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Prenatal Diagnosis: State of the art

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Abstract

Prenatal Diagnosis comprises a variety of obstetric and genetic techniques to determine the health and condition of an embryo or fetus. A high proportion of genetic disorders are associated with significant morbidity, mortality and intellectual disabilities, causing suffering and anxiety to expectant parents.

Milestones in its history include the development of invasive techniques, the progress of chromosome analysis and the emergence of a screening program. Although prenatal diagnosis has more than 40 years, the invasive techniques currently used in health programmes are still associated with a miscarriage risk and the screening methods available have poor accuracy to detect the majority of genetic disorders.

Thereby, when cell-free fetal DNA (cffDNA) was detected in the maternal circulation during pregnancy, a desire to avoid contact with the fetus grew out and a lot of techniques were developed in order to create the so-called non-invasive prenatal diagnosis (NIPD).

Moreover, as researchers are gaining knowledge about the genetic disorders, also genetic analysis has experienced an extraordinary evolution. Cytogenetic methodologies have improved its resolution of detecting chromosome abnormalities and have decreased the turnaround time. Molecular genetic analysis have highly contributed to the diagnostic capacities and the application of array chromosome genomic hybridization (aCGH) to prenatal diagnosis allowed the detection of genomic imbalances associated with congenital malformations and/or intellectual disabilities which were not identified by previous techniques.

The introduction of these new approaches into clinical practice brings clear benefits but also poses several ethical and social challenges.

The purpose of this review is to present some of the techniques currently available to diagnose genetical conditions in utero, invasive and non-invasive, and to evaluate the evolution of chromosomal analysis over the years.

Keywords: prenatal diagnosis; invasive prenatal diagnosis; screening methods; non-invasive prenatal diagnosis ; chromosome analysis ; array comparative genomic hybridization

Abbreviation List

aCGH	array Comparative Genomic Hybridization
ACOG	American College of Obstetricians and Gynecologists
AFP	Alpha- Fetoprotein
β- HCG	β- Human Chorionic Gonadotropin
cfDNA	cell-free DNA
cffDNA	cell-free fetal DNA
CVS	Chorionic Villus Sampling
FISH	Fluorescence *in situ* Hybridization
MLPA	Multiplex Ligation-Dependent Probe Amplification
MPS	Massively parallel sequencing
NIPD	Non-Invasive Prenatal Diagnosis
NIPT	Non-Invasive Prenatal Testing
NGS	Next Generation Sequencing
PAPP-A	Pregnancy-Associated Plasma Protein A
PCR	Polymerase Chain Reaction
PD	Prenatal Diagnosis
QF-PCR	Quantitative Fluorescence Polymerase Chain Reaction
RhD	Rhesus D
SMFM	Society for Maternal-Fetal Medicine
STR	Short Tandem Repeats
VOUS	Variants of Unknown Clinical Significance

Introduction

The history of prenatal diagnosis dates from 1968, when the first antenatal diagnosis of Down's syndrome was made [1].

Although some pregnancy disorders were previously reported using other methods, namely imagiologic ones, it was only in the early 1970s that amniocentesis became widely available, allowing the knowledge of fetal genetic status [2].

Ten years later, obstetricians developed an alternative method of collecting fetal cells, chorionic villus sampling. Despite amniocentesis being a very reliable procedure, the introduction of CVS allowed an earlier diagnosis, making the medical abortion more acceptable for some women [3].

By the time the first prenatal diagnosis was made, amniocentesis was already performed for other purposes (to treat polyhydramnios, for example). Thus, in 1970 it was already known that this procedure could lead to miscarriage [3].

Hence, amniocentesis and CVS were initially offered only to women of advanced maternal age at delivery. The relation between maternal age and Down syndrome was known since 1933, when the British geneticist Lionel Penrose published a study about the effect of parents' age in mongolism [4]. Since then, several studies were also reported, confirming Penrose's conclusions (figure 1).

When amniocentesis was introduced, biochemists looked for possible correlations of its chemical composition and congenital defects. Soon they noticed that women who carried fetuses with major neural tube defects, had a high concentration of alpha-fetoprotein (AFP) [3].

Few years later, they found that AFP and other substances were also in maternal serum and its concentration was related with other fetal anomalies, such as Down syndrome.

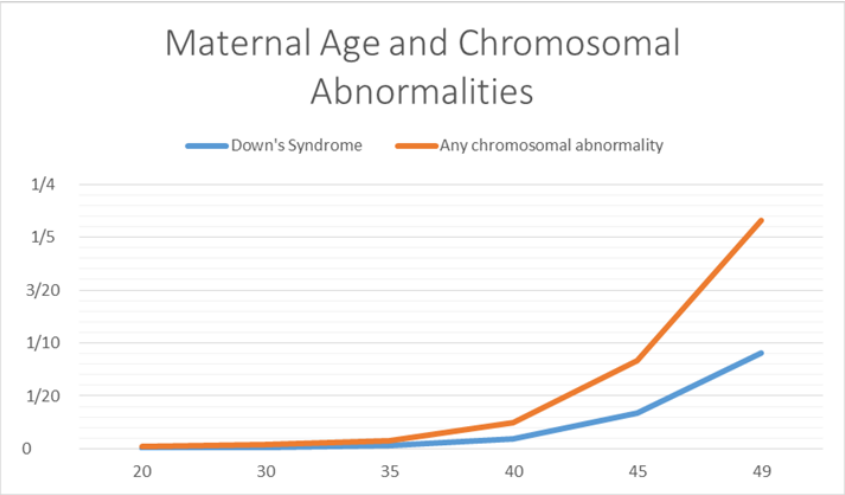

Figure 1- Risk of fetus with chromosomal abnormalities (live births) and maternal age adapted from Simpson [5].

In 1993, besides AFP, scientists discovered the relation between human chorionic gonadotropin and unconjugated estriol and trisomy 21, opening the door to a more complete screening test [3].

Currently, the invasive procedures responsible for the first prenatal diagnosis are still being used, despite the indications for its performance have been changing.

On the other hand, screening tests available have improved over the years. Nowadays, they include not only maternal age and serum analyses, but also ultrasound markers. However, these tests are primarily targeted to detect trisomy 21 and they have poor accuracy, with false-negative rates between 12% and 23% and false-positive rates between 1.9% and 5.2% [6].

Given the weaknesses of screening methods and the risks of invasive techniques, several researchers had tried to develop a more accurate, reliable and safe prenatal test.

After the discovery of cell-free fetal DNA in maternal plasma [7], several efforts have been made in order to detect fetal genetic diseases with a simple blood test, the so-called non-invasive prenatal diagnosis (NIPD) or testing (NIPT).

The introduction of these tests into clinical practice brings clear benefits but also poses several ethical and social challenges [8].

The purpose of this review is to present some of the techniques currently available to diagnose genetical conditions in utero, invasive and non-invasive, as well as chromosomal analysis and its evolution over the years (annex 1).

Invasive Prenatal Diagnostic Methods

These methods require the direct harvesting of fetal cells during pregnancy for subsequent karyotype and/ or genetic analyses [6]. Amniocentesis and CVS are more common techniques, although percutaneous umbilical blood sampling and other procedures can also be performed.

The common indications for a karyotype analysis in prenatal diagnosis are [9-11]:

- Advanced maternal age (≥35 years old);
- Previous child with chromosomal aneuploidy;
- Presence of structural chromosome abnormality in one of the parents;
- Following an abnormal maternal serum screening during the present gestation;
- Following an abnormal ultrasound;
- Family history of a genetic disorder that may be diagnosed or ruled out by biochemical or DNA analysis;
- Mother carrying an X-linked disorder;
- Known teratogen exposer during pregnancy (infection, drug or other).

1. Amniocentesis

Amniocentesis is the most worldwide used invasive prenatal diagnosis procedure. It is usually performed after 15 weeks of gestation and it is based on the insertion of a needle into the uterine cavity, aspirating around 1 ml of amniotic fluid per gestational week [6, 10].

This fluid contains fetal cells (amniocytes) which are originated from fetal urine, pulmonary secretions and skin [6] and could be used to determine fetal karyotyping and/or perform biochemical testing (table 1).

At present, the risk procedure-related losses after amniocentesis in singleton pregnancies is considered, in experienced hands, to be about 1 in 200 or less (table 1) [5]. Other

complications including leakage of amniotic fluid, infection and injury to the fetus by needle puncture are considered rare [9].

2. Chorionic Villus Sampling (CVS)

Chorionic villus sampling involves a biopsy of placental cells and can be performed transcervically or transabdominally, depending on placental site [6].

Although the procedure risk is higher than in amniocentesis (around 1:100), it is realized between 11 and 12 weeks, allowing an earlier diagnosis (table 1) [10].

However, in 1991, studies were published relating CVS with severe limb abnormalities [12]. Subsequently, numerous investigations were carried out in order to confirm this association but the results were not concordant [11, 12]. Nowadays, although still controversial, it is generally accepted that between 10 and 12 weeks, there is no increase in limb anomalies over the background risk [11].

As well as amniocentesis, it can be used to yield information on fetal chromosomes status, diagnose some single gene disorders or assay for biochemical disease.

	Amniocentesis	CVS
Advantages	• Low risk of miscarriage (≈1:200) • Measurement of AFP, detecting the risk of a neural tube defect	• Performed earlier in pregnancy (11- 12 weeks)
Disadvantages	• Performed later in pregnancy (usually after 15 weeks of gestation)	• Higher risk of miscarriage (≈1:100) • Ambiguous results due to chromosomal mosaicism (≈2% of the results) • Maternal contamination

Table 1- Amniocentesis or CVS: advantages and disadvantages adapted from Nussbaum et al and Binns et al [9, 11].

3. Percutaneous umbilical blood sampling (cordocentesis)

Cordocentesis or percutaneous umbilical blood sampling is a method of obtaining fetal blood from the umbilical vein [11], usually carried out around 20 weeks of gestation.

This method can be used to diagnose haematological disorders, congenital infections and to perform karyotype analysis. However, as it is a technically challenging method, it is usually realized only when amniocentesis or chorionic villus sampling cannot provide the required information or when these tests have inconclusive results.

The miscarriage risk associated with this procedure is, in theory, between 1-5%.

Screening Methods

Prenatal screening can be defined as the identification among apparently normal pregnancies of those at high risk for a specific fetal disorder which justify subsequent invasive and/or costly prenatal diagnostic test [6, 13].

Therefore, in prenatal care, the perfect screening test should:

- be used early in pregnancy;
- be safe;
- detect all fetal genetic conditions;
- have low false-positive and false-negative rates.

Since the discovery of alpha-fetoprotein, the screening methods have been progressing over the years. Nowadays, depending on the screening approach used, the detection rates are ranging between 75% and 96% [6].

In Portugal, as well as in most developed countries, the screening procedures currently used in the public health system are based on ultrasound signs and maternal serum biochemical markers. Usually, they are performed at two different times.

During the first trimester, the test is typically done at 11 to 14 weeks of gestation and it involves the sonographic nuchal translucency, and maternal serum biochemical testing (β – HCG and PAPP-A). Based on these results, as well as the mother's age, an adjusted risk of having an aneuploidy fetus can be given to the patient [6].

Between 15 and 20 weeks of gestation, four biomarkers are measured in the maternal blood: AFP, β – HCG, unconjugated estriol and inhibin-A. The same way, considering maternal age and serum results, an adjusted risk is calculated.

The first trimester risk assessment can be combined with second trimester serum analyse originating the integrated screen.

It is important to make women understand that the screening test does not give a positive or negative result. In other words, a “high-risk” fetus can be completely normal and for that reason, an invasive test is required in these situations (table 2).

Risk Factor	**Gestational Age** (weeks)	**Sensitivity** (%)
Maternal Age (Age≥35y)	----------	30
First trimester risk assessment	11-14	82-87
Second Trimester serum analyse (quad) screen	15-20	80
Integrated Screen	11-20	94-97

Table 2- Performance parameters of screening tests for fetal trisomy 21 adapted from Norwitz et al [6].

Chromosome Analysis

It was in 1959 that Lejeune and colleagues [14] reported that Down syndrome was caused by an extra copy of chromosome 21.

This technical innovation opened the way to the linking of abnormal numbers of chromosomes (aneuploidies) and chromosomal structural alterations associated with congenital anomalies [3].

As it was previously described, in the mid-1960s researchers showed that it was possible to diagnose aneuploidy in fetal cells floating in amniotic liquid.

Since then, cytogenetic analysis has been considered an indispensable diagnostic tool for the identification of chromosome abnormalities in prenatal diagnosis, improving its resolution year after year.

Although a high proportion of affected pregnancies end in spontaneous miscarriage, the incidence of major chromosomal abnormalities in newborns is still around 0.5% and 1% (table 3) [15].

Therefore, the prenatal diagnosis of these conditions can provide parents the birth of a healthy child avoiding the morbidity and mortality associated with some of these disorders.

Abnormality	Incidence per 10,000 Births
Autosomes	
Trisomy 13	2
Trisomy 18	3
Trisomy 21	15
Sex Chromosomes	
45, X	1-2
47, XXX	10
47, XXY	10
47, XYY	10
Other unbalanced chromosomal rearrangements	10
Balanced rearrangements	30

Table 3– Incidence of chromosome abnormalities in the newborn adapted from Turnpenny et al [15].

1. Karyotype Analysis

Karyotype analysis is based on the microscopic examination of the chromosomes. After an invasive procedure (amniocentesis, CVS or cordocentesis), cells are cultured for usually 8-14 days and then, the number of chromosomes is counted and their structure evaluated [11].

The diagnostic accuracy of karyotyping has been found to be 97.5% to 99.8% [6].

Conventional cytogenetic analysis requires a long turnaround time (8-21 days), conventional cytogenetic analysis and it cannot reliably detect rearrangements of genomic segments smaller than 5-10 Mb.

In order to overcome this resolution limitation there was a need to introduce a more efficient technique that could act at the molecular level: FISH.

2. Fluorescence In Situ Hybridization (FISH)

FISH was the first molecular test introduced to detect the presence or absence of microdeletions, microduplications and aneuploidy without the full effort associated with DNA sequencing or complete karyotype analysis [11].

The prenatal FISH panel typically targets chromosomes 13, 18, 21, X and Y and the results are usually available within 24 to 48 hours [6].

Additionally, a variety of probes can also be employed in order to detect other chromosomal structural rearrangements or aneuploidies. However, FISH analysis is limited because the sequence of specific genetic mutation must be known to apply the correct probe [11].

3. Quantitative Fluorescence Polymerase Chain Reaction (QF-PCR)

As FISH, QF-PCR emerged in order to allow rapid detection (1 or 2 days) of common aneuploidies at a lower cost and less labour intensive analysis.

In QF-PCR, highly polymorphic short tandem repeats (STR's) on chromosomes 13, 18, 21, X and Y are amplified using fluorescence primers and PCR in a multiplex assay, followed by the automated analysis of fluorescence intensity of the alleles in a genetic analyser.

Besides QF-PCR be a less expensive method, it also allows the simultaneous processing of much larger number of samples than FISH [16].

Since QF-PCR is designed to identify only the chromosomal abnormalities that are specifically being sought, it cannot detect most structural chromosomal abnormalities [16].

4. Multiplex Ligation-dependent Probe Amplification (MLPA)

MLPA was designed to detect gene dosage abnormalities in a wide range of conditions by the relative quantification of up 45 different DNA sequences in one reaction [16].

Although the results are typically available after 2-3 days, MLPA has the same limitations as QF-PCR in terms of targets. However it is less labour intensive and has a lower cost which is a good advantage in a dynamic laboratory and for the national health system.

5. Array Comparative Genomic Hybridization (aCGH)

Despite all the techniques described above, in clinical practice it is not uncommon that pregnancies at high risk for aneuploidy show a normal conventional chromosome analysis [17]. Some genomic imbalances associated with congenital malformations and/or intellectual disabilities are not detected using these techniques, which can give parents the false belief that they will have a healthy child.

In this context, a high-resolution technique emerged, whole-genome array comparative genomic hybridization (aCGH), which measures gains and losses of DNA throughout the human genome (figure 2) [18].

Currently, aCGH is considered the first tier genetic test for the investigation of children with unexplained intellectual disability, congenital anomalies, or autism spectrum disorder [18] since it offers significantly higher (15-20%) diagnostic yields than conventional karyotyping [19].

Besides the fact that aCGH analysis is more informative than karyotype and FISH [20], it also provides the result in a short turn-around time (3 to 5 days after DNA extraction) once it usually does not require cultured cells [17].

In addition, chromosomal microarray analysis is a standardized procedure that involves the use of computerized analysis, whereas karyotyping involves microscopic examination of banded chromosomes and may be more subjective and prone to human error [18].

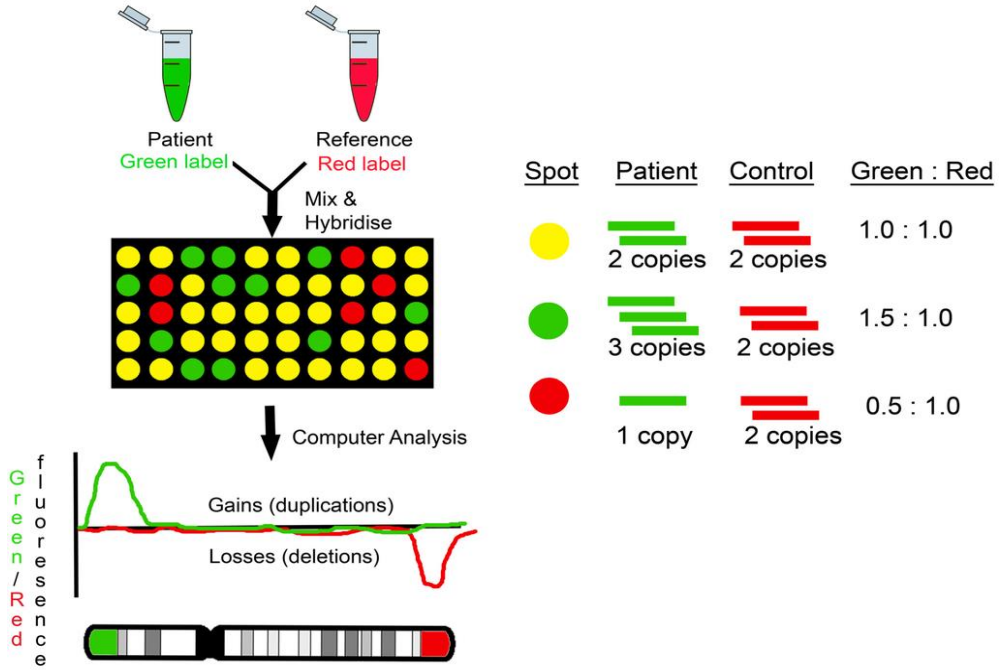

Figure 2- DNA is extracted from a control individual with a known, usually normal, karyotype and from the fetus. The two DNA specimens are differentially labelled with two different fluorochromes. Both sets of genomic DNA are hybridized to thousands of probes printed on a glass slide or microarray. Differences between the fluorescent intensities along the length of any given chromosome will reveal gains or losses of genome segments adapted from Karampetsou et al [21].

Therefore, in 2009, the American College of Obstetricians and Gynaecologists (ACOG) suggested that aCGH may be a useful adjunct in the prenatal diagnosis of multiple congenital

anomalies [20].

Since then, a lot of studies comparing aCGH and conventional karyotype were reported, proving the efficacy of the method. In pregnancies with abnormal ultrasound but normal karyotype, aCGH uncovers pathogenic imbalances in up to 5.2% of cases [20].

Nevertheless, this technique presents some limitations (table 4) and the major challenge appears to lie in the interpretation of the results [22]. The detection of variants of unknown clinical significance (VOUS) or incidental findings related to genetic abnormalities associated with adult-onset disorders, for example, raises several ethical problems due to its interpretation or lack of it [23].

VOUS describe DNA changes that have not yet been reliably characterized either as benign, pathogenic or associated with a variable phenotype (variable penetrance) [18].

These findings can result in substantial patient anxiety, which highlights the critical need for comprehensive patient pre-test and post-test genetic counselling from qualified personnel [18].

Nevertheless, the interpretation of results is expected to improve as knowledge of the human genome grows and the use of databases to link clinical findings with copy number variants becomes more robust [18].

Limitations
aCGH cannot detect:
• Triploidy • Balanced inversions • Balanced insertions • Balanced translocations • Low level of mosaicism
aCGH can detect:
• VOUS • Genetic abnormalities associated with adult-onset disorders

Table 4- Limitations of aCGH adapted from ACOG and SMFM recommendations [18].

Considering all the advantages as well as the limitations of aCGH, the American College of Obstetricians and Gynaecologists (ACOG) and the Society for Maternal-Fetal Medicine (SMFM) have jointly issued new guidelines (annex 2).

These recommendations underline the benefit of aCGH particularly when ultrasonographic examination identifies fetal structural anomalies, but also highlight the ethical issues surrounding the results' interpretation.

As far as incidental findings are concerned, there is no straight forward guideline on how this should be carried out. In Europe, the current tendency is to ask parents whether they want to be informed about treatable late-onset diseases or not [24]. Although opinions may differ about this point, all articles published about aCGH in prenatal diagnosis emphasize the need for pre- and post-test counselling.

In situations where a *de novo* balanced structural abnormality is identified, a 6% risk of intellectual disabilities is given to the patients. It has been described that approximately 37% of these cases have imbalances involving the same chromosome or other involved or not in the rearrangement that are detected by aCGH. Thereby it is recommended its use in these situations.

Non-invasive Prenatal Diagnosis

Although all medical advances in the last few decades, the gold standard methods in prenatal diagnosis are still invasive techniques (amniocentesis or CVS). For that reason, when Lo and colleagues [7] showed that cell-free fetal DNA (cffDNA) could be reliably detected in the maternal circulation during pregnancy, a desire to avoid contact with the fetus grew out and a lot of techniques are being developed ever since.

The cffDNA derives from apoptotic cells in placenta and it appears in maternal blood around 4 weeks of gestation [25]. Typically, it is smaller than maternal DNA (approximately 150 basepairs in length), but it represents all the fetus genome [26]. Though, non-invasive

prenatal diagnosis (NIPD) or, more precisely, non-invasive prenatal testing (NIPT) is referred to the analysis of these fragments of DNA.

Other interesting characteristic about fetal DNA is that it is rapidly cleared from the maternal circulation after delivery, which means it is pregnancy-specific. This fact is extremely important because it avoids that the DNA of previous pregnancies could be confounded with the DNA of subsequent pregnancies [27].

However, although it increases during pregnancy, the fetal fraction is only 10-20% of the total cell-free DNA [28]. Therefore, isolating cell-free fetal DNA was technically challenging and the first applications of NIPT were focused on the detection of sequences paternally inherited, such as Y-chromosome sequences used for fetal sex determination.

With recently technical advances, some of these barriers were exceeded allowing fetal sex determination in pregnancies at risk of a sex-linked disease, fetal rhesus D determination, the diagnosis of some monogenic diseases, and an accurate screening test for aneuploidies.

Nevertheless, the ease of access and safety of these tests, bring significant social and ethical issues that have to be discussed in order to avoid the misuse of this new technology.

Clinical Utility

- Fetal Sex Determination

As the Y chromosome of a male fetus can be easily distinguished from maternal DNA, the first clinical application of NIPT was the gender determination [8]. This test can be performed from 7 weeks' of gestation and it is usually based on the determination of *SRY* and *DYS14* genes [29].

The determination of fetal gender is offered when a male fetus is at risk of a sex-linked disease, such as haemophilia or Duchenne muscular dystrophy, when the development of

external genitalia is ambiguous and in some endocrine disorders, such as congenital adrenal hyperplasia.

With these new techniques, invasive procedures were reduced in almost 50% in pregnancies at risk of an X-linked condition because they are only needed when the fetus is male [8].

When female fetus are diagnosed with congenital adrenal hyperplasia, the administration of dexamethasone before 9 weeks' gestation can prevent fetal virilization.

A study realized in United Kingdom [30] revealed that this method does not entail additional costs when compared to invasive prenatal methods and avoid the risks of these techniques.

- Fetal Rhesus D determination

When a rhesus negative mother is carrying a rhesus positive fetus, she can develop antibodies against Rh antigen during the birth.

Usually, at the first pregnancy, this is not a problem because there is no sufficient antibodies causing damage to the fetus. However, in subsequent pregnancies, if the fetus are Rh negative, these antibodies can cross the placenta and cause the haemolytic disease of the newborn.

Currently, this pathology can be effectively prevented by the administration of anti-D antibodies during the first trimester of pregnancy. However it has been estimated that 40% of caucasian women receive unnecessary antenatal immunoglobulin, as they are carrying a rhesus negative fetus [31].

Invasive techniques are very effective in determining the RhD status of the fetus but besides the risks of miscarriage, they also provide maternal sensitization to RhD.

For these reasons, numerous efforts have been done regarding the determination of fetal RhD status from cffDNA. Nowadays, this test is currently available and it can be performed effectively from 11 weeks' of gestation with high levels of accuracy [8].

- Single gene disorders

As single gene disorders affect only 3.6 in 1000 live births [31], the progress of NIPT in this area has been slow. This represents a much smaller market than aneuploidies, for example, and it often requires the development of custom-made assays [32].

Thereby, clinical applications of NIPT to detect monogenic disorders are limited to autosomal dominant diseases where the father carries the mutant allele and autosomal recessive diseases where the parents carry different altered alleles.

Nonetheless, in the last few years, some techniques are being developed in order to determine if a fetus has inherited a maternal mutant allele.

In 2008, it was published a study based on digital PCR and relative mutation dosage that compare the quantity of mutant and non-mutant alleles in the maternal blood samples. More recently, a method based on massively parallel sequencing (MPS) and relative haplotype dosage was also reported [8]. Although some of these conditions are already described (table 5), studies must go on to overcome technical challenges.

Achondroplasia	**Paternally inherited or de novo autosomal dominant disorders**
Apert Syndrome	
Early onset primary dystonia I	
Thanatophoric dysplasia	
Huntington's disease	
α- thalassemia	**Autosomal recessive conditions where parents carry different altered alleles**
β- thalassemia	
Congenital Adrenal Hyperplasia	
Cystic Fibrosis	
Leber congenital Amaurosis	
Propionic Acidemia	
Frasers syndrome	
Autosomal recessive polycystic kidney disease	
Sickle cell anemia	**Autosomal recessive conditions where parents carry the same altered allele**
β- thalassemia	
Haemophilia	**X- linked conditions**

Table 5- List of NIPT for single gene disorders publications adapted from Daley et al [8].

- Aneuploidies

Unlike single gene disorders diagnosis, the development of NIPT for aneuploidy has been extremely fast due to its huge potential market. However, the ability to detect the increased chromosomal dosage resulting from fetal aneuploidy is technically challenging [6].

In 2008, the first proof of principle studies demonstrated that NIPT for Down's syndrome was possible using massively parallel sequencing [8]. Three years later, several studies were published and this technique was validated as a screening test for trisomy 21 with high sensitivities (98.6-100%) and specificities (97.9-100%) in high risk women [8].

Massively parallel sequencing is based on the sequencing of millions of short DNA molecules simultaneously, revealing both the identity and quantity of DNA fragments [33]. After sequencing, fragments are categorized by chromosome and the levels of these fragments are then compared with a reference euploid sample (figure 3) [8].

The other next-generation sequencing approach currently available is targeted sequencing. Unlike MPS, it is based on the amplification of only those chromosomal regions that are of interest (e.g. chromosomes 21, 18, 13, X and Y).

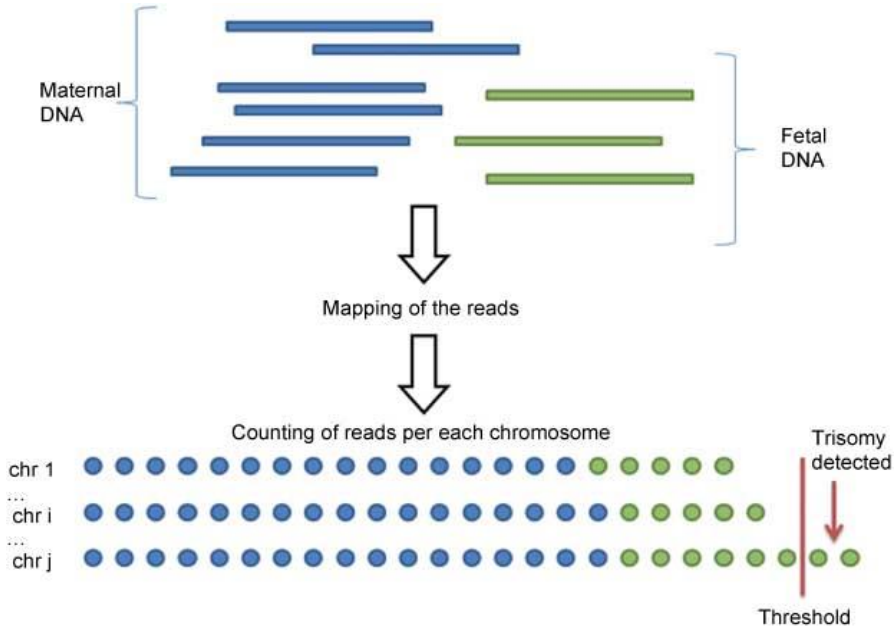

Figure 3. Massively parallel sequencing for the non-invasive prenatal detection of fetal chromosomal aneuploidy adapted from Norwitz et al [6].

Besides NGS, other techniques have been developed in order to detect fetal aneuploidies.

Methylated-DNA approaches are based on the difference in methylation pattern between mother's and fetus' genes and seem to be very promising as they are easy to perform, fast and inexpensive when compared to massively parallel sequencing [27].

Nowadays, the detection of trisomy 13, trisomy 18 and trisomy 21 using cffDNA is a reality and it can be performed as early as the 10th week of gestation [34]. However, some technical and biological factors can cause discordant results.

As it was explained previously, fetal fraction increases with gestational age, so, an early gestation age can cause false negative or inconclusive results. But this is not the only cause affecting fetal fraction. Studies showed that obese women have higher levels of maternal cfDNA originated from apoptosis and necrosis of adipose tissue which reduces the fetal fraction [8].

Furthermore, the type of aneuploidy can also influence the amount of fetal DNA. When compared to euploid samples, fetus with trisomy 21 have a higher fetal fraction. On the other hand, fetus with trisomy 18, trisomy 13 and monosomy X have a decreased fetal fraction [35].

Therefore, when NIPT shows a positive result, an invasive procedure is still required to confirm, revealing that more work is needed towards a higher efficiency of these tests.

Although there are some studies reporting high accuracy detection rates in low risk women [36], Society for Maternal-Fetal Medicine (SMFM) consider the available data insufficient to change current practice recommendations [37].

Therefore, at the present time, the American Congress of Obstetricians and Gynecologists (ACOG) [34] and the Society for Maternal-Fetal Medicine (SMFM) [37] consider NIPT more appropriate for high-risk women (table 6).

Despite this information, all pregnant women can access NIPT through various private testing companies. In Portugal, the test is done abroad and it costs around 495 and 670€, depending on the chromosomes included.

Indications for considering the use of NIPT
• Maternal age 35 years or older at delivery
• Fetal ultrasonographic findings indicating an increased risk for aneuploidy
• History of a prior pregnancy with a trisomy
• Positive test result for aneuploidy, including first trimester, sequential or integrated screen, or a quadruple screen
• Parental balanced robertsonian translocation with increased risk of fetal trisomy 13 or trisomy 21.

Table 6- Indications for considering the use of NIPT adapted from ACOG and SMFM recommendations [34].

Ethical issues

With recent advances in prenatal diagnosis particularly those concerning NIPD through maternal blood, it is impossible not to highlight some ethical concerns about the access of fetal genome. The facility with which a blood sample could be taken has the potential for the test to be viewed as a 'routine', placing in jeopardy the need for informed consent [38].

Furthermore, as soon or later these new approaches will make possible the diagnosis of any genetic disorder, it can be argued that it diminish the value of fetal life, increasing the number of abortions. It is also possible that the number of disabled children will decrease which

can be viewed as another outcome of these new prenatal techniques, since every family has the right to have a 'normal' child.

NIPT can also lead to sex selection for social reasons causing a gender disproportion, as it is already seen in China, for example, where the number of males under 20 years old exceeded in 25 million the number of females in 2005 [29,38].

Moreover, the use of comparative genomic hybridization in non-invasive prenatal diagnosis will allow screening of other genetic disorders including late-onset diseases like Parkinson or Alzheimer, which poses difficult ethical issues [39].

Conclusion

The discipline of prenatal diagnosis has evolved to encompass a wide ranging programme of screening and laboratory diagnosis that has become a routine part of antenatal care [40].

Since its implementation in the early 1970s, the technological advances, as well as the improvements in cytogenetics and cytogenomics, led to a greater sensitivity in the prenatal detection rates of fetal anomalies.

In Portugal, as in many developed countries, prenatal testing is divided into two categories: prenatal screening and prenatal diagnosis.

Prenatal screening is offered to all pregnant women as part of routine prenatal care to determine if the fetus is at substantial risk of having a particular disorder [31]. If it is considered to be at "high risk", an invasive technique (prenatal diagnosis) is offered to provide a definitive diagnosis.

After the sampling with amniocentesis, chorionic villus sampling or other technique, a karyotype analysis is performed. Over the years, molecular cytogenetic methodologies have

improved the resolution of detecting chromosome abnormalities and have decreased the turnaround time for chromosome enumeration [16].

First it emerged fluorescence *in situ* hybridization, a high resolution technique which allows the identification of microdeletions and microduplications. Few years later, quantitative fluorescence polymerase chain reaction and multiplex ligation-dependent probe amplification proved to be less labour intensive and less expensive methods when compared to fluorescence *in situ* hybridization.

Recently, a high-resolution whole-genome screening that can identify major chromosomal aneuploidy as well as the location and type of specific genetic changes that are too small to be detected by conventional karyotyping, shows promising data in prenatal diagnosis [18].

According to several studies, array comparative genomic hybridization identifies additional clinically significant abnormalities in approximately 6% of the fetuses with ultrasonographic abnormalities [18].

However, this test has the potential to identify copy number variants of unknown clinical significance (VOUS), consanguinity, non-paternity and genetic abnormalities associated with late-onset diseases.

Therefore, in December of 2013, the College and the Society for Maternal-Fetal Medicine published some recommendations helping geneticists and health care professionals to frame aCGH in the actual prenatal diagnosis [18].

Nonetheless, the remarkable advances in prenatal diagnosis were not only in cytogenetics field. Over the last two decades, numerous attempts have been made to find non-invasive methods to diagnose fetal genetic conditions [41].

Since the discovery of cell-free fetal DNA [7], non-invasive prenatal techniques have rapidly evolved with current clinical uses including fetal sex determination in pregnancies at

high risk, fetal rhesus D determination, the diagnosis of some single gene disorders, and a highly accurate screening test for aneuploidies [8].

Although they are safe for the pregnancy, currently available screening tests for fetal aneuploidy have poor accuracy with false-negative rates between 12% and 23% and false-positive rates between 1.9% and 5.2% [6].

On the other hand, studies evaluating the clinical utility of NIPT in high-risk women reported sensitivities for detection of trisomy 21 ranged from 98.6% to 100% and specificities from 99.7% to 100% [41].

However, this test presents some limitations and it is not considered a diagnostic test, at least not yet, which means that an invasive test is required when the result is positive.

Moreover, the Society for Maternal-Fetal Medicine (SMFM) and the American College of Obstetricians and Gynecologists (ACOG) do not recommend NIPT as a primary screening tool for all pregnant women [34,37], as there is no sufficient data to validate the method in low-risk women.

Besides all the constraints referred, the current prices of NIPT are unaffordable for many Portuguese women, ranging between 495 and 670€. Comparing to the actual screening approach, which is free in public health system and costs around 50€ in private sector, the implementation of this test for all pregnant women seems unfeasible.

At last, if NIPT with NGS and aCGH is ever to completely replace conventional cytogenetic analysis following an invasive approach, it will need to match the diagnostic accuracy as well as the scope of anomalies that can be detected.

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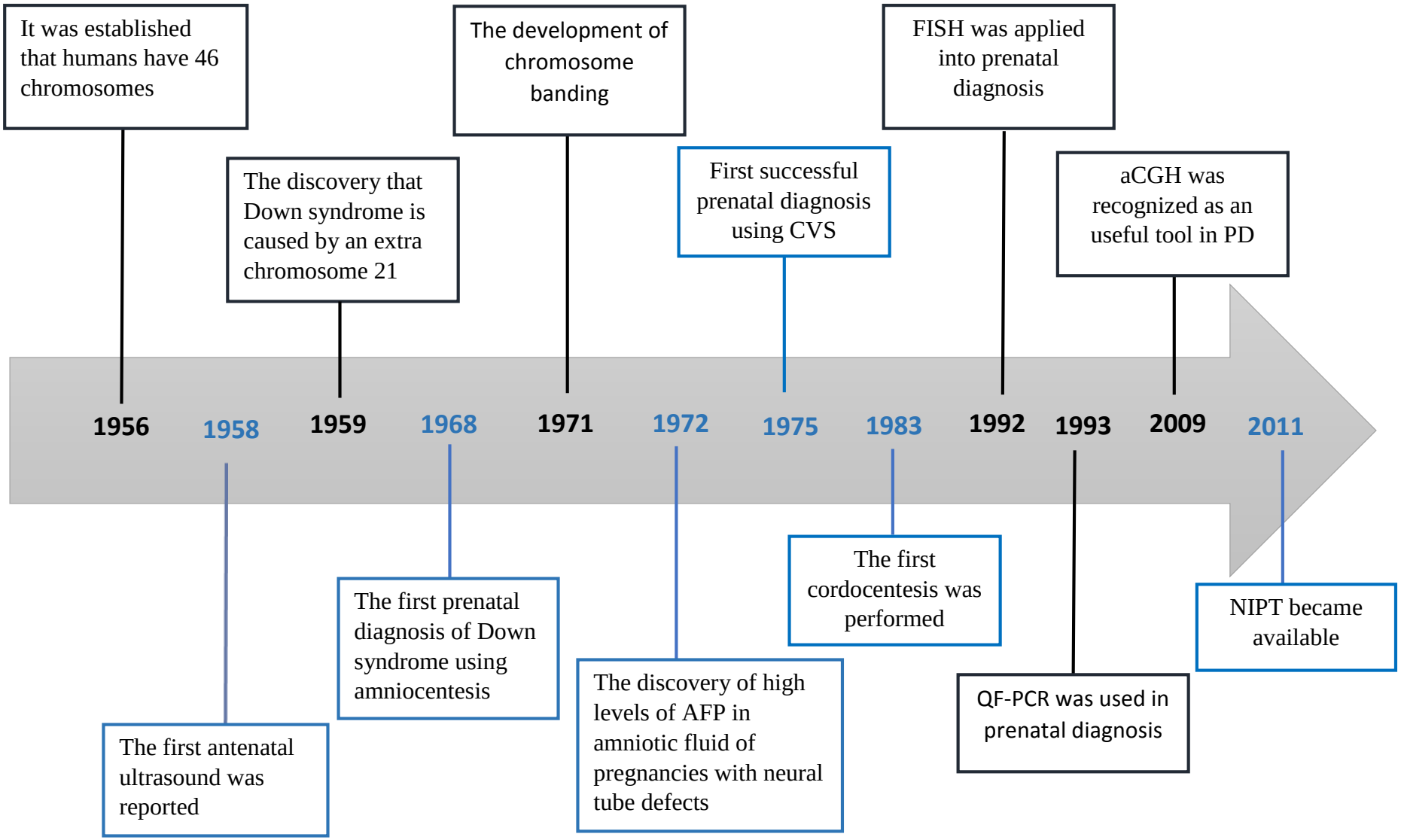

Annex 1 - The evolution of Prenatal Diagnosis [based on references 1; 3; 9; 14; 16; 42-44].

The College and the Society for Maternal-Fetal Medicine offer the following recommendations for the use of chromosomal microarray analysis in prenatal diagnosis:
• In patients with a fetus with one or more major structural abnormalities identified on ultrasonographic examination and who are undergoing invasive prenatal diagnosis, chromosomal microarray analysis is recommended. This test replaces the need for fetal karyotype.
• In patients with a structurally normal fetus undergoing invasive prenatal diagnostic testing, either fetal karyotyping or a chromosomal microarray analysis can be performed.
• Most genetic mutations identified by chromosomal microarray analysis are not associated with increasing maternal age; therefore, the use of this test for prenatal diagnosis should not be restricted to women aged 35 years and older.
• In cases of intrauterine fetal demise or stillbirth when further cytogenetic analysis is desired, chromosomal microarray analysis on fetal tissue (ie, amniotic fluid, placenta, or products of conception) is recommended because of its increased likelihood of obtaining results and improved detection of causative abnormalities.
• Limited data are available on the clinical utility of chromosomal microarray analysis to evaluate first-trimester and second-trimester pregnancy losses; therefore, this is not recommended at this time.
• Comprehensive patient pretest and posttest genetic counselling from qualified personnel such as a genetic counsellor or geneticist regarding the benefits, limitations, and results of chromosomal microarray analysis is essential.

- Chromosomal microarray analysis should not be ordered without informed consent, which should be documented in the medical record and include discussion of the potential to identify findings of uncertain significance, nonpaternity, consanguinity, and adult-onset disease.

Annex 2 – Guidelines on the use of chromosomal microarray analysis in PD adapted from ACOG and SMFM recommendations [18].