UNIVERSIDADE D
COIMBRA

Luís Miguel Lopes Marques Pires

NON-INVASIVE PRENATAL SCREENING FOR COMMON ANEUPLOIDIES

IMPLEMENTATION, CONSOLIDATION AND FUTURE

Dissertação no âmbito do Mestrado em Genética Clínica Laboratorial sob orientação da Professora Doutora Isabel Maria Marques Carreira, coorientação pela Mestre Ana Isabel Castro Jardim e apresentada à Faculdade de Medicina da Universidade de Coimbra

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“There are two ways to live:
you can live as if nothing is a miracle; you can live as if everything is a miracle.
Only a life lived for others is a life worthwhile.”

Albert Einstein

We performed the implementation and validation of NIPT test at the Laboratory of Cytogenetics and Genomics, Faculty of Medicine, University of Coimbra (LCG-FMUC) with the collaboration of Coimbra Hospital University Centre (CHUC) and technical support of Clarigo™ / Agilent Technologies.

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Resumo

Introdução e Objetivos: O diagnóstico pré-natal (DPN) é definido pelos procedimentos, médicos e laboratoriais, que permitam acompanhar o bom desenvolvimento do feto e estabelecer o diagnóstico precoce de eventuais anomalias congénitas. Efetuado em Portugal há mais de 3 décadas, o DPN é baseado em testes invasivos como a amniocentese e a biópsia das vilosidades coriónicas, complementado por testes não invasivos, como a ecografia e rastreio bioquímico, o que permite diagnosticar quase todas as condições com uma causa genética conhecida, com especial destaque para as aneuploidias mais comuns envolvendo os cromossomas 13, 18, 21, X e Y. Com a descoberta do DNA fetal em circulação (cfDNA) no sangue materno, aliada às tecnologias de sequenciação de nova geração (NGS), assistiu-se na última década a uma revolução no DPN, com a introdução do teste pré-natal não invasivo (NIPT). Apesar de se tratar de um teste de rastreio, e não de diagnóstico, o NIPT apresenta alta sensibilidade e alta especificidade na deteção de aneuploidias, com taxas de falsos positivos inferior a 0,1%, razão que levou o *American College of Obstetricians and Gynecologists* (ACOG) a recomendar, em 2020, o seu uso generalizado em todas as gestações. Neste trabalho apresentamos os resultados da implementação de um teste NIPT, através da sequenciação de cfDNA em circulação no sangue materno e utilizando tecnologias de NGS. Serão avaliados os seguintes pontos: 1) critérios de inclusão e de exclusão para a realização do teste; 2) eficácia, rentabilidade, limitações e perspetivas de melhoria; 3) Correlação dos resultados obtidos com parâmetros inerentes á amostragem selecionada; 4) Avaliação do impacto do NIPT na redução de exames invasivos e na redução de perdas fetais.

Materiais e Métodos: O trabalho foi dividido em duas partes: Fase 1 – Implementação do NIPT com descrição dos aspetos técnicos e laboratoriais da seleção do teste, procedimento laboratorial, validação, proficiência e implementação. Fase 2 – Consolidação do NIPT, com definição dos critérios de inclusão e exclusão, consentimento informado, logística de processamento, relatórios de análise e aconselhamento genético NIPT. Serão ainda apresentados os resultados do estudo

retrospetivo, das primeiras 1130 grávidas que realizaram o teste NIPT entre fevereiro de 2019 e janeiro de 2021, avaliando a taxa de deteção do teste, taxa de resultados inconclusivos, análise da estrutura populacional, tempos de resposta e os fatores significativos que condicionaram a obtenção de resultados conclusivos.

Resultados: O teste NIPT foi realizado em 1130 grávidas, tendo sido detetadas e confirmadas 8 trissomias 21 (0,7%) uma trissomia 13 (0,09%), 129 resultados inconclusivos (11,5%) e 992 com ausência/baixo risco de aneuploidia. A análise estatística aos resultados inconclusivos avaliando diferentes parâmetros da amostragem mostrou que são várias os fatores que que podem comprometer a obtenção de resultados com destaque da baixa fração fetal, índice de massa corporal da grávida e qualidade do cfDNA obtido. Para além das trissomias 21 e 13, foram ainda detetadas alterações incidentais no braço curto do cromossoma X e situações de mosaicismo envolvendo os cromossomas sexuais.

Conclusão: A implementação do NIPT permitiu oferecer um teste de rastreio a um número maior de grávidas com risco de aneuploidias, diminuindo a necessidade de recurso a testes invasivos. Em termos de eficácia, todas as aneuploidias identificadas foram confirmadas por amniocentese atestando a sua elevada sensibilidade, permitindo resultados fidedignos até a um mínimo de fração fetal de 3%. A sua aplicabilidade em grávidas de IMC elevado revelou-se válida, apenas comprometida em valores superiores a 40. As limitações do teste são a elevada taxa de inconclusivos (7 a 11,5%) e a não aplicabilidade em gestações de gémeos e de dadoras de ovócitos.

Palavras-Chave: Diagnóstico Pré-natal; Rastreio não invasivo; *cell-free* DNA; Aneuploidias; Sequenciação de nova geração.

Abstract

Introduction and Objectives: Prenatal diagnosis (PND) defines all medical and laboratory procedures that allow monitoring the normal development of the fetus and establishing the early diagnosis of any congenital anomalies. Performed in Portugal for over three decades, PND includes invasive tests like amniocentesis and chorionic villus sampling and non-invasive tests such as ultrasound and biochemical screening. The invasive non-invasive combination allows the diagnosis of almost all known genetic alterations, including the most common aneuploidies involving chromosomes 13, 18, 21, X, and Y. With the discovery of circulating fetal DNA (cfDNA) in maternal blood, combined with next-generation sequencing (NGS) technologies, the last decade has seen a revolution in PND with the introduction of non-invasive prenatal testing (NIPT). NIPT has high sensitivity and specificity in detecting aneuploidies, with false positive rates of less than 0.1%, a reason that led the American College of Obstetricians and Gynecologists (ACOG) to recommend, in 2020, its widespread use in all pregnancies. This work presents the results of implementing a NIPT test by sequencing circulating cfDNA in maternal blood using NGS technologies. The following points will be considered: 1) inclusion and exclusion criteria for carrying out the test; 2) effectiveness, profitability, limitations, and prospects for improvement; 3) Correlation of the results obtained with parameters inherent to the selected sampling; 4) Assessment of the impact of the NIPT in reducing invasive exams and fetal losses.

Materials and Methods: The work contains two parts: Phase 1 – Implementation of the NIPT with a description of the technical and laboratory aspects of test selection, laboratory procedure, validation, proficiency, and implementation. Phase 2 – Consolidation of the NIPT, defining the inclusion and exclusion criteria, informed consent, processing logistics, analysis reports, and NIPT genetic counseling. We also present the results of a retrospective study of 1130 pregnant women who underwent the NIPT test between February 2019 and January 2021, evaluating the test detection rate, rate of inconclusive results, analysis of population structure, response times, and the significant factors that conditioned the achievement of conclusive results.

Results: The NIPT test revealed eight trisomies of chromosome 21 (0.7%), one trisomy 13 (0.09%), 129 inconclusive results (11.5%), and 992 with absence/low risk of aneuploidy. The Statistical analysis of inconclusive results evaluating different sampling parameters showed that several factors could compromise the achievement of results, meager fetal fraction, pregnant body mass index, and quality of the cfDNA obtained. In addition to trisomies 21 and 13, we also detected unexpected maternal deletions and duplications in the short arm of the X chromosome and mosaicism involving the sex chromosomes.

Conclusion: The implementation of the NIPT made it possible to offer a screening test to more pregnant women at risk of aneuploidy, reducing the need for invasive tests. Regarding NIPT performance, all positive results were confirmed by amniocentesis, attesting to their high sensitivity, allowing reliable results up to a minimum fetal fraction of 3%. Its applicability in pregnant women with a high BMI proved valid, only compromised in values above 40. The test's limitations are the high rate of inconclusive results (7 to 11.5%) and its non-applicability in twin and egg donor pregnancies.

Key-words: Prenatal Diagnosis; Non-invasive testing; cell-free DNA; Aneuploidies; Next generation sequencing.

MSc Publications and Scientific Communications

Scientific Publications

Luís M Pires, Susana Ferreira, Almeida P, Val M, Lavoura N, Ramos F, Galhano E, Melo JB, Carreira IM. **Incidental detection of maternal Xp22.31 deletions and duplications in noninvasive prenatal testing**. **Proceeding abstracts Medicine** (**2021)**. 100:4:18. doi: 10.1097/ MD.0000000000023585. IF: 1.889; Q2 (Medicine)

(See supplemental data - **Pág. 68**)

Pinto M, **Pires LM**, Ferreira S, Paiva P, Jardim A, Melo JB, Carreira IM. **A NIPT aneuploidy suspition with normal QF-PCR and aCGH. Karyotype gives the answer. Proccedings of the SPGH 24Th Anual Meeting, Medicine (2020) 29:100** (4) (p e23585). doi.org/10.1097/ MD.0000000000023585. IF: 1.552; Q2 (Medicine)

(See supplemental data - **Pág. 69**)

Scientific Communication

October 2019

Oliveira D, Pita C, Sá J, Venâncio M, Ramos L, Abreu L, Branco M, Rei AI, Franco S, Coelho F, Goncalves H, **Pires LM**, Carreira IM, Saraiva JM, Galhano E, Ramos F. **Avaliação da Implementação de Teste Pré-Natal Não Invasivo no Centro de Diagnóstico Pré-Natal do Centro Hospitalar e Universitário de Coimbra**. Reunião Científica da Associação Portuguesa de Diagnóstico Pré-Natal. Viana do Castelo, 4 e 5 de outubro de 2019.

(See supplemental data - **Pág. 67**)

List of Abbreviations

aCGH - Array Comparative Genomic Hybridization

ACOG - American College of Obstetricians and Gynecologists

AF - Amniotic fluid

AFP – Alpha-fetoprotein

AMA – Advanced Maternal Age

Array-CGH – Array Comparative Genomic Hybridization

ASD - Autism spectrum disorders

BMI - Body Mass Index

cfDNA - Cell free deoxyribonucleic acid

cffDNA - Cell-free fetal deoxyribonucleic acid

CHUC - Coimbra Hospital University Centre

CRD - Cordocentesis

CVS - Chorionic villus sampling

DNA - Deoxyribonucleic acid

DS - Down Syndrome

EBMG - European Board of Medical Genetics

ESHG - European Society of Human Genetics

FF - Fetal fraction

FISH - Fluorescent in Situ Hybridization

GenQA - Genomics Quality Assessment

IVD - *In vitro* diagnostic

LCG-FMUC – Laboratory of Cytogenetics and Genomics, Faculty of Medicine, University of Coimbra

LFF - Low fetal fraction

MIDs - Molecular identifiers

MLPA - Multiplex Ligation-Dependent Probe Amplification

MPS - Massive parallel sequencing

NAC - Not Automatic Call

NIPT - Non-Invasive Prenatal testing

NIPS Non-Invasive Prenatal screening

NGS - Next Generation Sequencing

NT - Nuchal translucency

OMIM – Online Mendelian inheritance in man

ONTD - Open neural tube defect

PAPP-A - Pregnancy-associated plasma protein-A

PCR – Polymerase chain reaction

PND - Prenatal Diagnosis

QC - Quality Control

QF-PCR - Quantitative Fluorescence-Polymerase Chain Reaction

RhD - Rhesus D

ROC - Receiver operating characteristic curve

RT - Room Temperature

Sd - Standard deviation

SNPs - Single nucleotide polymorphisms

SPSS - Statistical Product and Service Solutions

VIP - Voluntary Interruption of Pregnancy

WGS - Whole genome sequencing

WHO - World Health Organization

β-hCG - β-human chorionic gonadotrophin

uE3 - Unconjugated estriol

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I - Introduction

1.1. Prenatal Diagnosis

Approximately 3% to 5% of pregnancies unexpectedly present congenital disabilities or genetic disorders in which chromosomal abnormalities are present in approximately 1 in 150 live births and include aneuploidies, translocations, duplications, and deletions[1]. According to the World Health Organization (WHO), prenatal diagnosis (PND) means "all actions taken before birth, which aim to diagnose a congenital anomaly (morphological, structural, functional or molecular) present at birth or that will manifest later, external or internal, sporadic or family, hereditary or not, single or multiple…"[2]. The main objective of the PND is to allow couples to control their health and that of the fetus and determine whether the fetus has genetic abnormalities[1,3].

Chromosomal aneuploidies are one of the more frequent human genetic abnormalities and are defined as an alteration in the number of a whole or part of a chromosome. Its incidence is variable, but Hsu and colleagues reported values of 1 in 154 liveborn (0.65%)[4].

There are two main types of tests performed in PND, screening and diagnostic tests, selected according to the age of gestation, fetal development, and the genetic condition in question. PND screening tests include biochemical tests and ultrasound performed during the first or second trimester[1,3,5].

In Portugal, PND began in 1984 with the publication of the first legislation authorizing the voluntary termination of pregnancy due to genetic causes. Initially limited to the large urban centers of Porto and Lisbon, this type of diagnosis gradually created centers dispersed throughout the country[6].

PND and screening have evolved significantly in the last forty years through gradual technical methodologies, equipment's and public health awareness and recommendations[3,5].

Compared with invasive tests (amniocentesis or chorionic villus sampling), non-invasive approaches using maternal blood or biochemical screening usually integrated with ultrasound and maternal age, are more accepted due to the great advantage of not presenting a risk of miscarriage. The discovery of cell-free fetal deoxyribonucleic acid (cffDNA) in maternal plasma and recent advances in next-generation sequencing (NGS) improved both the accuracy and variety of non-invasive prenatal testing (NIPT) for genetic diseases. In most centres these tests are incorporated into clinical care, for fetal aneuploidy screening. However, these advances bring new technical and financial challenges associated with ethical issues and public opinion[3,5].

1.1.1. Common Aneuploidies

Aneuploidies are the occurrence of usually one extra or a missing chromosome leading to an unbalanced chromosome complement. They constitute one of the major categories of human genetic disorders. The most common aneuploidy is trisomy 21 (Down syndrome), with an incidence that ranges from of 1 per 650 to 1 per 1000 live births, followed by Trisomies 13 and 18 and less frequent sex chromosome aneuploidies, with monosomy X standing out as the only viable monosomy (**Table-I.1** and **Table-I.2**)[1,29,38, 39,40].

Table I.1 – Incidence of Human common aneuploidies

Incidence of common aneuploidies	
Trisomy 21	1 in 800 live births
Trisomy 18	1 in 7500 live births
Trisomy 13	1 in 15,000 live births
Monosomy X (Turner syndrome)	1 in 5000 girls
Trisomy X	1 in 1000 girls
XXY (Klinefelter syndrome)	1 in 1000 boys
XYY	1 in 1000 boys

Adapted from: Thompson & Thompson genetics in medicine. 7th edition. Philadelphia: Saunders/Elsevier; 2007.

Table I.2 – Features of Autosomal aneuploidies compatible with postnatal survival.

Feature	Trisomy 21	Trisomy 18	Trisomy 13
Incidence (live births)	1 in 850	1 in 6,000-8,000	1 in 12,000-20,000
Clinical presentation	Hypotonia, short stature, loose skin on nape, palmar crease, clinodactyly	Hypertonia, prenatal growth deficiency, characteristic fist clench, rocker-bottom feet	Microcephaly, sloping forehead, characteristic fist clench, rocker-bottom feet, polydactyly
Dysmorphic facial features	Flat occiput, epicanthal folds, Brushfield spots	Receding jaw, low-set ears	Ocular abnormalities, cleft lip and palate
Intellectual disability	Moderate to mild	Severe	Severe
Other common features	Congenital heart disease Duodenal atresia Risk for leukemia Risk for premature dementia	Severe heart malformations Feeding difficulties	Severe CNS malformations Congenital heart defects
Life expectancy	55 yr	Typically less than a few months; almost all <1 yr	50% die within first month, >90% within first year

Adapted from: Thompson & Thompson genetics in medicine, 8th edition, Philadelphia: Elsevier; 2016 [40].

A screening test tells whether the fetus is at an increased risk of having a specific condition. In contrast, a diagnostic test usually tells whether the anomaly exists[7,24].

1.1.2. Invasive Prenatal Diagnosis

There are three PND invasive procedures available during pregnancy: Amniocentesis, chorionic villus sampling (CVS) (**Fig.1**), and cordocentesis (CRD); although the latter is nowadays being used much less due to the improvement of laboratory technologies, and so, CRD is not addressed in this work. These procedures have been performed globally since the 1970s, and 1980s[8,9], respectively, and both have high sensitivity in the detection of chromosomal aneuploidies. Amniocentesis, can be performed since the 14th week of gestation although many obstetric prefer to do it around the 16th-17th week, and it involves the collection of cells from the amniotic fluid (AF). Chorionic villus sampling is usually

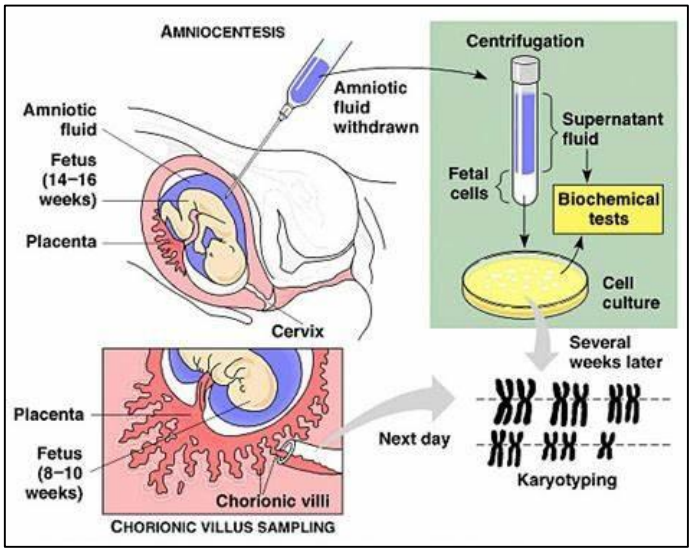


Figure 1 – Amniocentesis and Chorionic Villus Sampling. In both techniques, collected fetal cells are used to determine the probability of a fetus being a carrier of a common aneuploidy (trisomy 21, 18 or 13). Source: CanadaQBank.com.

performed between 10th and 12th -week gestation, is the processing of fetal cells from chorionic villi tissue, which is part of the placenta. Both techniques allow the isolation of fetal cells that can be directly analysed or cultured in the laboratory for karyotyping, FISH (Fluorescence in Situ Hybridization), microarray analysis, or another molecular testing[7]. These methods are reliable with a 99% accuracy. Previously, only women considered "high risk" were advised for invasive procedures to determine the probability of a fetus being a carrier of aneuploidy[10].

However, both tests are invasive, with some discomfort for the pregnant woman, and there is a risk of miscarriage (0.3 - 1%), regardless of whether the fetus has an anomaly[11,29].

1.1.3. Non-Invasive Prenatal Screening

Compared with amniocentesis or chorionic villus sampling, non-invasive approaches are widely accepted due to the great advantage of not being associated with a risk of miscarriage.

For the assessment of the risk of chromosomal aneuploidies, there are three screening options[11]: 1- First-trimester screening; 2- Second-trimester screening; and 3- Morphological ultrasonography.

We will briefly discuss each of them, considering their advantages and limitations to contextualize and compare them with the implementation of NIPT, which is the central theme of this thesis.

First-trimester screening

First-trimester screening became widely used in the 1990s, when it was realized that the great majority of fetuses, with major aneuploidies, can be identified by a combination of maternal age and ultrasound markers like increased fetal nuchal translucency (NT) (**Fig.2**), and maternal serum proteins β-hCG (β-human chorionic gonadotrophin) and PAPP-A (pregnancy-associated plasma protein-A). First-trimester screening performed

between 11th and 14th week of gestation, is associated in 33% of the cases with a chromosomal abnormality, of which, 75% is trisomy 21[9].

The blood marker screening measures the relative amounts of PAPP-A and β-hCG. In trisomy 21 pregnancies, the fetus has β-hCG increased, and PAPP-A is decreased (**Table I.3**). For trisomy 13 and 18, β-hCG and PAPP-A are decreased[9,12,29,39].

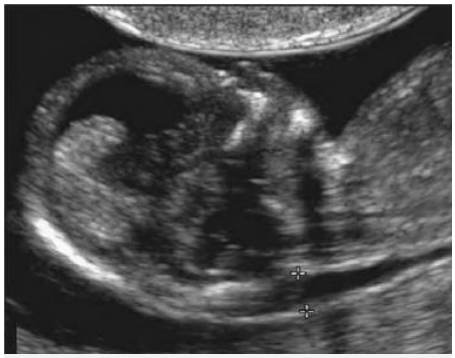


Figure 2 – Increased NT and trisomy 21.
Nuchal translucency exceeding 3 mm.
Adapted from: Nyberg et al. (2006)[13]

Table I.3 – Elevation and depression of parameters used in 1st and 2nd trimester screening tests.

	First-Trimester Screen			Second-Trimester Screen			
	Nuchal Translucency	PAPP-A	Free β-hCG	uE_3	AFP	hCG	Inhibin A
Trisomy 21	↑	↓	↑	↓	↓	↑	↑
Trisomy 18	↑	↓	↓	↓	↓	↓	—
Trisomy 13	↑	↓	↓	↓	↓	↓	—
Neural tube defect	—	—	—	—	↑↑	—	—

AFP, Alpha-fetoprotein; β-hCG, human chorionic gonadotropin β subunit; PAPP-A, pregnancy-associated plasma protein A; uE_3, unconjugated estriol.

Adapted from: Thompson & Thompson genetics in medicine, 8th edition, Philadelphia: Elsevier; 2016 [40].

First-trimester screening detects 85-90% of cases of Down syndrome, with a false positive rate of around 5% [9,40].

Biochemical markers screening – "quadruple screening"

This screening test is done in the second trimester (15-20 weeks) to determine a relative risk for an open neural tube defect (ONTD), Down syndrome, and Trisomy 18. The risk is determined from the mother blood levels of alpha-fetoprotein (AFP), total β-hCG, unconjugated estriol (uE3), and Inhibin-A. For example, low levels of AFP, β-hCG, and uE3

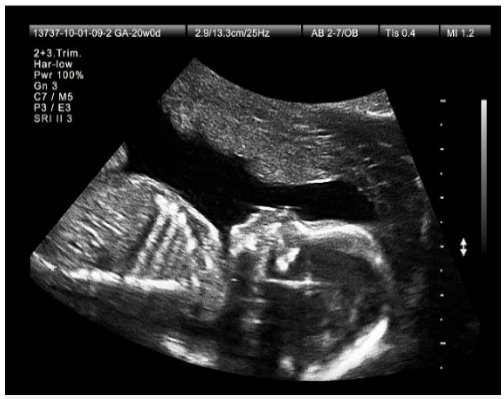


Figure 3 – 20th week ultrasound
Adapted from: Today´s Parent.com

(**Table I.3**) indicate an increased risk for Trisomy 18 [12].

"Quadruple screening" detects about 81% of cases of Down Syndrome, 60% of Trisomy 18 and 60% of ONTDs with a 5% false positive rate [9,40].

Ultrasonography

There are three types of ultrasound examinations: Standard, limited and detailed. The detailed or specialized are performed when there is a suspicion of fetal abnormality and can include fetal Doppler, fetal echocardiography, or even a detailed anatomy ultrasound, usually performed between 18th and 21th week gestation (**Fig.3**).

By Portuguese legislation, every pregnant woman should be offered at least three ultrasound sessions; in the 1st trimester (10th - 13th week) necessary for NT evaluation, morphological ultrasound (20th - 22th week); and a late ultrasound for labor preparation. Anatomy ultrasound can detect 50% of cases of Down Syndrome and more than 90% of cases of spina bifida and anencephaly[7,40].

Cell-Free fetal DNA Non-invasive prenatal screening

The study of cell-free fetal DNA as a rapid molecular aneuploidy (MRA) screening, often known as non-invasive prenatal screening, became commercially available in the USA in 2011. This relatively recent technology involves collecting a maternal serum sample, in which fetal cell-free DNA (cffDNA) fragments are isolated[1]. In 2020, ACOG issued guidelines recommending that NIPT be offered to all patients regardless of maternal age or baseline risk. NIPT has a Down Syndrome detection rate of 99% and a false positive rate of less than 1%.

1.2. Non-Invasive Prenatal Testing

The risk of miscarriage when performing invasive tests has stimulated non-invasive options in Prenatal testing. The existence of fetal cells circulating in the bloodstream and its use for PND has been considered since 1969. However, the process for the

isolation of these intact fetal cells was very complicated due to their small number and the aggravating factor that they persist in the blood from previous pregnancies, invalidating its use for PND[14,29].

Fetal ultrasound analyses coupled with screening of biochemical markers have been considered important forms of non-invasive screening. More recently, non-invasive prenatal testing (NIPT) has been introduced, it refers to the use of cffDNA for common aneuploidies screening, and has been shown to be a reliable, easy, non-invasive approach which is the reason of the present work.

1.2.1. Cell free fetal DNA discovery and the NIPT evolution

When Dennis Lo and his team discovered, in 1997, the existence of fetal cfDNA in circulation in the mother's blood, a reliable methodology for non-invasive prenatal diagnosis started[15,16,26,29].

cfDNA consists of extracellular DNA fragments of 50 to 200bp, resulting from apoptosis and cell death, released into the blood current. In the case of a pregnant woman, in addition to the cfDNA from her genome, there is also fetal cfDNA of placental origin released in maternal blood from apoptotic trophoblasts. The cffDNA is not constant, increasing its quantity during pregnancy. The proportion of cffDNA in mother circulation, called fetal fraction (FF), reaches values of about 10% from the 10th week of gestation, varying between 6.0% and 20% during pregnancy[1,29]. Lo detected fetal Y chromosome DNA in 70 to 80% of maternal plasma from mothers of male fetuses. The concentration of cffDNA was estimated at 3.4% in the first trimester and 6.2% during the second trimester[17,18]. The FF is essential in performing NIPT and can be affected by different factors, mainly by the gestational age, maternal weight, number of pregnancies, ethnic origin, and the presence of fetal aneuploidy. In the analysis of the results, the validity of most analysis techniques depends on the FF. Therefore, the process of extraction and efficient enrichment of cffDNA is critical[18,29].

In 1999, fetal DNA in maternal circulation was used to determine fetal rhesus D (RhD)[18].

In 2004, Gupta *et al.* found that fetal DNA and RNA were in a cell-free form in the maternal plasma, had its origin from the placenta. Achondroplasia was detected using

NIPT in 2007. In 2006, Tong *et al.* used it to detect fetal aneuploidy, based on epigenetic allelic ratio analysis for chromosome 18. Tong's discovery began the use of NIPT for fetal aneuploidy detection, now offered clinically for Trisomy 21, Trisomy 18, Trisomy 13, and sex chromosome aneuploidy[18,20].

1.2.2. cfDNA NIPT Technology Platforms

There are different technology platforms used for NIPT. Among them are whole genome sequencing, or targeted to some chromosomes using next-generation sequencing (NGS). The most suitable option for each laboratory should consider the number of samples per week, data generation and analysis, laboratory workflow, and the resulting clinical implications.

Microarray Analysis

In this type of NIPT, a microarray or chip (glass slide printed with thousands of DNA oligonucleotides), specific for chromosomes 13, 18, 21, X and Y, is used. The cfDNA fragments (maternal + fetal) are first amplified by PCR, labeled with fluorochrome dyes, and bound to the NIPT microarray (**Fig.4**). Both light intensity and binding position indicate the relative amount of DNA and the presence or absence of the target, respectively. Deviations from the expected fluorescent counts indicate aneuploidy [21].

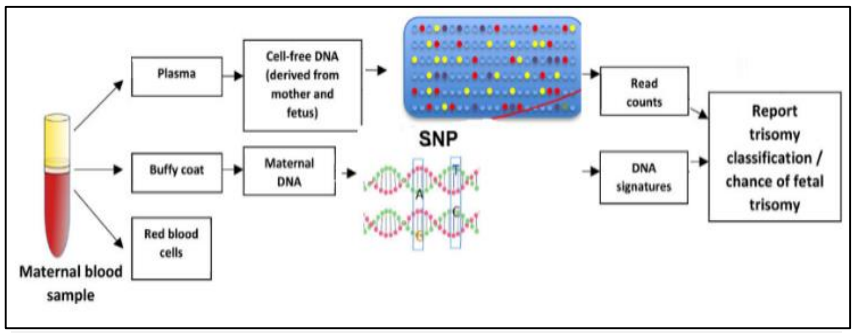


Figure 4 – NIPT Technology based on microarray analysis.
Adapted from: Geppert *et al.*

Rolling Circle Amplification

Rolling circle amplification is a new NIPT technology targeting relevant chromosomes. It is performed without PCR neither MPS, based on digital molecular quantification in a 96-well microplate. The method converts targeted specific cfDNA fragments into a circular template. It replicates them by a rolling mechanism (**Fig.5**). The normalized ratio between the number of chromosome-specific objects is then used to calculate the z-score and mapped to a post-test risk. Deviations in expected fluorescent counts indicate aneuploidy[22].

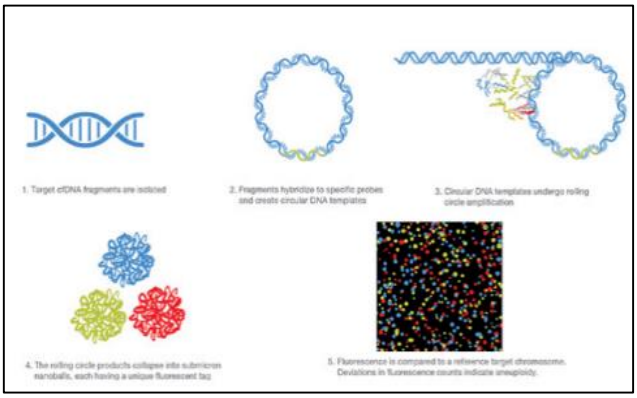


Figure 5 – NIPT Technology based on Rolling circle amplification.
Source: Illumina.

SNP analysis – Genotyping Method

Single nucleotide polymorphisms (SNPs) are genetic variations among individuals. NIPT based on SNPs uses targeted PCR amplification and MPS sequencing of SNPs on specific chromosomes of interest rather than a quantitative or 'counting' method that involves whole genome sequencing (WGS) or targeted sequencing of nonpolymorphic loci. After SNP genotyping, an algorithm determines abnormalities in expected allele frequencies between mother and child, allowing the fetus determination of copy number (**Fig.6**)[23].

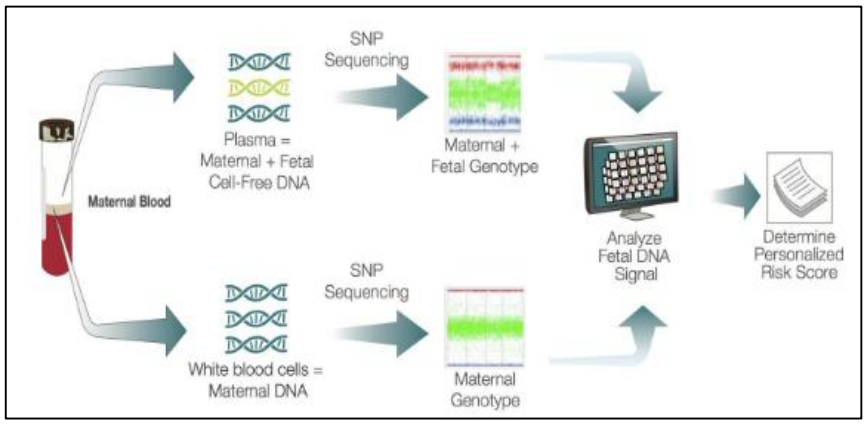


Figure 6 – NIPT Technology based on SNPs Genotyping using MPS.
Adapted from: Ryan *et al.*[23]

Whole-Genome Sequencing – Counting Method

NIPT using WGS technology allows for more informative results from a view of the entire genome. Consensually has lower failure rates than targeted sequencing or array-based platforms, but it is also the most expensive as it requires sequencing equipment with greater capacity. There are different strategies to determine chromosomal aneuploidy, but the most common is the counting method of all cfDNA fragments[25]. Both maternal and fetal DNA segments are sequenced simultaneously, each piece mapped to the chromosome of origin. If the percentage of cfDNA fragments from each chromosome is as expected, the fetus has a reduced risk of having a chromosomal condition (negative test result). If the percentage of cfDNA fragments from a specific chromosome is more significant than expected, the fetus is more likely to have a trisomy condition (positive test result) (**Fig.7**). To reliably detect these differences, however, the FF present needs to be adequate as differences between disomy and trisomy are challenging to determine with too little fetal DNA[14].

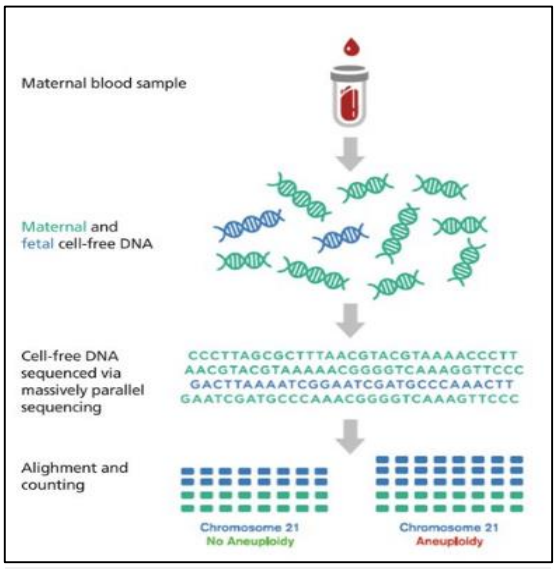


Figure 7 – NIPT Technology based on WGS counting method.
Adapted from: Rink et al.[14]

1.3. LCG-FMUC and PND

The work of this thesis took place in the Laboratory of Cytogenetics and Genomics of Faculty of Medicine of Coimbra University (LCG-FMUC).

The LCG-FMUC, located in Coimbra, began its activity in Prenatal Diagnosis in 1992. Benefiting from being located at the University of Coimbra, side by side with a reference university hospital, LCG-FMUC is involved in several research projects, including collaborations with research units from different Portuguese and foreign hospitals and

universities. It has in its team specialists in Genetics recognized in Portugal by the Central Administration of the Health System (ACSS) and internationally as European Clinical Laboratory Geneticists (ErCLG) specialists titled by the European Board of Medical Genetics (EBMG) of the European Society of Human Genetics (ESGH). It performs prenatal in an extensive list of tests that include: conventional cytogenetics, molecular cytogenetics, molecular biology, genomics, and NGS. All these technologies are also used for postnatal studies. LCG-FMUC is ISO 9001:2015 certified and annually participates in Genomics Quality Assessment (GenQA) external quality control programs with excellent ratings.

1.3.1. PND Timeline in LCG-FMUC

1992-2008 – Karyotype "Golden Age"

The LCG-FMUC started its activity with the PND by studying the karyotype in amniotic fluid. From 1992 to 2000, conventional cytogenetics was the primary PND test, reaching 1000 cases per year at the end of the millennium (**Fig. 8**). From 2000 until 2008, the karyotype continued in its "golden phase", reaching two thousand tests/year at the end of 2008 (**Fig.8**).

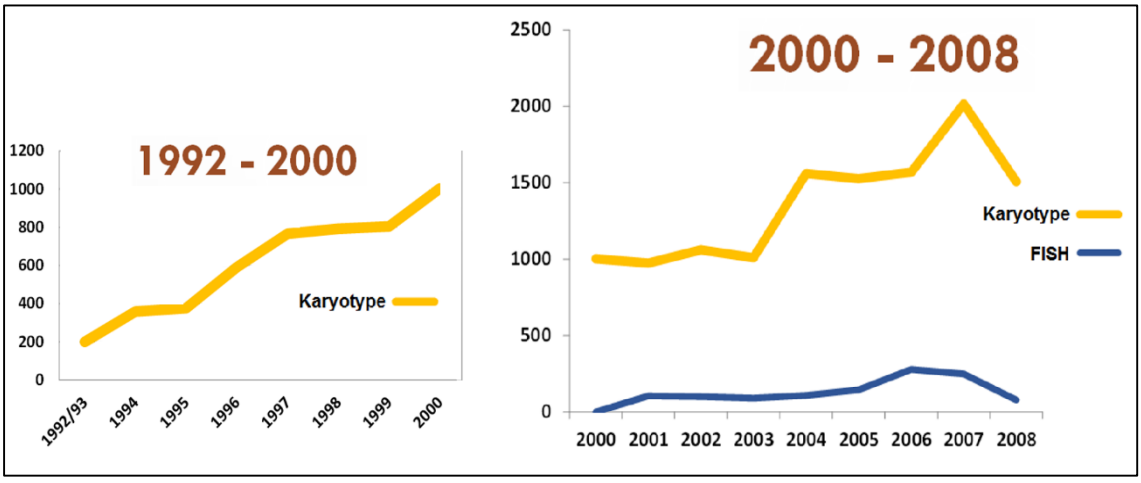


Figure 8 – Conventional Cytogenetics and Molecular Cytogenetics Tests performed by LCG-FMUC between 1992 and 2008.
Left 1992-2000 Right 2000-2008 Source: LCG-FMUC (2022)

The rapid screening tests for aneuploidies by FISH were initiated in 2002, reaching the maximum in 2007, with an average of 300 cases per year (**Fig.8**).

2008-2014 – Introduction of Molecular Biology and Array-CGH

In 2008, LCG FMUC implemented MLPA (Multiplex Ligation-Dependent Probe Amplification) for the rapid screening of common aneuploidies.

In 2014, the limitations of MLPA in diagnosing female triploidy (69,XXX) led to the QF-PCR (Quantitative Fluorescence-Polymerase Chain Reaction) implementation as the primary test for MRA. In 2012, the Array-CGH was introduced as a postnatal test in the genomic study of cases of intellectual disability and autism spectrum disorders (ASD), being used for some years as the 1st tier test in these cases.

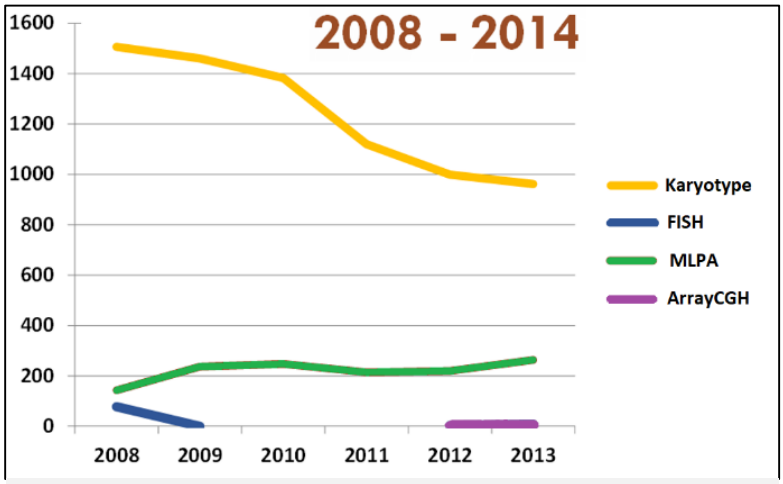


Figure 9 – Karyotype, FISH, MLPA and ArrayCGH number of tests performed by LCG-FMUC between 2008 and 2014.
Source: LCG-FMUC (2022)

With the new technologies, karyotype decreases to an average of 1000 cases per year, but still keeping the test with the highest annual volume at the end of 2014.

2015-Present – The Rise of NIPT

With the implementation of NIPT in the LCG-FMUC, and the use of aCGH for cases with relevant ultrasound alterations and Normal QF-PCR, the karyotype decreased (**Fig. 10**).

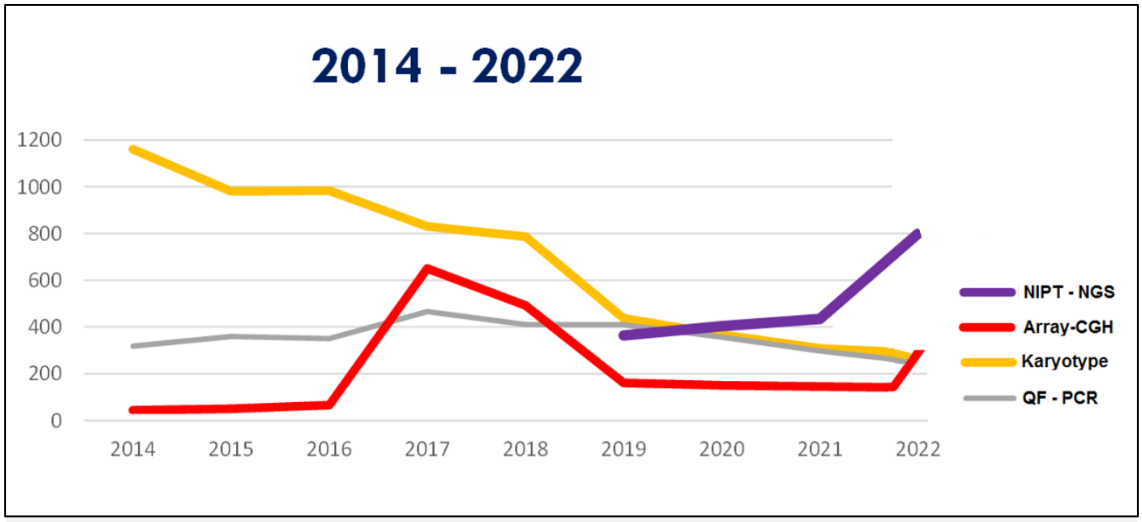


Figure 10 – LCG-FMUC Volume of PND tests performed between 2014 and 2022.
In 2020, the NIPT is rising, and for the first time in the history of the LCG-FMUC, karyotype is no longer the test with the highest volume in PND.
Source: LCG-FMUC (2022)

The aim of this work was the implementation of NIPT as a screening test in PND and evaluate its sensitivity, specificity, and impact on the Lab workflow.

2 - Framework

Non-invasive prenatal testing, based on massive parallel sequencing of cfDNA, allows the screening for the most common fetal aneuploidies from a maternal blood sample. Used in the USA since 2011, it has rapidly become widespread in Western Europe and China as a first-line screening test.

In 2020, ACOG has issued a new set of guidelines, recommending that prenatal aneuploidy screening be offered to all pregnant people regardless of their age or other risk factors.

In Portugal, although there are no official guidelines yet for carrying out the NIPT, there has been an increase in the request for this test in pregnant women. In this context, the LCG-FMUC invested in implementing and validating a NIPT using cfDNA sequencing strategy, assessing the feasibility, advantages, and disadvantages of this test.

Based on the results obtained, an attempt was made to determine its effectiveness, profitability, and improvement possibilities.

3 - Objectives

- Description of the preparation and validation stages that led to implementing a NIPT at LCG-FMUC based on MPS using cffDNA circulating in maternal plasma.

- Analysis and interpretation of the results obtained, determining sensitivity and specificity for aneuploidies.

- Correlation of the results with different parameters: Body Mass Index (BMI), Fetal Fraction (FF), quantity/quality of extracted cfDNA, and sample quality.

- Evaluation of the impact of NIPT on reducing invasive tests and the possibility of adjusting the inclusion and exclusion criteria.

4 - Materials and Methods

4.1. Study Design

We present a study describing NIPT implementation process in our laboratory as a first-line prenatal diagnostic screening test for the most common aneuploidies. During the study, the NIPT was offered to pregnant women selected in the CHUC obstetrics consultation according to the established inclusion and exclusion criteria, which we will describe below. The first part describes the technical and laboratory implementation process: sample collection, sample processing, and analysis methodology and reporting. The second part, assumed as a consolidation phase, describes aspects of the logistics and offer of the screening as inclusion criteria, counselling, reporting and limitations of the test analysis.

4.2. Implementation Phase

In order to accommodate our technologies and knowledge we opted for a NIPT based on SNPs genotyping, using cfDNA NGS sequencing (Clarigo™ – Agilent Technologies).
Clarigo™ is an in vitro diagnostic (IVD) medical device, CE-marked intended to screen for the fetal trisomy status of chromosomes 21, 18, and 13, using cfDNA. The technology is based on target amplification of cfDNA present in the maternal blood using a multiplex polymerase chain reaction (PCR) procedure followed by massive parallel sequencing on Illumina (MiSeq®, HiSeq®, or NextSeq®) systems. Generated sequencing data (*FastQ* files) are uploaded to a bioinformatics pipeline software (Clarigo™ Reporter) for data analysis, determining the ploidy status of chromosomes 21, 18, and 13, the fetal fraction, and optionally the fetal gender.
Clarigo NIPT cannot accurately determine fetal ploidy status in the case of: twins gestation, Egg donation IVF pregnancies, placental mosaicism, fetal chimerism, partial fetal chromosomal aneuploidy, vanishing twins, samples with a fetal fraction less than 3%, fetal triploidy or fetal sex chromosomes aneuploidy.

Clarigo™ workflow

The entire Clarigo™ methodology workflow, is divided into five main steps: Sample preparation, Target Amplification, Preparation for sequencing, Sequencing, and Data Analysis (**Fig.11**). Given the length of the protocol, we will only describe a few key points of the protocol.

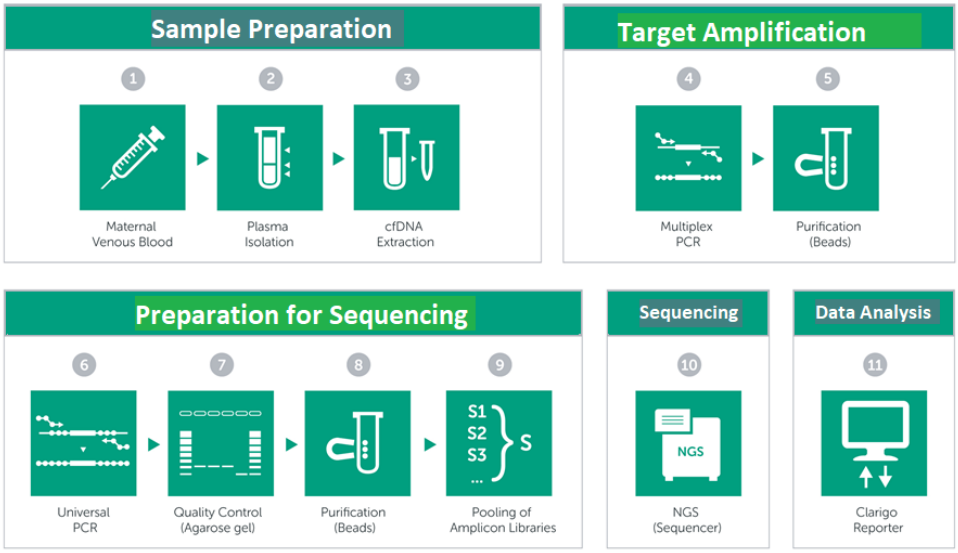


Figure 11 – Clarigo NIPT Workflow
Sample Preparation – Steps 1-3; Target Amplification – Steps 4 and 5;
Preparation for Sequencing – Steps 6-9; Sequencing – 10 and Data Analysis – Step 11.
Adapted from: Clarigo™ / Agilent Technologies.

Sample Preparation (Steps 1 to 3)

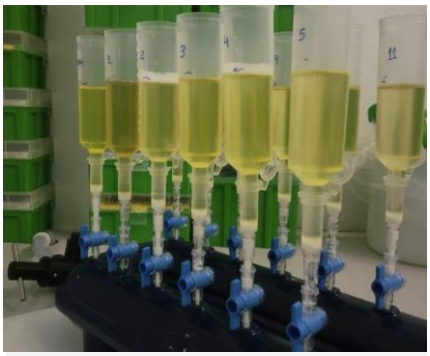


Figure 12 – cfDNA Extraction using QIAVac 24 plus vacuum system.
Source: LCG – FMUC (2020)

For extraction and quantification of cfDNA, approximately 10mL of blood was collected in Streck Cell-Free DNA BCT® (Streck, Nebraska) and processed within 24 hours of harvest. The plasma is isolated by two consecutive centrifugations (1600 x g for 10 minutes and 3200 x g for 20 min at RT, and stored in 2 mL aliquots at -80 °C until analysis. The extraction of cfDNA from plasma samples was performed with the QIAamp Circulating Nucleic Acid

Kit (Qiagen, Germany). In the first stages of the nucleic acid extraction process (digestion, precipitation, and washing), the *QIAVac24 plus* vacuum system is used instead of centrifugation, avoiding cfDNA damage (**Fig.12**).

The total amount of cfDNA was quantified with the Invitrogen Qubit dsDNA HS Assay Kit and Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, California, USA). The measured concentration should be at least 0.10 ng/µl to proceed for next steps.

<u>Target Amplification</u> – Multiplex PCR (Steps 4 and 5)

Each Clarigo experiment includes batches of 12 samples plus a negative control. For each sample, about 6 thousand regions containing SNPs specific to chromosomes 13, 18, 21, X, and Y are amplified in a targeted single tube Multiplex PCR amplification reaction (**Fig.13**).

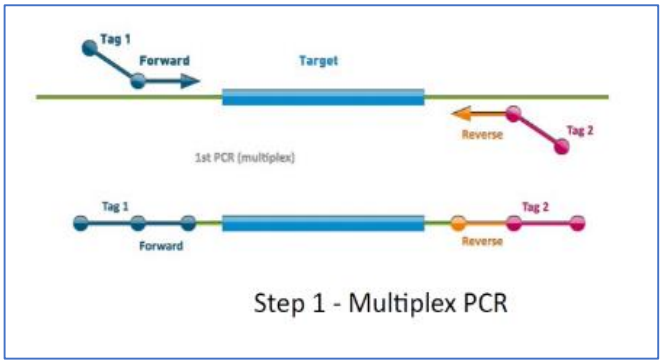


Figure 13 – Multiplex PCR.
In this step, aprox. 6000 SNPs regions are amplified and "tagged" with a specific Illumina adaptor.
Adapted from: Clarigo™ / Agilent Technologies.

After amplification, small residual DNA fragments are removed (**Fig.14**) using Agencourt® AMPure® XP magnetic beads (Beckman Coulter Genomics, USA), used to "capture" NIPT library amplicons, removing inespecific or large genomic DNA fragments. Each Clarigo experiment includes batches of 12 samples plus a negative control. For each sample, about 6 thousand regions containing SNPs specific to chromosomes 13, 18, 21, X, and Y are amplified in a targeted single tube Multiplex PCR amplification reaction (**Fig.13**).

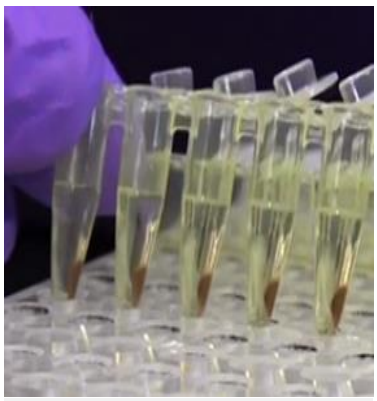


Figure 14 – Multiplex PCR Product purification.
Source: Agilent Technologies

After amplification, small residual DNA fragments are removed (**Fig.14**) using Agencourt® AMPure® XP

magnetic beads (Beckman Coulter Genomics, USA), used to "capture" NIPT library amplicons, removing inespecific or large genomic DNA fragments.

Preparation for Sequencing

Universal PCR (Step 6)

In this phase, a universal PCR is performed to tag all amplicons, from each sample, with specific molecular identifiers (MIDs) and p5 and p7 adaptors (required for sequencing on Illumina next-generation sequencing (NGS) systems) (**Fig.15**).

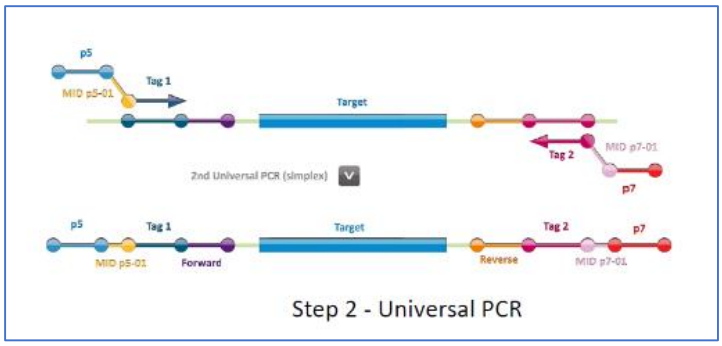


Figure 15 – Universal PCR.
In this step, each sample is tagged with a unique p5/p7 combination. Adapted from: Agilent Technologies.

Quality Control and Purification of libraries (Steps 7 and 8)

In order to assess the success of the amplification of the libraries, a quality control step is performed running 2µl of each library on 2% agarose gel or in Agilent TapeStation 4100 automatic DNA QC system (Agilent Technologies, USA).

Successful amplification is detected as a clearly visible but dispersed band around 200bp on the agarose gel (**Fig.16**).

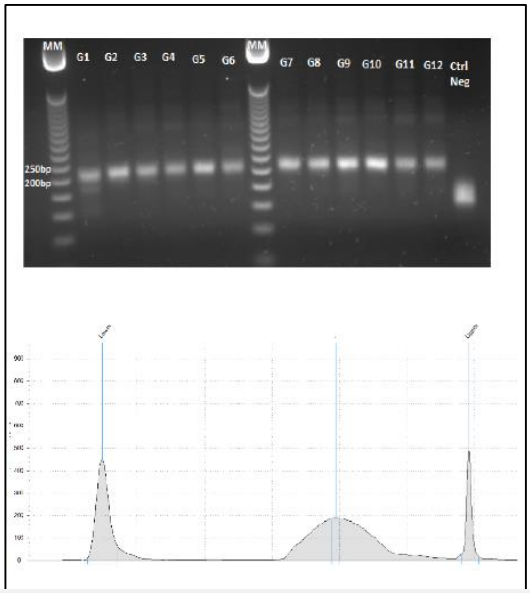


Figure 16 – Clarigo Library QC control.
In this step, the quality of the library is evaluated, through the confirmation of the ideal size of the fragments (216 bp) and the absence of unspecific products. Up – Agarose 2% Gel, Down – TapeStation QC
Source: LCG - FMUC

Pooling and Sequencing

Each amplicon library is purified with magnetic beads to remove small residual DNA fragments.

After purification, all the libraries of the bench (10-12) are quantified with the Invitrogen Qubit dsDNA HS Assay Kit using Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, California, USA). Libraries with a concentration below 5 ng/µl cannot be sequenced, being necessary to repeat the process or even a new blood sample. For the final amplicon pool, the input of each sample is calculated using the equation:

$$\text{Amplicon pool conc [nM]} = \frac{\text{Amplicon pool conc [ng/µl]} \times 10^6}{656.6 \times 216 \text{ bp}}$$

To make the calculation of volumes easier, and to guarantee equimolarity for all samples, an Excel table is used (**Fig.17**).

Sample ID	C° 1 (ng/µL)	C° 2 (ng/µL)	C° 3 (ng/µL) (Optional)	Average	Volume per library	ng per library
Ni126	18.8	19.6		19.2	5.11	98.1
Ni127	18.9	19.3		19.1	5.14	98.1
Ni129	18.2	17.8		18.0	5.45	98.1
Ni130	24.6	24.9		24.8	3.96	98.1
Ni131	21.1	21.2		21.2	4.64	98.1
Ni133	18.5	19.3		18.9	5.19	98.1
Ni134	19.6	20.2		19.9	4.93	98.1
Ni135	19.8	19.1		19.5	5.04	98.1
Ni136	15.9	16.8		16.4	6.00	98.1
Ni137	18.1	17.6		17.9	5.50	98.1
Ni138	16.9	16.6		16.8	5.86	98.1
Ni139	20.4	21.4		20.9	4.69	98.1
Insert rows above						

Total ng	Total volume		Theoretical Pool C° **(ng/ul)**	Average Amplicon Size	Theoretical Pool C° **(nM)**
1177.2	61.51		19.14	216	134.95
C° 1 (ng/µL)	C° 2 (ng/µL)	C° 3 (ng/µL) (Optional)	Measured Pool C° **(ng/ul)**	Average Amplicon Size	Measured Pool C° **(nM)**
19.23	19.48		19.36	216	136.47

Pool C° (nM)	136.47	**4**
Volume of previous Pool/Dilution	NA	10.00
Volume TE (µL)	NA	331.18

Figure 17 - Excel Sample sheet example for Library final pool calculation

After preparation of the final pool, diluted to 5.3pM to achieve optimal sequencing density, so that the *flowcell* is not overloaded, the library is run on the MiSeq® platform (Illumina) in a single read regime of 76 cycles, with an approximate duration of 10 hours. (**Fig.18**).

Figure 18 – MiSeq NGS System (Illumina)
Source: LCG-FMUC (2022)

Clarigo™ Reporter - Data analysis

After sequencing, the result files are generated in *FastQ* format. These files include, in addition to the sequences, a quality control score, assigning confidence to a particular base within a read. These files are uploaded to the Clarigo Reporter, a dedicated software tool for automated analysis. The valid sequences are aligned with the human reference genome, and variants (SNPs in our case) are identified. In this step, the most crucial parameter is the number of reads that cover a given sequence, also known as sequence coverage; the higher, the better. After the alignment and annotation of the variants, it is then possible to obtain information on the alignment's quality and mapping quality and compare the maternal and fetal genotypes. The results are generated in the format of a report (**Fig.19**), which contains essential parameters such as FF, aneuploidies calculated risk, and gender of the fetus. The risk of aneuploidies on chromosomes 13, 18, and 21 is determined through a statistical calculation of the Z-score. The Z-score provides evidence or not for the presence of fetal trisomy.

Clarigo

Sample Ni129_S3_L001_R1_001

Test Results Agilent

Fetal fraction*: 6.0%

Condition tested	Trisomy Call*	Trisomy Evidence*	Z-score*
Trisomy 13	negative	-5.4	-0.9
Trisomy 18	negative	-4.4	0.5
Trisomy 21	negative	-6.1	-0.9

Gender evidence*: 0.99. Gender call*: female.

Process parameters

*Sample coverage**:	2.571M reads
*Sample correlation**:	99.58%
*Sample correlation σ-fold**:	0.3
*Classified read fraction**:	96.86%
*Primer dimer read fraction**:	2.56%
*Homozygous coverage fraction**:	54.89%

Figure 19 – Clarigo Analisys Report
Source: LCG-FMUC (2022)

We develop this matter further in section 4.3.5, dedicated to the analysis and reporting procedure.

LCG-FMUC Clarigo™ Implementation

Implementing this NIPT test at the LCG-FMUC was a relatively quick process. It required a series of preparatory steps (**Fig.20**). In September 2018, the training phase occurred at the supplier's facilities for familiarization, execution, and approval of the workflow.

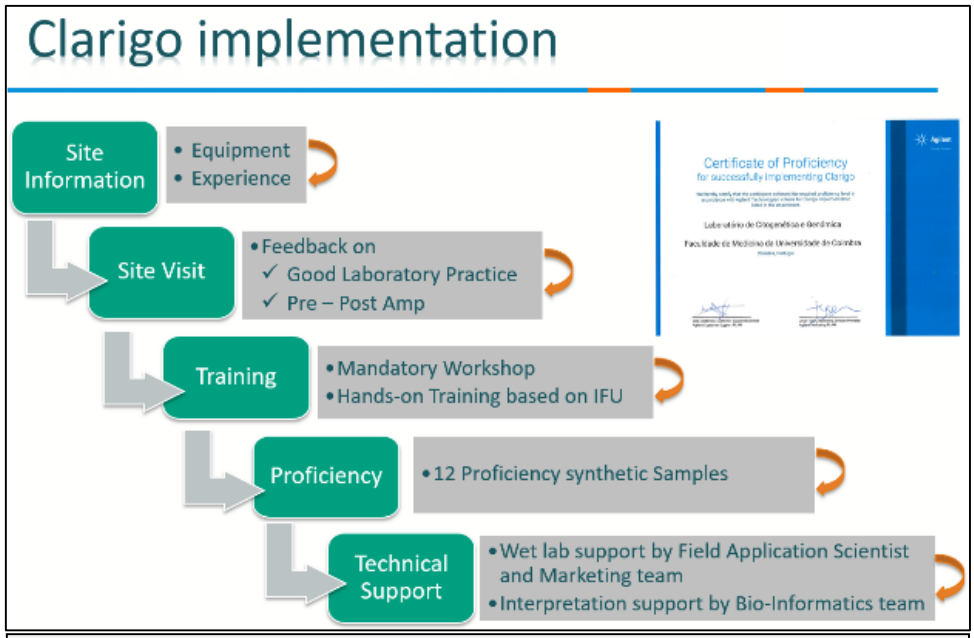


Figure 20 - Implementation process of Clarigo NIPT in LCG-FMUC.

At the end of 2018, the proficiency stage at the LCG-FMUC took place. It included a run of 12 synthetic workflow validation samples performed in the presence of Application Scientist evaluators. In collaboration with CHUC, 24 pregnant volunteers with known invasive results were selected to validate the platform and create references for the informatics pipeline. In January 2019, the certificate and proficiency approval was granted to the LCG-FMUC, officially allowing its use after rechecking the entire workflow.

4.3. Consolidation Phase

4.3.1. Information and Counselling

During the first-trimester obstetrics consultation, and according to the established inclusion criteria, pregnant women were informed and asked about the possibility of performing a non-invasive screening test. The candidates were clarified by a genetic counselor who informed the couple of the advantages and limitations of prenatal screening, the possibility of positive results that would need to be confirmed by invasive tests, and the possibility of incidental findings not expected of the pregnant genome. After an Informed Consent (See Supplemental Data – **Pag. 65**), a sample was collected.

4.3.2. Inclusion and Exclusion criteria

Defined Inclusion criteria for NIPT:

- Unifetal pregnancies,
- Combined 1st or 2nd trimester screening with increased risk of aneuploidy ≥ 1/300 for trisomy 21,
- Family history or previous pregnancy with chromosome 13/18/21 aneuploidy,
- Member of the couple carrier of a Robertsonian translocation involving chromosomes 13 and 21,
- Couples with Turner or Klinefelter syndrome.

Defined Exclusion criteria for NIPT:

- Twin pregnancies,
- Advanced maternal age without combined screening,
- Significant obesity (BMI ≥40 kg/m2),
- Member of the couple with Robertsonian translocation involving chromosomes 14 and 15,
- Fetal NT measurement greater than P99 or with a *major* malformation.

Participants under 18 years, women with a current malignancy, who received blood transfusions in the last three months, and who had stem cell therapy or organ transplantation were excluded.

4.3.3. Sample collection

10 ml maternal blood samples were collected in one Cell-Free DNA BCT CE tube (Streck, La Vista, NE, USA) (**Fig.21**), according to the pre-established procedures, conserved at room temperature, and sent to LCG-FMUC in the 1st 24 hours after collection and always accompanied by the respective requisition and term of responsibility.

Figure 21 - Streck cfDNA Blood collection Tubes processing.
Source: LCG – FMUC (2020)

4.3.4. Laboratory Sample Registration and Processing

The reception and registration of samples at the LCG-FMUC obeys the pre-established and certified workflow that includes: 1- Computer registration of the user's data in the database; 2- Creation of anonymized internal code and printing of labels for marking the samples received; 3- Internal control of the laboratory procedure at all stages.

4.3.5. NIPT analysis, Quality Control and Aneuploidy Risk Reporting

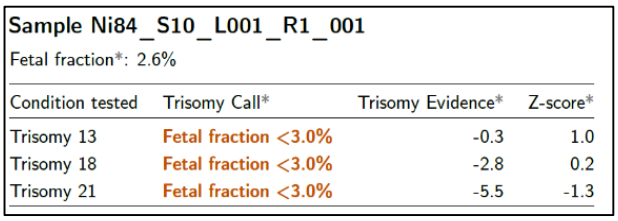
Clarigo Reporter is an online computing pipeline designed and optimized for Clarigo 2.0 data obtained by NGS, facilitating rapid and reliable determination of fetal ploidy status for chromosomes 21, 18, and 13. The analysis is done online through a password-protected account on the analysis computer.

4.3.5.1. NIPT analysis

The *FastQ* files to be analyzed, for each pregnancy, are downloaded directly from the Illumina *BaseSpace* cloud or directly from the NGS equipment used, copying the files

from the output folder selected in the run configuration. After completing the automatic analysis process, the results generated are printed as well as the quality control report. For each of the samples under analysis, the following parameters are evaluated:

Fetal Fraction (FF)



Sample Ni84_S10_L001_R1_001

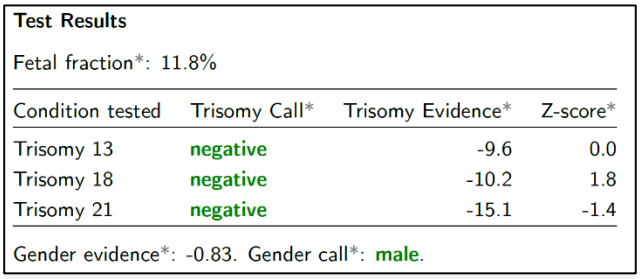
Fetal fraction*: 2.6%

Condition tested	Trisomy Call*	Trisomy Evidence*	Z-score*
Trisomy 13	Fetal fraction <3.0%	-0.3	1.0
Trisomy 18	Fetal fraction <3.0%	-2.8	0.2
Trisomy 21	Fetal fraction <3.0%	-5.5	-1.3

Figure 22 - Clarigo output Report for Low FF.
Source: LCG – FMUC (2022)

The FF value that is calculated automatically and must be greater than 3% (**Fig.22**). Otherwise the result will be inconclusive due to low fetal fraction (LFF).

Trisomy Call



Test Results

Fetal fraction*: 11.8%

Condition tested	Trisomy Call*	Trisomy Evidence*	Z-score*
Trisomy 13	negative	-9.6	0.0
Trisomy 18	negative	-10.2	1.8
Trisomy 21	negative	-15.1	-1.4

Gender evidence*: -0.83. Gender call*: male.

Figure 23 - Clarigo output Report for Trisomy call
Normal Result in this Example Source: LCG – FMUC (2022)

The classification limits of the results for the presence of fetal aneuploidy are established in a score (Z-Score) optimized to achieve high sensitivity and specificity (**Fig.23**). Due to differences associated to biological differences between the target chromosomes, different Z-Score thresholds were defined for chromosomes 21,18, and 13:

<u>Chromosome 18 and 21</u>

Negative for aneuploidy if: Z-score is < 3.5 and trisomy evidence is ≤ −0.5

Positive for tisomy if Z-score is > 4 and trisomy evidence is ≥ 2.0

Chromosome 13

Negative for aneuploidy if: Z-score is < 4 and trisomy evidence is ≤ −0.5

Positive for tisomy if Z-score is > 3.5 and trisomy evidence is ≥ 2.0

Evidence Chart for Trisomies 13, 18 or 21

To facilitate interpretation, the analysis includes an evidence chart that combines the FF value (vertical axis) with the score of evidence (horizontal axis), where the dotted line indicates the Z-score value. The blue dot indicates the sample to which the analysis refers (**Fig.24**). A dot in the "green" zone will be a sample with a negative result for trisomy, and in the "red" zone will be a sample with a high risk for trisomy 21. Out of the "green" or "red" zones, the result is inconclusive.

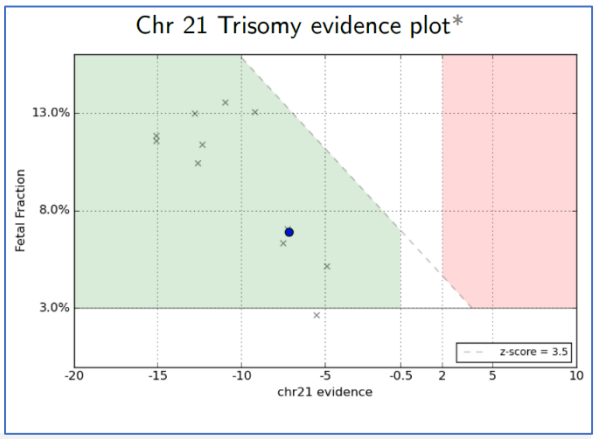


Figure 24 - Clarigo Reporter Evidence chart of T21
Normal Result in this Example Source: LCG – FMUC (2022)

Gender information

Fetal sex is determined from the coverage of informative SNPs on the X and Y chromosomes. If a non-maternal X chromosome and the absence of the Y chromosome are detected, the fetus is female. If a non-maternal X chromosome and a Y chromosome are detected, the fetus is male (**Fig.25**).

Male - Gender evidence < -0.3

Female – Gender evidence > 0.3

The evidence plot for fetal gender determination combines the FF value (vertical

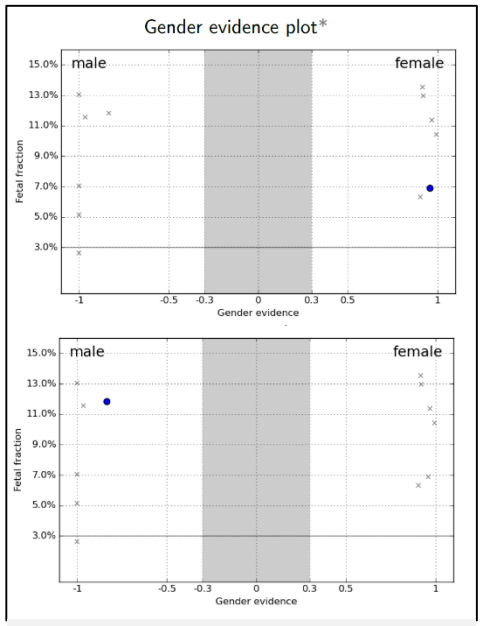


Figure 25 - Clarigo Reporter Gender plot
Up – Female Down - Male
Source: LCG – FMUC (2022)

axis) with the gender score representation (horizontal axis). The blue dot indicates the sample to which the analysis refers. If a dot is in the “male” zone,
it will be a male sample. If it is in the “female” zone, it will be a female sample. Results outside the “male” or “female” zones do not allow a conclusive result.

Result “Not Automatically Called”

Whenever it is not possible to automatically arrive at a conclusive result, the generated report will be of not automatically called (NAC). It arises when the fetal fraction is less than 3% or when the quality control parameters (QC Report) are not achieved.
Samples with a NAC result will have to be re-evaluated, and if the result does not allow the establishment of a conclusive result, and an Inconclusive result will be reported.

4.3.5.2. Quality Control

Processing parameters for each of the samples individually in the NGS run are verified as quality control criteria. For each of the samples under analysis, there must be a compliance in each one of the following parameters:

Sample Parameters

Sample coverage - Defined as the total number of reads per sample coincident with the targets. Clarigo panel v2 reference, ideally above 2.4M reads (**Fig.26**).

Process parameters	
Sample coverage:*	2.776M reads
Sample correlation:*	99.65%
Sample correlation σ-fold:*	-0.2
Classified read fraction:*	96.98%
Primer dimer read fraction:*	2.21%
Homozygous coverage fraction:*	56.65%

Figure 26 – Sample Parameters QC
Source: LCG – FMUC (2022)

Sample correlation - Indicates the degree of correlation between the sample and the other samples analyzed in the same run. Values greater than 95% indicate a strong correlation and robustness in the results.

Sample correlation (σ-fold) - Defines the degree of sample divergence with the correlation average of all the others.

Primer dimer read fraction - Indicates the fraction of reads in a sample corresponding to primer dimers (not used). Ideally, it should be below 5%.

Homozygous coverage fraction - Defines the DNA fraction of a sample in the homozygous state, allowing determining the number of genomes present in the sample. The result will be between 50 and 60%. Values below 50% may indicate a third source of DNA (contamination) from a vanishing twin, egg donation or external.

Run Parameters (Run)

Number of rejected samples - Indicates the number of samples who did not pass the run QC (**Fig.27**).

Sample coverage - Should be higher than 2.4Mb.

Average Sample correlation – Ideally greater than 98%,

Average Primer dimer read fraction - Should be less than 5%.

Graph Read Count – Shows the distribution of the total number of reads per sample in the run, including the percentage of reads due to primer-dimers or unranked, allowing quick visualization of the robustness and heterogeneity of the samples.

Run Ni 108 22
Process parameters

Created by:	luismiguel.pires
Sequencing device:	MiSeq
User provided Lot number:*	2005190
User provided srMID lot number:*	2008033
Reference runs:	NI105-22 NI107-22 NI_104_NI 106 22
Sample count in current run;	12;
Rejected samples due to low coverage:	0
Rejected samples due to low correlation:	0
Average Sample coverage:*	2.565M ± 0.280M reads
Average Sample correlation:*	99.66% ± 0.04%
Average Classified read fraction:*	94.59%
Average Bad quality read fraction:*	0.00%
Average Primer dimer read fraction:*	4.71%
Average Unclassified read fraction:*	0.70%

Figure 27 – Run QC parameters
Source: LCG – FMUC (2022)

4.3.5.3. Aneuploidy Risk Reporting

After finishing the analysis and confirming the results, a final report of the risk of aneuploidy and fetal sex is sent to the requesting physician, indicating one of three possible results:

- Negative for the presence of aneuploidies.
- Positive with high risk for the presence of aneuploidy. Recommending an invasive test (amniocentesis) for confirmation. (See supplemental data, **Pag. 66**)
- Inconclusive for the presence of aneuploidies. Depending on the cause (Low fetal fraction, 3rd DNA source, or other), blood resample for NIPT repetition may be suggested.

4.3.6. Statistical Analysis of NIPT Results

In order to evaluate the performance of NIPT, and the impact of specific factors inherent to the samples: fetal fraction, body mass index, and sample quality, in obtaining inconclusive results, a statistical analysis was done.

Quantitative data was presented using mean and standard deviation (+sd) and median [quartile 1; quartile 3], and qualitative data was presented using absolute and relative frequencies. For the samples with uninformative results, comparisons between each group or type of inconclusive results with the conclusive ones were performed using the Mann-Whitney U test due to the lack of normality of sample data (evaluated through the Shapiro-Wilk test) while the association between those groups and qualitative data was performed using the Fisher exact test and odds ratio were computed. Adjustment for multiple comparisons was not performed as those comparisons were used in an exploratory step to identify features for classification. Confidence intervals (95%) were determined whenever possible.

Afterward, receiver operating characteristic curve (ROC) analysis was performed in order to assess threshold values for discriminating each type of inconclusive results for every feature identified as statistically significant in the prior step. Then multivariate logistic regression models were applied, considering all the previous identified features,

but none converged. When either weight and BMI were identified, only BMI was considered in order to avoid multicollinearity.

This analysis was replicated for the set group of inconclusive tests that were repeated once. Only descriptive statistics were presented for the second repetition due to the small sample size.

Analysis was performed in SPSS (V.27) and was analysed at a 5% significance level.

5 - Results

5.1. Sample Characterization

A total of 1130 pregnant women that took the NIPT test were evaluated and the demographic structure of the sample studied, considering age, BMI, gestation week, and their impact on FF.

5.1.1. Age Distribution

The Sample's age ranged between 17 and 56 years, with a mean value of 35.8 (sd 5.3) and a median of 33 years [32, 40] (**Fig.28**).

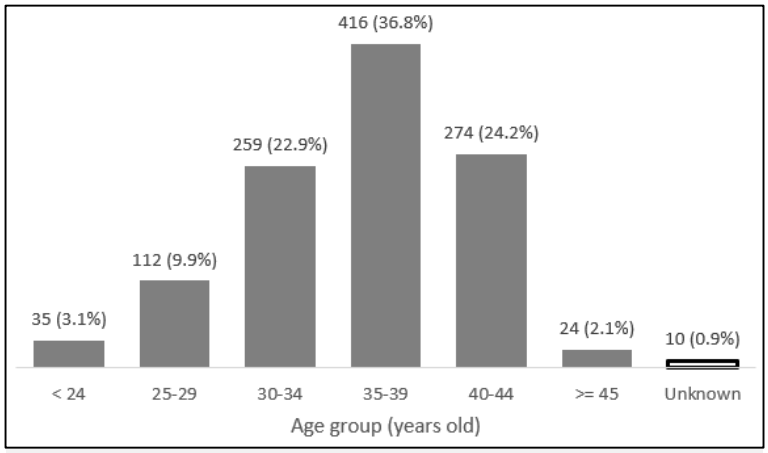


Figure 28 – Sample distribution per age – Media age= 35.8 (sd 5.3); Mediana age = 33. Age group [35-39] is the more representative.

As expected, the sample distribution by age is higher for pregnant women between 35 and 39 years old, representing more than a third of the sample.

5.1.2. Gestation Age

Gestation age varied between 66 and 224 days (corresponding to 9.4 and 32 weeks), with a mean value of 106.4 days (± 17.4) which corresponds to 15.4 weeks (± 2.5). The median was 102 days [95, 115], equivalent to 14.6 weeks [13.6, 16.4] (**Fig.29**).

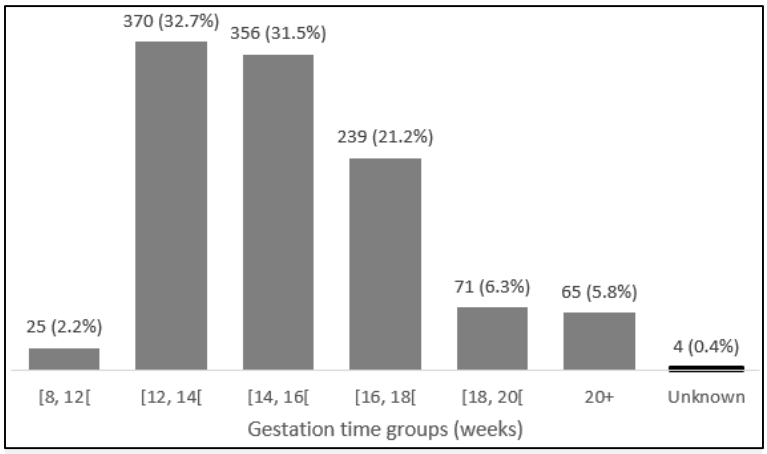


Figure 29– Distribution by Gestation age – Mean value of 106,4 days (15w+1d) and Median of 102 days (14w+4d).

The NIPT was performed mainly between 12 and 16 weeks, corresponding to the theoretically ideal period to have a high enough FF and before the end of the second trimester to allow timely pregnancy management.

5.1.3. Weight and Body Mass Index

Pregnant women weight and body mass index in this sample varied respectively between 38-120 kg and 13.9 – 46.8 kg/m2, presenting mean values of 69.2 (+ 13.6) kg and 26.2 (+ 5.0) kg/m2. Half of the pregnant women had a weight below 66 kg and presented almost normal BMI at the time of the test (median 25.2 kg/m2 and quartiles [22.5, 29.34]). 50.8% of these women were overweight (28.3%) or obese (22.5%) (**Fig.30**).

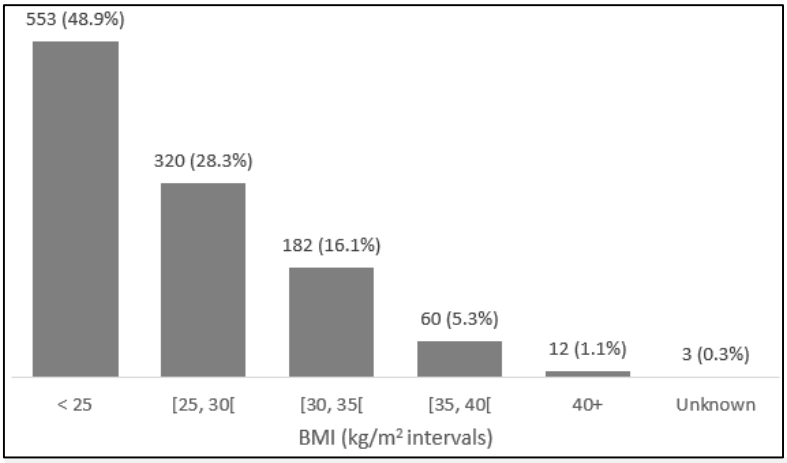


Figure 30 – Weight and Body mass Index Distribution - Half of the pregnant women had their weight below 66 kg and presented almost normal BMI at the time of the NIPT.

5.1.4. Fetal Fraction

Fetal fraction ranged from 0% (FF undetermined) to 23.5%. The mean and median values for this variable were respectively 7.5% (±3.4%) and 7.1% [5.3%, 7.3%] (**Fig.31**).

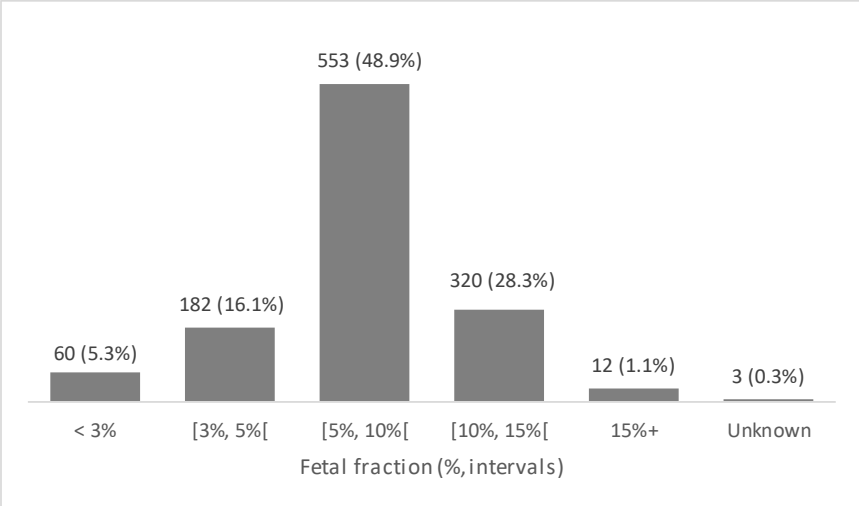


Figure 31– Fetal Fraction distribution – More than 90% of the samples with expected FF between 3 and 15%. FF Media = 7.5% (+ 3.4%). FF median = 7.15.

5.2. High Risk Aneuploidy Results

Of the received NIPT samples, 9 (0,8%) had positive high risk for aneuploidy, 992 (87,8%) were negative (low risk for aneuploidy), and 129 (11.4%) were inconclusive (**Fig. 32**). We identified eight trisomies of chromosome 21 (six females and two males) and one female trisomy 13. The female/male ratio for trisomy 21 was 6:2, but due to the small sample size for positive results, any generalization would not have sufficient statistical power.

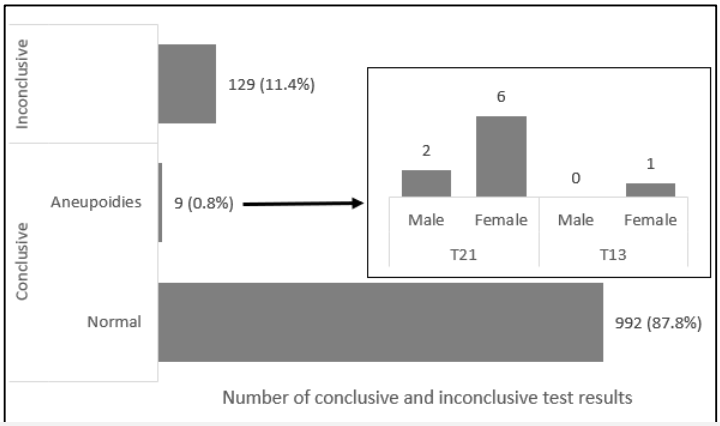


Figure 32– High Risk Aneuploidies and Incidence in the Sample
Left: Distribution of Aneuploidy, Normal and Inconclusive Results.
Up/Right: T21 and T13 Distribution

Table III.1 - High Risk Aneuploidy Cases - Motifs

ID	NIPT Result	FF	Plasma	Mother Age	BMI	NIPT Motif
NI13_19	Trisomy 21 Male	8%	OK	32	23.42	1:164 T21 Risk
NI40_19	Trisomy 21 Male	4.5%	OK	40	36.73	1:50 T21 Risk
Ni75_19	Trisomy 21 Female	9.9%	OK	19	25.32	1:340 T21 Risk
Ni160_19	Trisomy 21 Female	8.8%	OK	35	27.72	1:28 T21 Risk
Ni190_19	Trisomy 13 Female	5.9%	OK	29	23.44	1:4 T21 Risk; 1:8 T13 Risk
Ni358_19	Trisomy 21 Female	7.8%	OK	41	20.83	1:4 T21 Risk
NI198_20	Trisomy 21 Female	4.4%	OK	41	31.64	1:12 T21 Risk
NI271_20	Trisomy 21 Female	6.2%	Haemolysed	41	27.72	1:72 T21 Risk
NI39_21	Trisomy 21 Female	6.9%	OK	38	27.1	1:11 T21 Risk

Regarding pregnant women with a positive result for aneuploidy (**Table III.1**), 66% of pregnant women are of advanced maternal age. On the other hand, one of the pregnant women, with a T21 female fetus, was only 19 years old, showing the importance of NIPT screening being affordable to be used at any age. Relatively to the case NI 190_19 (trisomy 13), it presented a very high risk for both T21 and T13, which the NIPT confirmed.

5.3. Inconclusive Results and Failure rate

In 129 cases studied by NIPT, it was impossible to reach a conclusive result, representing a failure rate of 11.4%. Of these 129, 66 women repeated a second blood collection and a new analysis, showed 15 cases with a second inconclusive NIPT, 51 with a low aneuploidy risk, decreasing our failure rate to 6.9%.

To try assess the causes of our high failure rate, we subdivided the initial sample of 129 inconclusive cases in four subgroups by inconclusive testing reasons: Low fetal fraction (LFF), Non-automatic call (NAC), presence of third DNA source (3rd DNA) and unknown causes (Other).

5.3.1. Inconclusive NIPT Causes

Table III.2 – Inconclusive NIPT distribution by causes

Inconclusive Nipts - Causes	n (%)
LFF (Solo)	24 (18.5%)
LFF (Combined with other causes)	60 (46,5%)
NAC	37 (29.2%)
3rdDNA	16 (12.3%)
Other	52 (40.0%)

LFF – Low Fetal Fraction (< 3%); **NAC** – No Automatic Call; **3rdDNA** – Contamination with a 3rd source of DNA; **Other** – Unknown.

Table III.2 shows that the causes of inconclusive results vary, and it is not always easy to address a cause. However, we can easily conclude that almost half (40%) is due to a lack of amplification or short sequencing that may reflect unknown cfDNA contaminants. On the other hand, we can also say that 60% of the cases had a known justification since the samples had LFF or there was a NAC, and in 16 cases, we could ascertain the possibility of a 3rd source of DNA, that is assumed limitations of this type of NIPT.

5.3.2. Sample group of Inconclusive NIPT characterization

Table III.3 - Women characterization

	N	Min - Max	Mean (SD)	Median [Q1, Q3]
Age	129	21 - 48	35.6 (5.6)	36 [31; 40]
Weight (kg)	129	45 - 114	72.5 (16.3)	70 [59; 81]
Height (m)	129	1.5 - 1.8	1.6 (0.1)	1.6 [1.6; 1.7]
BMI (kg/m2)	129	17.6 - 46.7	27.5 (6)	26.3 [23.4; 29.7]
Gestation age (days)	128	70 - 179	106.3 (17.2)	103 [95; 114]
Gestation age (weeks)	128	10 - 25.6	15.2 (2.5)	14.7 [13.6; 16.3]

Among those 129 women who presented inconclusive results, the media age was 35.6 and the gestation age 106.3 days (**Fig.28** and **Fig.29**). Considering BMI, media was slightly higher (27,5 kg/m^2), compared to the total population (25,2 kg/m^2), with 53 women (41.1%) presenting normal BMI (below 25 kg/m^2), 44 (34,1%) were overweight and 32 (24.8%) were obese (BMI above 30 kg/m^2) (**Fig.33**).

If we compare the distribution of inconclusive cases caused by LFF (**Table III.2**) with BMI distribution in the inconclusive group, it is clear that LFF is more frequent in the Obese Subgroup with an incidence of 37,5% (**Fig. 33**), as we will discuss with statistical support.

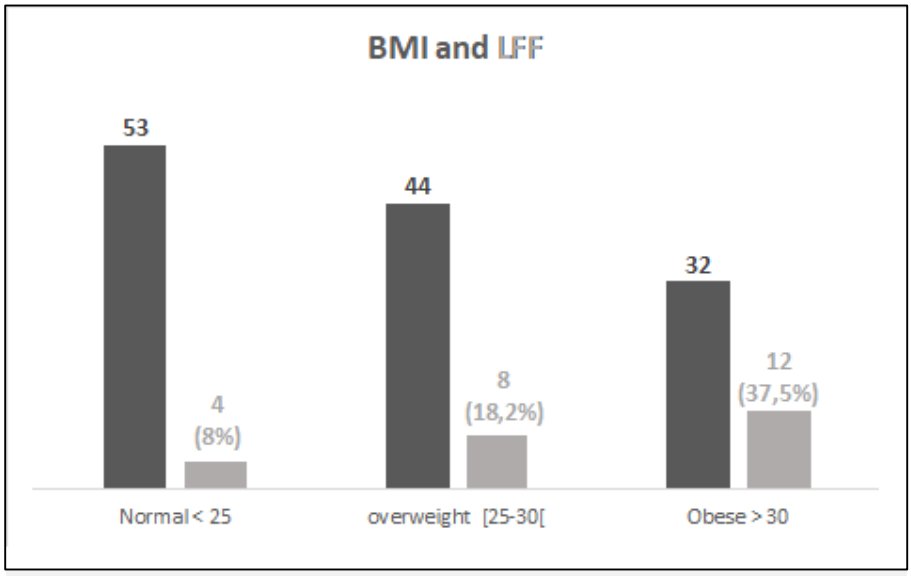


Figure 33– BMI distribution in the Inconclusive group and LFF correlation.
Normal (41,1%); Overweight (34,1%); Obese (24,8%).

5.3.3. Blood Sample Quality and cfDNA extracted

In the 129 cases with inconclusive results, blood characterization according to fetal fraction, the volume of plasma collected, and cfDNA concentration is presented in Table III.4.

Table III.4 - Blood characterization

	n	Min - Max	Mean (SD)	Median [Q1, Q3]
FF	129	0 - 13.9	3.5 (3.3)	3.1 [0; 5.8]
Plasma (ml)	129	4 - 5	4.5 (0.1)	4.5 [4.5; 4.5]
[cfDNA] (ng/µl)	129	0.2 - 2.3	0.7 (0.4)	0.7 [0.5; 0.9]

The fetal fraction was below 3% in 61 valid 129 samples analyzed (47.3%). The fetal fraction was within 3%-5% for 31 women (24.0%), within 5%-10% for 31 women (24.0%) and within 10%-15% for 6 women (4,6%) (Data not shown).

The media of cfDNA concentration obtained was 0.7 ng/µl.

The frequency of haemolyzed blood in the total sampling was 9,6% and clotted blood was 0.6%. If compared with 129 women of inconclusive group, haemolyzed blood was detected in 33 (25.6%) samples, clotted blood was detected in 4 (3.1%), which is considerably significant (**Fig. 34**). Haemolysis or blood clotting is an essential factor in NIPT. Of these 37 samples which did not present good plasma, 17 repeated blood collection, and NIPT analysis, 13 had conclusive results (76,5%) (Data not shown).

Table III.5 – Distribution of inconclusive tests according to cfDNA concentration

cfDNA [concentration] (ng/µl)		N (%)
[0.2, 0.5[	Ideal cfDNA [ ]	39 (30.0%)
[0.5, 0.8[	Good cfDNA [ ]	45 (34.6%)
[0.8, 1.2[	(cfDNA probably contaminated with genomic DNA)	31 (23.8%)
$\geq$ 1.2	(Bad cfDNA High probability of contaminated with genomic DNA)	14 (10.8%)

The cfDNA concentration obtained from the 129 women in inconclusive group had a good or ideal concentration in more than 64% of the cases (**Table III.5**).

5.4. Features related to inconclusive test results

Observed and expected rates for each variable according to the type of inconclusive test, were calculated using Fisher exact test. Odds ratios were also computed for statistically significance between variables (**Fig. 34**).

It seems that Inconclusive tests due to LFF, NAC, or other causes seem to be related to the presence of haemolyzed blood. However, clotted blood may be mainly related to LFF inconclusive tests. As the number of samples presenting clotted blood is too small, this result must be carefully interpreted.

It seems from our results that, in the presence of haemolyzed blood, the chance of obtaining an inconclusive test increases about 4 times for LFF inconclusive tests, about 7.4 times for NAC inconclusive tests, and about 5.1 times for other types of inconclusive

tests. The chance of obtaining an LFF inconclusive test is approximately 19 times higher in the presence of clotted blood.

Pregnant women's blood which turns on an inconclusive test classified as LFF, seems to have higher BMI than those who have conclusive tests (**Table III.6**; median differences of 10.5kg and 5.2 kg/m2). Another characteristic is that the fetal fraction seems to be smaller (almost 5%) and cfDNA concentration higher than those presenting conclusive tests.

Variable	Group	n (%)	IC95%	p	Odds ratio
Haemolyzed blood	Comclusive	76 (7.2%)	5.7% - 8.8%		
	INC LFF	7 (24.1%)	8.6% - 39.7%	**0.005**	4.1
	INC NAC	15 (36.6%)	21.8% - 51.3%	**< 0.001**	7.4
	INC 3rdDNA	1 (5.6%)	0% - 16.1%	1.000	-
	INC other	16 (28.6%)	16.7% - 40.4%	**< 0.001**	5.1
Clotted Blood	Comclusive	2 (0.2%)	0% - 0.5%		
	INC LFF	1 (3.4%)	0% - 10.1%	**0.007**	18.8
	INC NAC	1 (2.4%)	0% - 7.2%	0.142	-
	INC 3rdDNA	0 (0%)	0% - 0%	1.000	-
	INC other	1 (1.8%)	0% - 5.3%	0.188	-
Heamolized or clotted blod	Comclusive	78 (7.4%)	5.8% - 9%		
	INC LFF	8 (27.6%)	11.3% - 43.9%	**< 0.001**	4.8
	INC NAC	16 (39%)	24.1% - 54%	**< 0.001**	8
	INC 3rdDNA	1 (5.6%)	0% - 16.1%	1.000	-
	INC other	17 (30.4%)	18.3% - 42.4%	**< 0.001**	5.4

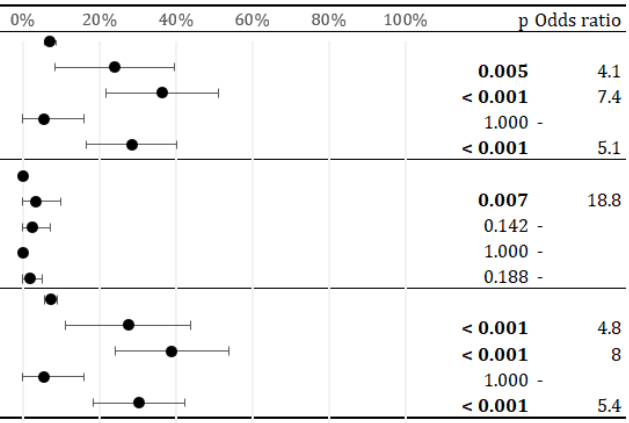


Figure 34 – Observed and expected rates (95% confidence) for each variable according to the type of inconclusive test and p-values for the Fisher exact test. Odds ratios were computed for statistically significant

NAC inconclusive tests are characterized by a low fetal fraction compared to conclusive tests.

Inconclusive results due to the presence of 3rdDNA seem to occur in women with a lower weight. However, no statistically significant differences were found in the BMI of both groups.

At the same time, inconclusive results due to other causes seem to occur in younger women with lower fetal fractions and higher concentrations of fetal fraction compared to conclusive tests.

Table III.6 – Descriptive and comparisons (Mann-Whitney U test) between each major group of inconclusive tests (LFF, NAC, 3rd DNA, other) and conclusive results

Variable	Group	N	Min – Max	Mean (SD)	Median [Q1, Q3]	P
Age (years)	CONC	991	17 - 56	35.8 (5.3)	37 [33; 40]	
	INC LFF	24	28 - 46	36 (5.1)	36 [31.3; 39]	0.544
	INC NAC	38	28 - 44	37.3 (4.8)	38 [33; 42]	0.121
	INC 3rd	16	24 - 43	36.2 (5.1)	37 [34; 39]	0.815
	INC Other	51	21 - 48	33.9 (6.1)	35.5 [29; 38]	**0.022**
Weight (kg)	CONC	998	38 - 120	68.7 (13.5)	66 [59; 77]	
	INC LFF	24	58 - 114	83.8 (16.8)	76.5 [70; 98.4]	**< 0.001**
	INC NAC	38	45 - 114	70.9 (16.3)	68 [58; 78]	0.421
	INC 3rd	16	47 - 83.5	60.9 (10.2)	58 [55; 68.8]	**0.022**
	INC Other	51	48 - 108	72.1 (14.8)	69 [61.3; 80.5]	0.119
Height (m)	CONC	999	1.5 - 1.8	1.6 (0.1)	1.6 [1.6; 1.7]	
	INC LFF	24	1.5 - 1.8	1.6 (0.1)	1.6 [1.6; 1.7]	0.145
	INC NAC	38	1.5 - 1.8	1.6 (0.1)	1.6 [1.6; 1.7]	0.939
	INC 3rd	16	1.5 - 1.7	1.6 (0.1)	1.6 [1.6; 1.7]	0.221
	INC Other	51	1.5 - 1.7	1.6 (0.1)	1.6 [1.6; 1.7]	0.535
BMI (kg/m^2)	CONC	998	14 - 44.1	26 (4.9)	25 [22.5; 29.3]	
	INC LFF	24	23.3 - 46.7	32.3 (6.5)	30.2 [27.5; 38.4]	**< 0.001**
	INC NAC	38	19 - 39.4	26.7 (5.2)	26 [22.4; 29]	0.284
	INC 3rd	16	19.3 - 27.9	23.5 (2.5)	23.8 [20.8; 25.4]	0.083
	INC Other	51	17.6 - 41.2	27.2 (5.8)	26.2 [22.5; 30.3]	0.194
Gestacion age (days)	CONC	998	66 - 224	106.4 (17.4)	102 [95; 115]	
	INC LFF	24	85 - 142	106.6 (18)	101 [92; 123]	0.831
	INC NAC	38	86 - 179	111.4 (20.1)	109 [97; 124]	0.100
	INC 3rd	16	78 - 139	101.7 (15.4)	98.5 [94.3; 106.5]	0.592
	INC Other	50	70 - 150	103.7 (14.2)	102 [94.8; 112.3]	0.552
FF (%)	CONC	998	3 - 23.3	8 (3.1)	7.5 [5.7; 9.6]	
	INC LFF	24	0 - 3	2.2 (1)	2.7 [2.3; 2.9]	**< 0.001**
	INC NAC	35	0 - 7.7	0.2 (1.3)	0 [0; 0]	**< 0.001**
	INC 3rd	16	0 - 12.9	6 (3.6)	7.3 [3.8; 7.8]	0.176
	INC Other	51	0 - 13.9	5.6 (2.8)	4.6 [3.7; 6.8]	**< 0.001**
[cfDNA] (ng/µl)	CONC	1000	0.1 - 3.3	0.6 (0.3)	0.6 [0.4; 0.7]	
	INC LFF	24	0.4 - 2.3	1 (0.4)	0.9 [0.8; 1.2]	**< 0.001**
	INC NAC	38	0.3 - 2.2	0.7 (0.4)	0.5 [0.4; 0.9]	0.873
	INC 3rd	16	0.2 - 1.2	0.5 (0.3)	0.5 [0.3; 0.7]	0.152
	INC Other	51	0.2 - 1.8	0.7 (0.3)	0.7 [0.5; 0.9]	**0.016**

p-values computed from the Mann-Whitney test between conclusive results and each inconclusive type of result. CONC – conclusive tests; INC_LFF – inconclusive test due to low fetal fraction, INC_NAC – Not Automatic Call; INC_3rdDNA – contamination with another DNA; INC_other – No amplification or correlation.

We have not found any meaningful correlation between the above variables, except for the obvious one between weight and BMI.

Considering quantitative features that presented statistically significant differences in the previous section, it was possible to define thresholds that discriminate the type of inconclusive test from the conclusive ones. Therefore, and according to **Table III.7**, women heavier than 93.75 kg or presenting BMI above 27.34 kg/m^2 or with a cfDNA

concentration above 0.7245 are more likely to present inconclusive tests due to low fetal fraction. However, the separation considering the weight has low sensitivity (high rate of false negatives).

Inconclusive tests due to the presence of 3rd DNA are more likely present in women with a maximum weight of 62.25kg.

Table III.7 – Area under the ROC curve (AUC) and thresholds for positive discrimination between groups of inconclusive tests

Variable	LFF			NAC			3rdDNA			Other		
	AUC (95%CI)	Cut-off	Sens Spec	AUC (95%CI)	Cut-off	Sens Spec	AUC (95%CI)	Cut-off	Sens Spec	AUC (95%CI)	Cut-off	Sens Spec
Age (years)							0.484 (0.360, 0.608) p = 0.815			0.591 (0.510, 0.672) **p = 0.022**	≤ 31.5	35.7% 79.5%
Weight (kg)	0.777 (0.694, 0.861) **p < 0.001**	≥ 93.75	50.0% 94.6%				0.657 (0.535, 0.778) **p = 0.022**	≤ 62.25	66.7% 61.5%			
BMI (kg/m²)	0.798 (0.718, 0.878) **p < 0.001**	≥ 27.34	82.1% 66.4%									
FF (%)	1.0 (1.0, 1.0) **p < 0.001**	≤ 3.05%	100.0% 99.5%	0.986 (0.958, 1.0) **p < 0.001**	≤ 1.50%	97.4% 100.0%				0.758 (0.686, 0.831) **p < 0.001**	≤ 4.65%	63.8% 88.7%
[cfDNA] (ng(ul)	0.796 (0.707, 0.886) **p < 0.001**	≥ 0.7245	75.8% 77.4%							0.595 (0.51, 0.681) **p = 0.016**	≥ 0.731	44.6% 77.9%

Unknown causes of inconclusive tests are likely to occur in younger women, at least 31 years old, or presenting a maximum FF of 4.65% or a minimum cfDNA concentration of 0.731ng/µl.

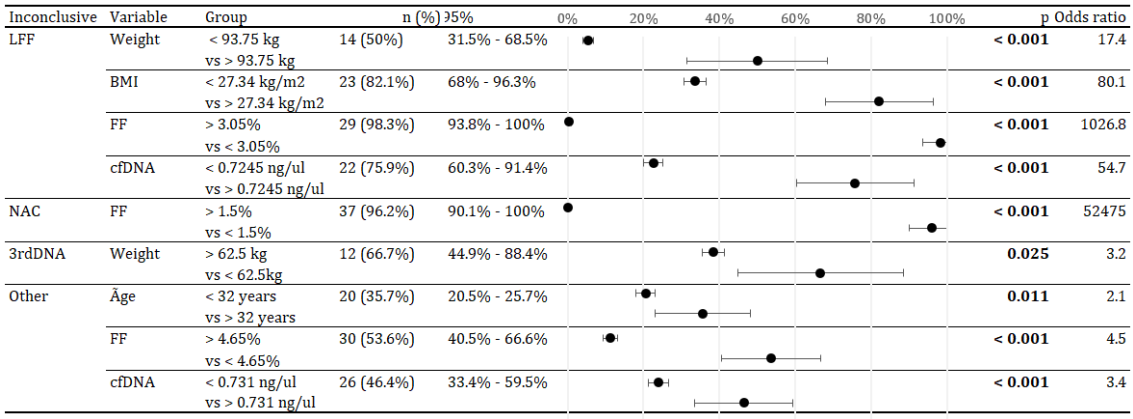


Figure 35 – observed and expected rates (95% confidence) for each variable according to the type of inconclusive test and p-values for the Fisher exact test. Odds ratios were computed for statistically significant associations.

In fact, the dichotomization of the previous variables according to their optimal threshold always presents an increased chance of obtaining an inconclusive result (**Fig.35**).

5.5. Sexual chromosomes aneuploidy detection

One of the limitations of our test is that it is intended to screen for fetal aneuploidy status exclusively on chromosomes 21, 18, and 13 and is IVD validated only for these chromosomes. Therefore, concerning sex chromosomes, the analysis algorithm also defines ploidy but in a semi-quantitative way, only for fetal sex determination. For this reason, all ploidy alterations found involving the sex chromosomes had to be evaluated very carefully. All cases with suspected alterations involving the sex chromosomes were discussed with the requesting physician to decide on the need for repeat testing, ultrasound follow-up, or invasive studies.

In three cases with suspected aneuploidies involving the sex chromosomes, a third source of DNA was identified, suggesting a vanishing twin situation, two were decided not to carry out further studies, and, in one of the cases, which we will describe below, (**Fig.36**), it was decided to confirm the result by invasive testing.

LCG-FMUC ID: Ni95_20 and L111/20

Clinical History: 13 weeks + 2 days' pregnancy; 38 Years old; Combined Risk for T21 1/112; BMI 23.6; plasma and cfDNA within normal values.

NIPT Result: Low Risk for Aneuploidies. Female (No Y detected). X ploidy Inconclusive.

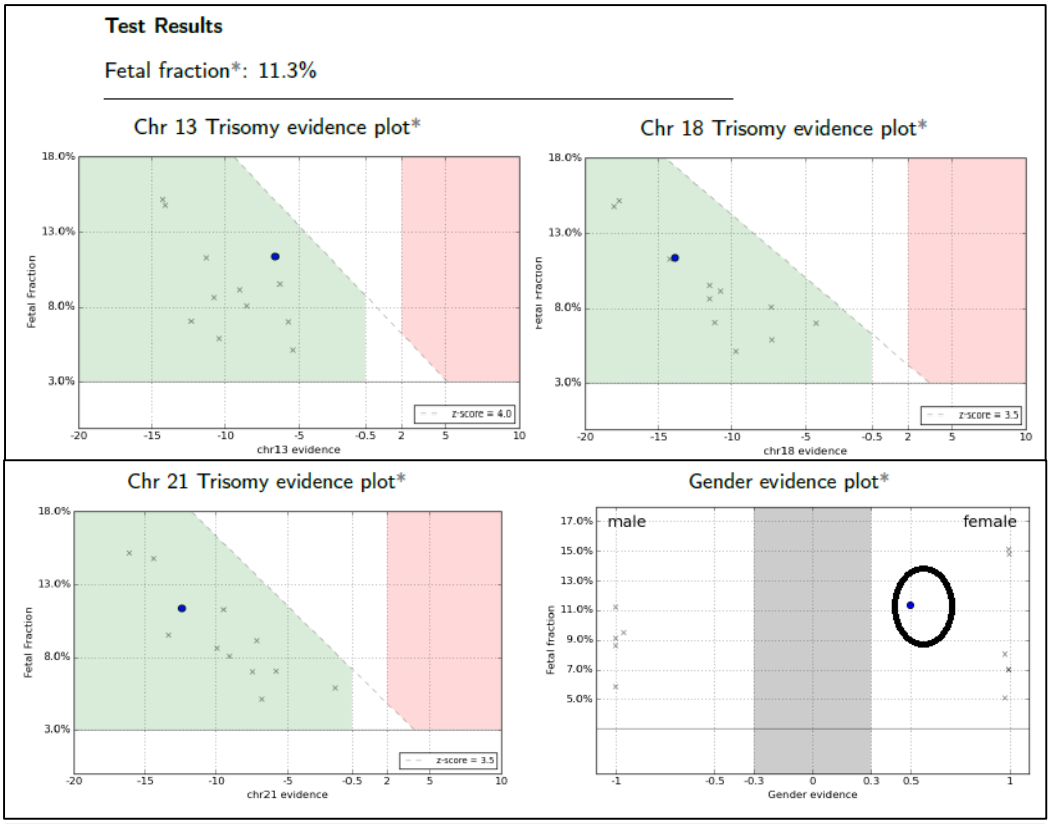


Figure 36 – **Clarigo Reporter trisomy Evidence Results for NI95_20** – Low Risk for Aneuploidy 13/18/21. Chromosome X Inconclusive. Possible Mosaicism or Aneuploidy.
Source: LCG-FMUC (2022)

After genetic counseling with the couple, it was decided to clarify the NIPT's inconclusive result through an amniocentesis performed at 17 weeks of gestation. Since it was also intended to exclude the possibility of mosaicism, three different tests were required by prioritization according to the result obtained. First QF-PCR, Second Array-CGH, and if both are Normal, the karyotype would also be studied to screen for a possible mosaic involving the X chromosome.

QF-PCR Studies

The MRA diagnostic test for common aneuploidies was performed with DNA extracted from uncultured AF cells using QF-PCR (Devyser Complete v2).

The obtained results were negative for aneuploidies involving chromosome X (**Fig.37**).

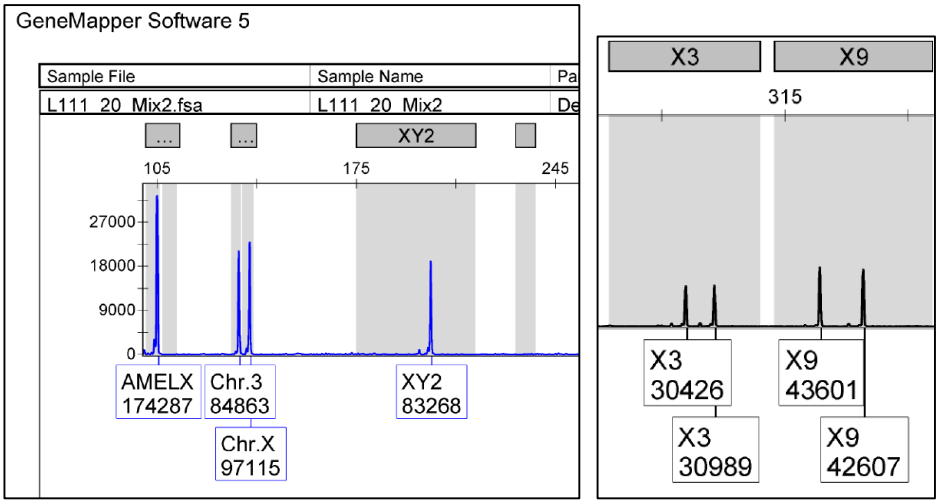


Figure 37 – **QF-PCR Devyser Results for L111_20** – Compatible with a Female Fetus with 2 copies forchromossome X. Result (ISCN-2020): rsa(13,18,21,X)x2
Source: LCG-FMUC (2020)

Array-CGH studies

Array-CGH studies were performed on DNA extracted from the amniotic fluid culture. No microdeletions, microduplications, or unbalanced rearrangements were found (**Fig.38**).

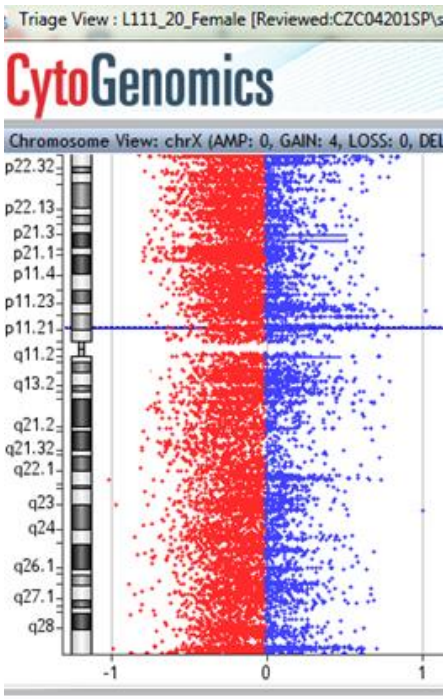


Figure 38 - Cytogenomics ArrayCGH chromosome view for L111_20.
Result (ISCN-2020): arr(1-22,X)x2, Compatible with a Normal Female Fetus. Source: LCG-FMUC (2020)

After these two negative results, the possibility of a false NIPT positive result derived from mosaicism confined to the placenta was raised. Karyotype studies revealed a mosaic of two cell lines compatible with NIPT results (**Fig.39**). One line with 45,X karyotype and the other with 47,XXX, with approximated ratios 1:1 justifying the impossibility of detection by aCGH or QF-PCR.

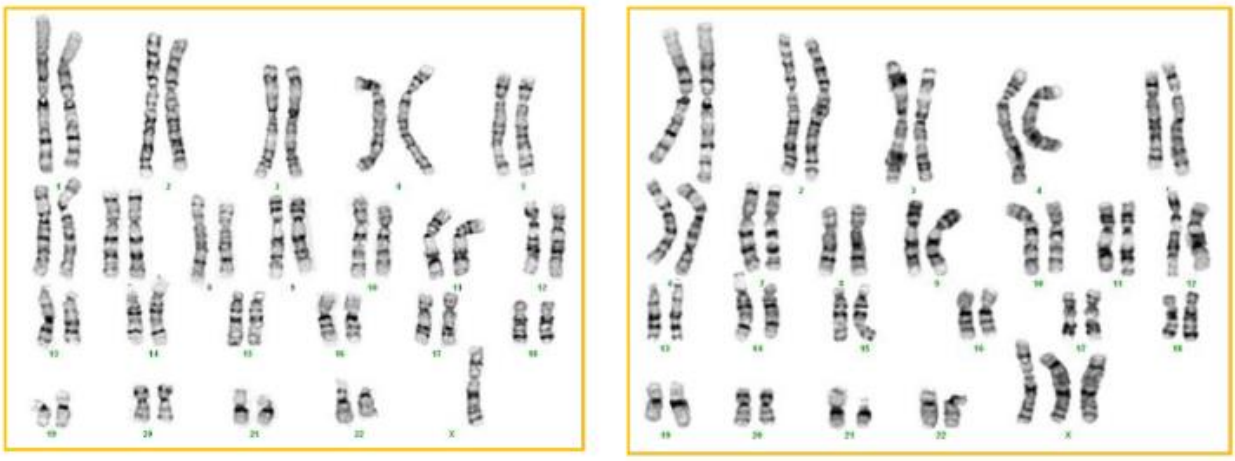


Figure 39 – Conventional Cytogenetics Results. Karyotype studies revealed a mosaic of two abnormal cell lines: Resultado (ISCN-2020): mos 45,X[11]/47,XXX[9].
Source: LCG-FMUC (2020)

5.6. Maternal alterations and Incidental Findings

Chromosome X incidental maternal alterations were identified in 14 cases (1,2%) with a low fetal risk for aneuploidies: one deletion Xp (**Fig.41**), 12 duplications Xp (**Fig.43**), and 45,X mosaicism (data not shown).

Deletion Xp

LCG-FMUC ID: Ni267_20

Clinical History: 12 weeks + 4 days pregnancy; 37 Years old ; Combined Risk for T21 1/268; BMI 28; plasma and cfDNA within normal values.

NIPT Result: Low Risk for Aneuploidies. Male (**Fig.40**)

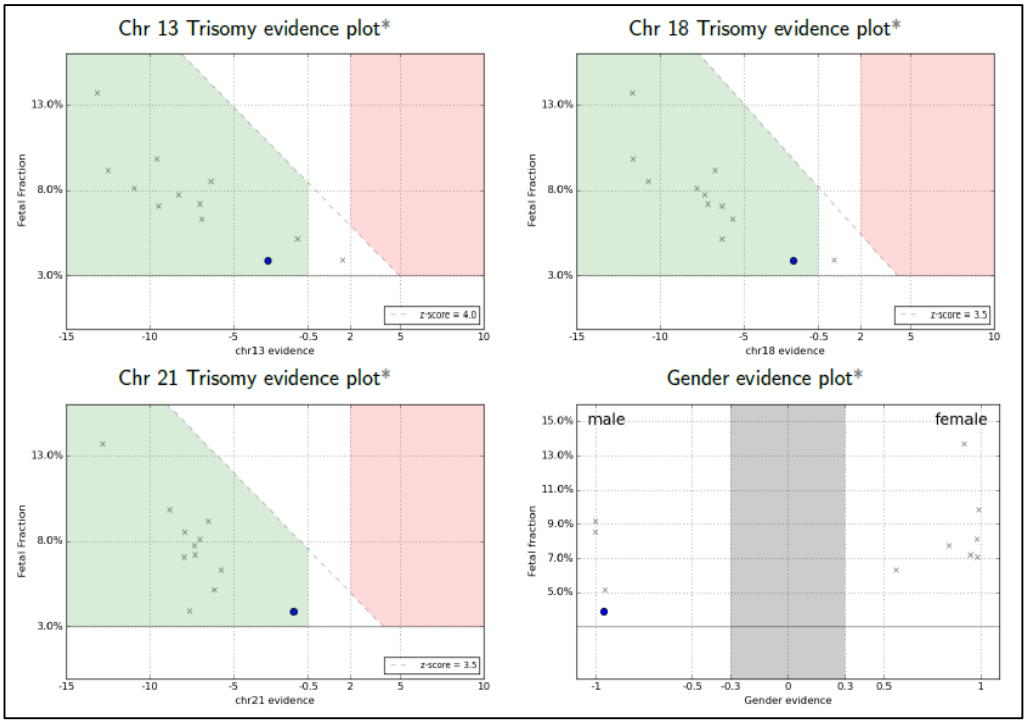


Figure 40 – **Clarigo Reporter trisomy Evidence Result** – Male fetus with low Risk for Aneuploidy 13/18/21.
Source: LCG-FMUC (2020)

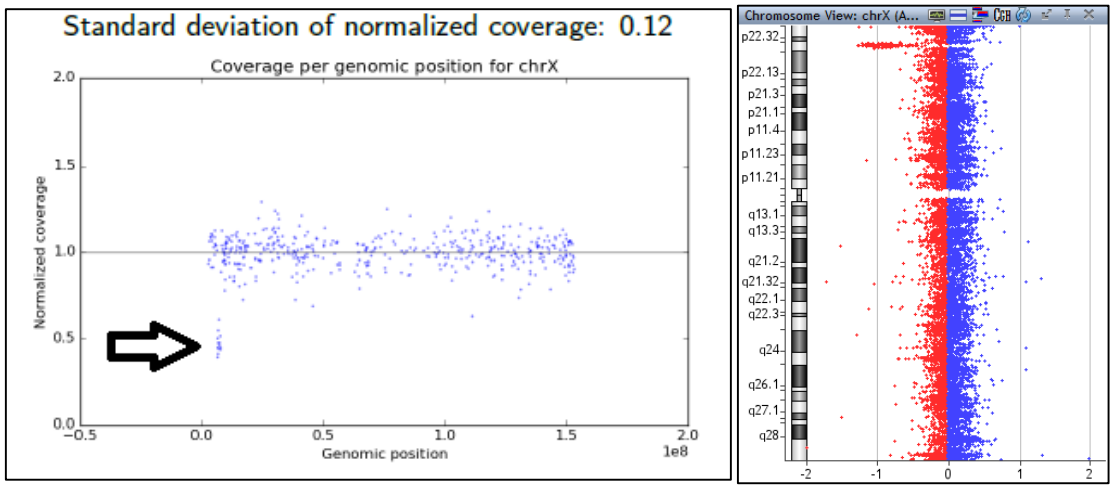


Figure 41 – **Maternal Xp Deletion identified by NIPT and confirmed by Array-CGH.**
LEFT: Clarigo Reporter Chr.X SNPs Genomic Coverage with SNPs low representation.
RIGHT: Cytogenomics ArrayCGH chromosome view confirming Xp Deletion.
Source: LCG-FMUC (2020)

In all the NIPT cases, a quality control (QC) analysis of data is doubled check before reporting final results. The QC analysis of genomic X SNPs coverage revealed a maternal Xp deletion (**Fig.41**).

This incidental finding was discussed with the clinical team, and under informed consent, confirmation by aCGH was decided. As expected, aCGH confirmed a deletion in Xp22.31, (**Fig.41**) with a size of 1.46 Mb, which included four Online Mendelian Inheritance in Man (OMIM) genes (OMIM ID: 306480 -*PUDP*, 300747-*STS*, 300229-*VCX* and 300102-*PNPLA4*) (**Fig.42**).

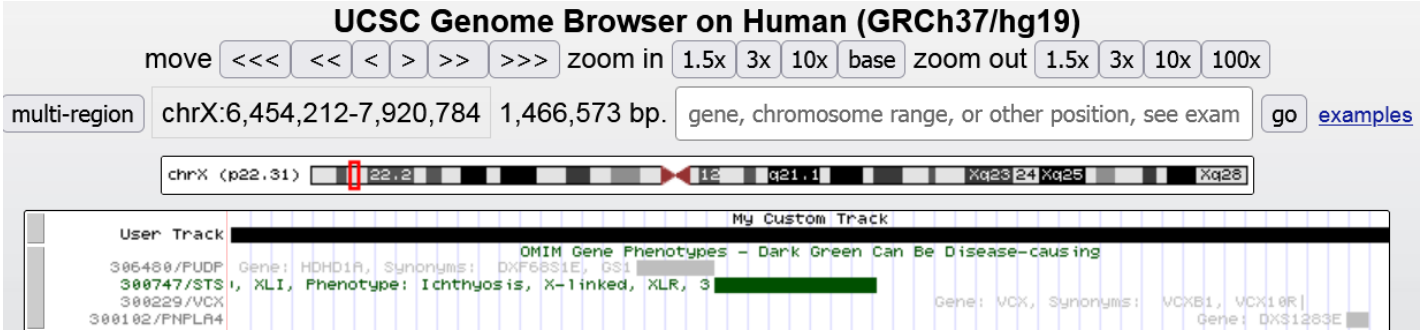


Figure 42 – **UCSC genome browser view of the maternal Xp Deletion.**
Gene content and OMIM Morbid Map genes (dark grey bar).The red box indicates the deleted region in chromosome X ideogram.
Source: UCSC Genome Browser (2022)

The impact of this deletion on the mother is conditioned by the pattern of inactivation of the X chromosome. Being pregnant with a male fetus implies a 50% risk of transmission to the fetus.

The child, a boy, at birth showed signs of ichthyosis but, after 9 months of preprogrammed treatment he is currently healthy, apparently with a normal development.

Duplications Xp

In addition to the deletion described above, we found 12 duplications involving the same region. This duplication is already reported in the databases classified as benign.

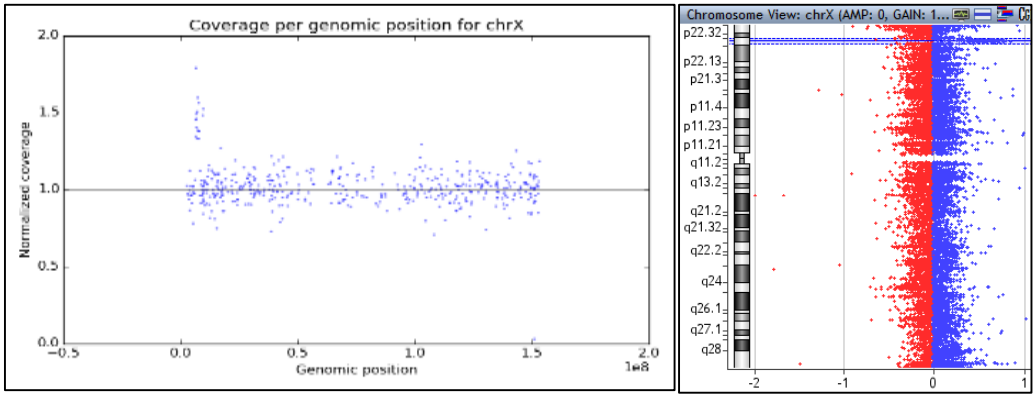


Figure 43 – **Maternal Xp Duplication identified by NIPT and confirmed by Array-CGH.**
LEFT: Clarigo Reporter Chr.X SNPs Genomic Coverage with SNPs high representation.
RIGHT: Cytogenomics ArrayCGH chromosome view confirming Xp Duplication.
Source: LCG-FMUC (2020)

5.7. NIPT Confirmation

Of the 9 cases with High risk for aneuploidy, seven NIPT positive results were validated by amniocentesis. In one case, the mother decided to continue the pregnancy, and in one case, there was a spontaneous fetal loss (T13) confirmed in skin biopsy by QF-PCR (**Fig.43**) (specificity = 100%). There weren´t known false-negative results (sensitivity = 100%).

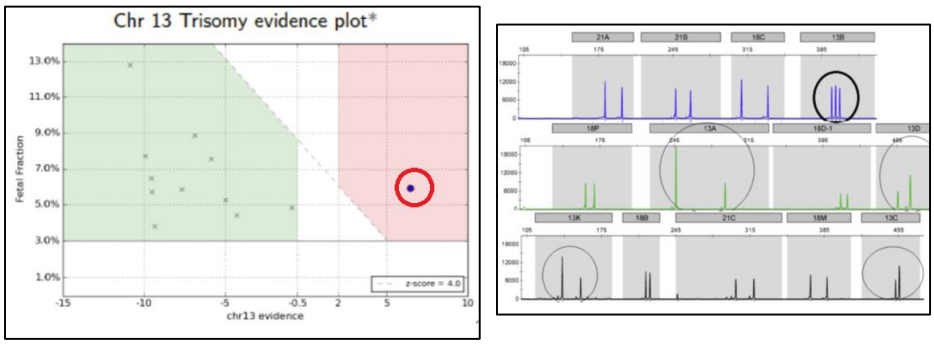


Figure 44 – Confirmation of a T13 NIPT Result by QF-PCR
Left: Trisomy 13 Positive evidence plot. Right: QF-PCR profile confirming the T13 in fetal DNA extracted from skin biopsy. Source: LCG-FMUC (2022).

6 - Discussion of Results

The results demonstrate that the NIPT is highly effective in screening for the most common fetal aneuploidies (13, 18, 21, X, and Y), becoming a clinical reality in our national health service, following what is already happening in many other countries that have applied MPS of cfDNA, using WGS, targeted, or SNP-based sequencing methods [27].

The sensitivity and specificity of the test are high compared with traditional fetal aneuploidy screening methods (based on ultrasound, maternal biochemistry and age). However, based on recent studies in a meta-analysis performed in 2014 by Gil *et al.*, NIPT results defined sensitivity values of 99% and specificity of 99.92% for trisomy 21; sensitivity of 96.8% and specificity of 99.85% for trisomy 18; and 92.1% sensitivity and 99.80% specificity for trisomy 13, while false-positive rates for trisomy 21, 18 and 13 were 0.08%, 0.15%, and 0.20%, respectively [27,28], proving the robustness of the test.

In our sample, sensitivity and specificity was 100%, but we are conscient that we do not yet have a sufficient sample size to allow us to define rates with statistical confidence. Of the 1130 samples received, we identified nine positive results (0.8%) for a high risk of aneuploidy, eight corresponding to trisomy 21 (six females and two males), one trisomy 13 and no trisomy 18 (**Fig.32**). These results agree with the expected incidences for the general population (**Table I.1**), where trisomy 13 or 18 incidence is 10-20 times lower than trisomy 21. The 3:1 female/male ratio of trisomy 21 detected in our samples is not statistically significant due to the yet small sample size.

A prospective analysis of the impact of NIPT among pregnant women evaluated in PND consultation at CHUC after identification of a high risk of aneuploidy by Oliveira et al. revealed an acceptance rate of 87.1% and an estimated reduction of invasive techniques by 80.1% (See Supplemental data – **Pág.67**).

From the 129 samples (11.4%), where it was not possible to obtain a conclusive result (**Fig.32)**, 66 successfully repeated the test, reducing the test failure rate to 6.9%.

Although the failure rate is in line with data reported by other groups [28,31,32,33], it was essential to make a deeper evaluation about its causes. Inconclusive results

unnecessarily increase the couple's concern and anxiety, making genetic counselling before (and after) NIPT very important.

The average FF obtained in our sample was 7.5% from an average volume of 4.5ml of plasma (**Fig.31**). 94.4% of the samples presented values greater than 3%, the minimum value allowed by our analysis algorithm for calculating aneuploidy risk. A conclusive result was not possible for 60 samples (5.3%), due to directly low FF (< 3%), representing 46% of the 129 samples with no conclusive results.

As already mentioned, FF below 3% does not allow the risk of fetal aneuploidy assessment, being one of several parameters that can compromise a NIPT result.

A low FF can occur from different causes: gestational age, cfDNA contamination with maternal genomic DNA, blood sample quality and high BMI [34].

In respect to the maternal age, 61% of the women were aged between 35 and 44 years old, tending to advanced maternal age (AMA) (**Fig.28**). Most had no previous history of fetus with aneuploidy (94.8%). Considering gestational age, two-thirds of the pregnant woman did NIPT before 16th week of gestation (**Fig.29**). However, it is worth mentioning a unique test carried out late at 32 weeks of gestation, in a 24-year-old woman, that had no obstetric evaluation and was very anxious.

A Kruskal-Wallis test was used to determine whether or not there was a statistically significant difference between major group of inconclusive tests (LFF, NAC, 3rd DNA, other) and conclusive results group (**Table III.6**). From the results, there is no correlation between FF and maternal age neither with gestational age confirming data already reported by other groups [30,36]

Regarding BMI, in our sample, 49.2% of women had a "normal" value (BMI < 30) at the time of the test (**Fig. 30**). The other 50.8% of women were considered overweight (28.3 %) or obese (22.5%). **Table III.6** shows a strong correlation between these variables and LFF ($p < 0.001$), in line with Hestand *et al.*, and other reports that conclude that there is a negative correlation between FF and BMI[34]. High BMI can be considered as a critical factor for the failure rate of NIPT by LFF.

Haemolysis and clotted blood change the constitution of plasma by contamination with maternal genomic DNA, which indirectly dilutes fetal cfDNA and decreases FF. From p Odds ratios (**Fig.34**) the chance of obtaining an inconclusive test increase about 4 times

for LFF, 7.4 times for NAC, and about 5.1 times for other types of inconclusive tests. For clotted blood, the chance of obtaining an LFF inconclusive test is approximately 19 times higher.

Finally, the quality of cfDNA extracted was also evaluated. Thirty-one cases (23.8%) had cfDNA concentrations above 0.8ng/µl, indicating a probable contamination with genomic DNA (23.8%). In 14 cases, the concentrations were above 1.2 ng/µl, strongly suggesting contamination with maternal genomic DNA, made up of large molecules of DNA, originated from the lysis of maternal white blood cells in test samples. The increase in maternal DNA level has resulted in a decrease in fetal fraction[41]. Nevertheless, 64,6% of the cases had a good or ideal cfDNA extraction under 0.8 ng/µl (**Table III.5**). From **Table III.6** analysis, it is clear that cfDNA concentration is strongly correlated with LFF inconclusive cases.

Considering the quantitative characteristics that showed statistically significant differences (FF, BMI, Weight, age, and cfDNA), it was possible to statistically define thresholds that discriminate the type of inconclusive test from the conclusive ones (**Table III.7**). For example, if we consider a BMI threshold cut-off > 27.34 Kg/m^2, we will have for LFF a positive discrimination with 82,1% sensitivity and 66,4% specificity statistically significant (**Table III.7** and **Fig.35**).

From our results, the statistical analysis of inconclusive results evaluating different parameters showed that several factors could compromise the achievement of results: low fetal fraction, high pregnant body mass index, and low quality of the cfDNA obtained from plasma.

As advantages of the implemented test, in addition to trisomies 21, 18, and 13, our NIPT can also detect, with some limitations, sex chromosomes aneuploidies, including mosaicism. It works well with relatively low FFs (up to the 3% cutoff), even in pregnant women with high BMI, although we have statistically proven that BMI > 40 Kg/m^2 increases the NIPT failure rate. The high failure rate of NIPT in the initial stages of the study, allowed us to make some optimizations in sample collection that reduced the frequency of clotted samples and decreased the failure rate.

Nevertheless, the platform used in this study cannot be used in twin pregnancies or for pregnancies resulting from oocyte donors.

NIPT methods vary from laboratory to laboratory, and therefore each should independently optimize its FF detection methods by establishing other reliable quality control metrics[30].

In near future, since FF is often limiting, the possibility of using FF enrichment methodologies could also be evaluated, especially for pregnant women with high BMI or history of early pregnancy loss[37]. The announced development for non-invasive testing that also detects relevant microdeletions and duplications will firmly have significant implications for public health. However, established recommendations should always be followed.

NIPT tends to be an increasingly safe and effective non-invasive option, with greater sensitivity and specificity than current options.

7 - Conclusion and Future Perspectives

This work has confirmed that NIPT implemented in the LCG_FMUC is a reliable and robust screening test for detecting fetal trisomies 21, 18, and 13 with high and proven sensitivity and specificity, limiting and reducing the need to perform invasive tests, resulting in better management of hospital resources and safety for pregnant women.
As a screening test, based on cffDNA of placental origin, false positives may occur due to confined placental mosaicism, and confirmation of positive results by invasive diagnostic tests remains necessary.
The implemented NIPT test, based on SNPs, proved to be suitable for screening the most common aneuploidies, with a specificity and sensitivity of 100% to date. However, the corrected rate of inconclusive tests, around 7%, is still very high and requires reflection on ways to reduce it. Finally, due to its technical limitations, this NIPT cannot be used in twin pregnancies or in oocyte donors, which will have to be done through an alternative NIPT test (based on WGS).

Given the expected increase in the annual volume of samples, which is not compatible with a manual process and the expected need of transition to IVD class C platform, accordingly to EU new published *In Vitro Diagnostic Regulation* (IVDR)[42], NIPT based on WGS, will require investment in more robust NGS equipment, as well as the need to use automated systems for cfDNA extraction and library preparation.

In the near Future, developing a non-invasive test that detects relevant microdeletions and duplications will also have significant public health implications. High-risk pregnancies can be reclassified after a negative NIPT result, decreasing the need for unnecessary, additional invasive tests and decreasing couples' anxiety. Also, reducing the number of invasive tests will save time for other clinical priorities.

The NIPT implementation in the routine of prenatal care has been changing reproductive and public health strategies in Portugal and globally. It will also be essential to update the non-invasive prenatal screening guidelines to increase the scope of the technique and standardize procedures in line with global trends for the benefit of pregnant women.

Supplemental Data

Informed Consent

Consentimento Informado da grávida

1- Fui informada sobre os benefícios e limitações deste teste, como se encontram descritos no folheto informativo do teste. Tive oportunidade de solicitar esclarecimentos adicionais ao meu médico.

2- Compreendo que este teste se destina à deteção das trissomias 21, 18 e 13 a partir das 10 semanas de gestação. Outros testes mais apropriados poderão ser indicados quando existe um risco aumentado para outras alterações genéticas.

3- No caso de um resultado normal, a probabilidade do feto poder ter trissomia 21, 18 ou 13 é muito baixa, mas não pode ser excluída. Um resultado anormal deve ser sempre confirmado por um teste pré-natal invasivo (biópsia das vilosidades coriónicas ou amniocentese).

4- Não pode ser utilizado em gestações de gémeos, gestações por doação de óvulos ou quando a mãe foi sujeita a transfusão sanguínea, terapia com células estaminais, imunoterapia, ou transplante de órgãos no período de 3 meses antes da gravidez.

5- O teste NIPT, apesar de ter uma elevada precisão, não permite obter resultados conclusivos em situações de fração fetal (FF) inferior a 3%.

6- O teste NIPT permite identificar o sexo do feto mas não permite identificar aneuploidias envolvendo os cromossomas sexuais, sendo apenas analisada a presença ou ausência do cromossoma Y.

7- O teste NIPT não permite detetar rearranjos equilibrados, microdeleções, microduplicações, aneuploidias parciais, situações de mosaicismo ou doenças monogénicas.

8 - Em casos muito raros, o teste de NIPT pode identificar anomalias cromossómicas clinicamente relevantes na mãe.

Compreendi a informação acima e aceito que o teste pré-natal não invasivo seja realizado nestas condições.

Grávida:

Nome:_____

Data: ____/____/_____

Assinatura:_____

High Risk Aneuploidy Model Report

LCG LABORATÓRIO DE CITOGENÉTICA E GENÓMICA
Faculdade de Medicina da Universidade de Coimbra
Direcção Técnica - Prof. Doutora I. Marques Carreira

Nº interno amostra: NI
Tipo de amostra: Sangue
Data de colheita:

Nome:
Data de nascimento:
Sexo: Feminino
Morada:

Nº Processo Externo:
Entidade requisitante: CENTRO HOSPITALAR E UNIVERSITÁRIO DE COIMBRA, E.P.E.
Serviço/consulta:

Médico requisitante:
Data de requisição:
Data de entrada:
Data de saída:

Análise solicitada: Teste Pré-Natal Não Invasivo (NIPT) para a pesquisa das aneuploidias mais comuns.

Indicação clínica:

Descrição do Método: Extração e purificação de DNA livre em circulação (cfDNA) a partir de amostra de sangue materno. Amplificação *target* por *multiplex polymerase chain reaction* (PCR) seguido de análise por sequenciação de nova geração (NGS) para deteção de aneuploidias fetais dos cromossomas 13, 18 e 21. Na sequenciação NGS foi utilizada a plataforma MiSeq (Illumina) e na análise dos dados recorreu-se à *pipeline* informática Clarigo Reporter software V2 (CE/IVD).

Resultado: Risco Muito Elevado para Trissomia do Cromossoma 21.
Não foram detetadas aneuploidias para os cromossomas 13 e 18. Sexo masculino.

Interpretação: Através da técnica de NIPT utilizada, foi detetada uma alteração no número de cópias do cromossoma 21, tendo os outros cromossomas analisados um padrão normal. O Feto é do sexo masculino.
Neste ensaio, a fração fetal (FF) estimada foi de 8%.

Conclusão: Estes resultados indicam que este feto do sexo masculino tem um resultado compatível com uma trissomia do cromossoma 21, associado à Síndrome de Down. Este resultado tem de ser confirmado através de um teste de diagnóstico invasivo, preferencialmente por amniocentese.

Comentários:
(1) Um resultado de teste NIPT alterado ou inconclusivo deverá ser sempre confirmado por um teste de diagnóstico invasivo, como a amniocentese (preferencialmente) ou pelo estudo de vilosidades coriónicas. Da mesma forma, se o feto for portador de anomalias ecográficas é sempre recomendável a realização de um teste de diagnóstico invasivo.
(2) O teste NIPT permite identificar o sexo do feto mas não permite identificar aneuploidias envolvendo os cromossomas sexuais, sendo apenas analisada a presença ou ausência do cromossoma Y.
(3) O teste NIPT não permite detetar rearranjos equilibrados, microdeleções, microduplicações, aneuploidias parciais, situações de mosaicismo ou doenças monogénicas.
(4) Em casos muito raros, o teste de NIPT pode identificar anomalias cromossómicas clinicamente relevantes na mãe.

Notas complementares:
(a) O teste NIPT, apesar de ter uma elevada precisão, é apenas um teste de rastreio. Em situações raras, pode levar a resultados inconclusivos ou discordantes devido a diversos fatores tais como: baixa FF, mosaicismo confinado à placenta, feto sobrevivente numa gravidez gemelar com gémeo "absorvido", mosaicismo materno, neoplasia materna e parâmetros estatísticos desviantes no *software* de análise. É menos sensível em gestações inferiores a 10 semanas. Não pode ser utilizado em gestações de gémeos, gestações por doação de óvulos ou quando a mãe foi sujeita a transfusão sanguínea, terapia com células estaminais, imunoterapia, ou transplante de órgãos no período de 3 meses antes da gravidez.

(b) A análise e elaboração do relatório de resultados foram feitas de acordo com as orientações de Deans et al, *Prenat Diagn* 2017: 37,699-704; da tomada de posição da American College of Medical Genetics and Genomics - Gregg et al, *Genet Med* 2016 e do Committee opinion do The American College of Obstetricians and Gynecologists (AOCG) e Society for Maternal Fetal Medicine – Committee Opinion No. 640 - 2015.

(c) Sensibilidade do teste: Trissomia 21, 13, 18 – 100%. Especificidade do teste: Trissomia 21 – 99.93%, Trissomia 18 – 99.93%, Trissomia 13 – 99.86%.

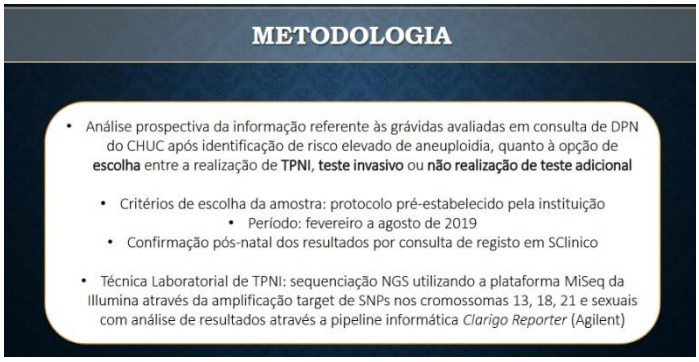
(d) O LCG-FMUC participa num programa de Avaliação Externa da Qualidade (www.ccneqas.org.uk) em diagnóstico pré e pós-natal, nomeadamente nas suas vertentes de citogenética convencional, diagnóstico molecular e array-CGH.

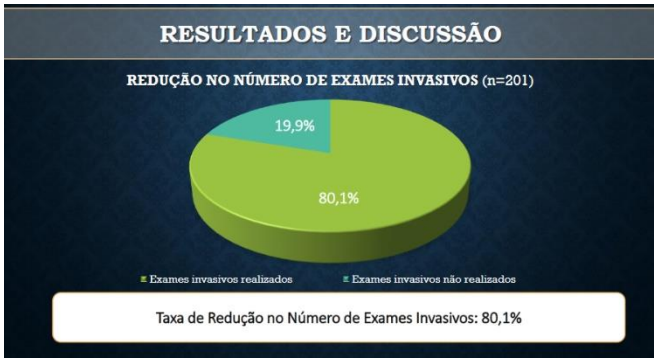
(e) O LCG-FMUC possui um Sistema de Gestão da Qualidade certificado pela NP EN ISO 9001:2015, com o certificado número 2016/CEP.5027 emitido pela APCER.

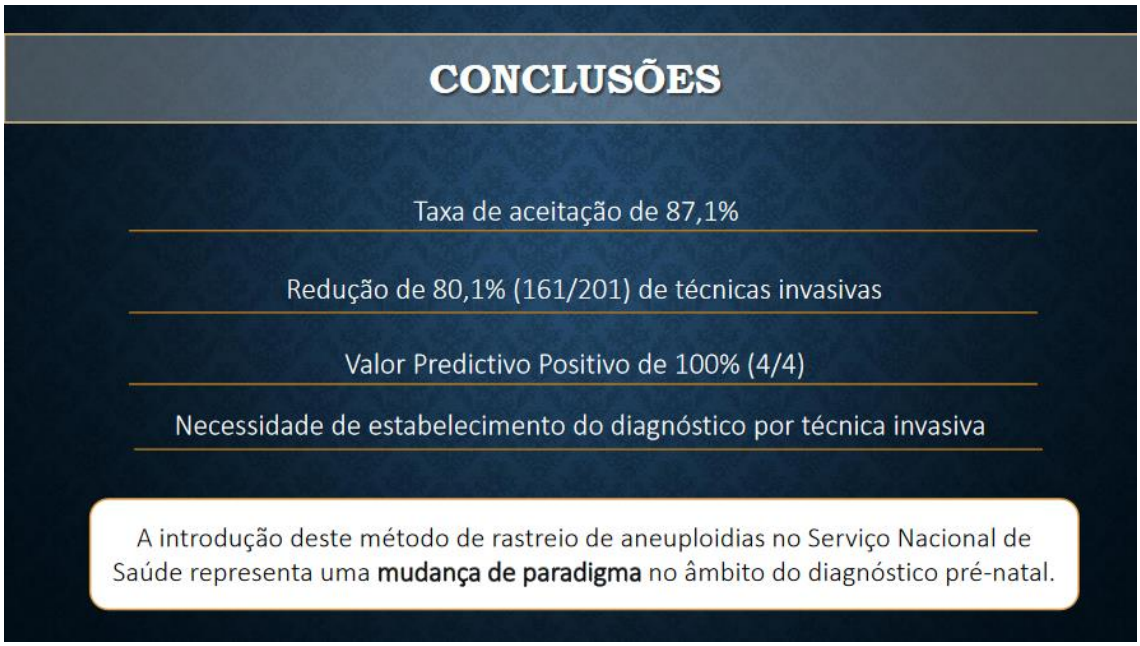
Análise efetuada por: **A Coordenadora:**

MSc Publications and Scientific Comunications - I

Avaliação da Implementação de Teste Pré-Natal Não Invasivo no Centro de Diagnóstico Pré-Natal do Centro Hospitalar e Universitário de Coimbra. Reunião Científica da Associação Portuguesa de Diagnóstico Pré-Natal. Viana do Castelo, 4 e 5 de outubro de 2019.







MSc Publications and Scientific Comunications - II

Incidental detection of maternal Xp22.31 deletions and duplications in noninvasive prenatal testing.
Proceeding abstracts Medicine (2021). 100:4:18.
doi: 10.1097/ MD.0000000000023585. IF: 1.889; Q2 (Medicine)

P32 – INCIDENTAL DETECTION OF MATERNAL Xp22.31 DELETIONS AND DUPLICATIONS IN NONINVASIVE PRENATAL TESTING

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Introduction: Non-invasive prenatal testing (NIPT) has been widely used to detect common fetal chromosome aneuploidies (T13, T18 and T21) and, eventually, sex chromosome aneuploidies. However, NIPT is only a screening method and therefore can lead to false positive or false negative results. (This is directly due to the NIPT techniques used that analyzes all the cfDNA circulating in the blood (maternal and fetal)). The free fetal cfDNA that circulates in maternal blood is of placental origin and may, in very rare situations, represent chromosomal alterations limited to the placenta, being the fetal karyotype normal. In the same way, maternal chromosomal abnormalities can also be detected, including X chromosome alterations with increased risks of pathogenic phenotype in male fetuses. In this work we report relevant sex maternal chromosomal abnormalities detected in our NIPTcohort.

Methodology: A retrospective analysis of 619 singleton pregnancies, with moderate risk for common fetal chromosome aneuploidies was performed.

Results: In this cohort, we identified 8 duplications in Xp22.31 and 1 deletion in the same region. This deletion is a 1.46M maternal Xp22.31 deletion, confirmed by aCGH, involving the *STS* ichthyosis gene linked to the X chromosome and reported on the OMIM Morbid Map. The child, at birth, showed signs of ichthyosis but, after 9 months of preprogrammed treatment he is currently healthy, apparently with normal development. The duplication in Xp22.31 region, were also confirmed by aCGH, with a frequency of 1.3% in our cohort. This is an interesting result, and the possibility of this variant having a high representation in the Portuguese population cannot be ruled out.

Discussion: We can conclude that NIPT is currently the best screening test to detect fetal common aneuploidies, with high specificity, but anomalies confined to the placenta, tumor abnormalities and incidental maternal genetic abnormalities can be a disadvantage leading to false positive or false negative results. For this reason, all abnormal NIPT results, including maternal incidental findings must always be confirmed by invasive diagnostic methods.

MSc Publications and Scientific Comunications - III

A NIPT aneuploidy suspition with normal QF-PCR and aCGH. Karyotype gives the answer.
Proccedings of the SPGH 24Th Anual Meeting, Medicine (2020) 29:100 (4) (p e23585).
doi.org/10.1097/ MD.0000000000023585. IF: 1.552; Q2 (Medicine)

P50 – A NIPT ANEUPLOIDY SUSPITION WITH NORMAL QF-PCR AND aCGH. KARYOTYPE GIVES THE ANSWER

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Introduction: Advances in molecular tests brought greater capacity for genetic diagnosis. Nevertheless, even with these higher resolution tests, the identification of mosaics may still be a complex process, remaining the mosaicism a challenge for clinical diagnosis and genetic counselling. We report a mos 45,X/47, XXX fetus with an inconclusive result after a non invasive prenatal test (NIPT) and normal results by QF-PCR and oligoarray-CGH (aCGH).

Case Report: A pregnant woman was referred for NIPT due to an increased risk for trisomy after first trimester screening test. Normal results were obtained for chromosomes 13, 18 and 21 but were inconclusive for sexual chromosomes, indicating a possible aneuploidy involving the X chromosome in a female fetus. The geneticist decided to request QF-PCR for common aneuploidies and aCGH in an amniotic fluid sample, which revealed, in both tests, a female fetus with normal results. Since there was a strong suspicion of sexual aneuploidy, as laboratory internal quality control, metaphases were analysed revealing a mos 45,X[11]/47,XXX[9] karyotype.

Discussion and Conclusions: The NIPT result, although inconclusive for the sexual chromosomes, it was suggestive of an aneuploidy, partial or complete, involving the X chr in a female fetus. The karyotype result explains the inconclusive NIPT and also explains the normal results observed with the other two molecular techniques, since it identified a 50:50 X/XXX mosaic. The QF-PCR showed two alleles for the X chr and the percentage of each cell line explains why no aneuploidy was identified. The normal result by aCGH is, also, due to normalization of ratios between the patient and control samples.

This case point out the fact that despite we are in the genomic era, the karyotype is still an important tool and, in this particular case, it allowed a genetic diagnosis that, otherwise, would be missed.

The case also highlights the role of Clinical Laboratory Geneticist (CLG) to assist the Medical Geneticist in the best laboratory strategy of study in order to achieve the most accurate diagnosis and genetic counselling.

Special Acknowledgments

I would like to thank in a very special way all the pregnant women who agreed to participate and contribute to the implementation of this NIPT test in our laboratory. I would also like to thank the wonderful medical and nursing team of the two maternity hospitals of Coimbra Hospital University Centre for their patience and availability to sending or allow the collection of samples. Finally, thanks to Clarigo and Agilent's technical support in the validation and re-verify of the most challenging results, often during off-hours, including weekends and holidays.

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