMC5551541/?report=abstract%20https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5551541/.

[97] J. Campisi and F. D'Adda Di Fagagna, "Cellular senescence: when bad things happen to good cells," *Nature Reviews Molecular Cell Biology 2007 8:9*, vol. 8, no. 9, pp. 729–740,

Sep. 2007, ISSN: 1471-0080. DOI: 10.1038/nrm2233. [Online]. Available: https://www.nature.com/articles/nrm2233.

Acronyms

AF	Amniotic Fluid.
AKH Wien	Allgemeines Krankenhaus Wien (University Hospital Vienna).
Array-CGH/ aCGH	Array-based Comparative Genomic Hybridisation.
AS	Angelman Syndrome.
bp	Base Pair.
BWS	Beckwith-Wiedemann Syndrome.
cfDNA	cell-free DNA.
cff-DNA	cell-free fetal DNA.
CNV	Copy Number Variation.
CVS	Chorionic Villus Sampling.
DNA	Deoxyribonucleic Acid.
FISH	Fluorescence In Situ Hybridisation.
GA	Gestational Age.
gDNA	genomic DNA.
gf-DNA	Genomic Fetal DNA.
MLPA	Multiplex Ligation-depended Probe Amplification.

MS-MLPA	Methylation Specific Multiplex Ligation-depended Probe Amplification.
NGS	Next Generation Sequencing.
NIPT	Non-Invasive Prenatal Testing.
NTC	No Template Control.
PCR	Polymerase Chain Reaction.
PWS	Prader-Willi Syndrome.
QC	Quality Control.
QIAamp CNA kit	QIAamp Circulating Nucleic Acid kit.
qPCR	Quantitative Real-Time Polymerase Chain Reaction.
seqPCR	Sequencing Polymerase Chain Reaction.
STR-Marker	Short Tandem Repeat Marker.
WES	Whole Exome Sequencing.
WG	Week of Gestation.