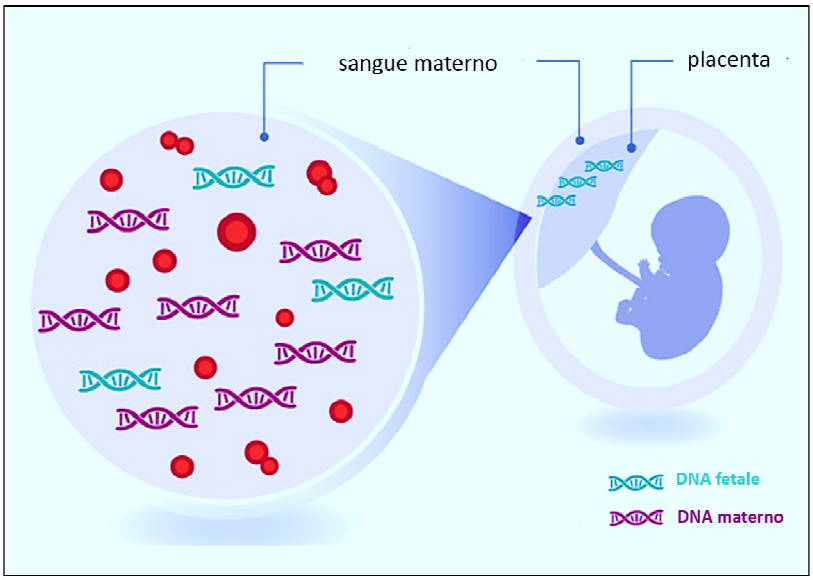
Ministry of Health

Higher Health Council

Section I

Guidelines

DNA-based Non-Invasive Prenatal Testing – NIPT



Fetal nucleated cells and cell-free fetal DNA (cffDNA) originating from placental cells are present in maternal plasma during pregnancy.

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Higher Health Council of Italy (Consiglio Superiore di Sanità - CSS)
(Chair: Pr Roberta Siliquini)

Working group

"DNA-based Non-Invasive Prenatal Testing – NIPT"

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Higher Health Conucil of Italy, Section I

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"DNA-based Non-Invasive Prenatal Testing - NIPT"

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1. Introduction

Techniques for prenatal diagnosis include instrumental and laboratory tests, which have been developed over the past 50 years with the aim of monitoring the unborn baby, from the early stages of embryonic development until just before birth.

Prenatal ultrasound, i.e. ultrasound (US) monitoring of pregnancy, represents the most important and widespread non-invasive technique for prenatal diagnosis, currently used to monitor the embryo and fetus development, to check its state of health and monitor pregnancy progression, as well as to provide support to invasive procedures of fetal tissues sampling. The extraordinary spread of prenatal US is due to its non-invasiveness and safety that allow its repetition throughout pregnancy, and to the high degree of resolution obtained with the last generation instruments. For this reason, prenatal US monitoring is performed in almost all pregnancies in developed countries and is a major technique for prenatal diagnosis. The potential of this technique correlates directly to the gestational stage at which it is performed, to the instrument resolution and the operator's skills.

Amniocentesis is the most common invasive technique for prenatal diagnosis (>100.000 samples per year in Italy) aimed at sampling amniotic fluid through an ultrasonographic-guided transabdominal puncture, ideally around 15-16 weeks of amennorhea. The risk of abortion related to the invasiveness of this technique has been estimated at around 1:200, although it varies with the operator's experience. Amniotic fluid contains non-corpuscolar components, which are cell-free and can be isolated by centrifugation of the sample, and corpuscular components composed of amniocytes, which are cells originating from fetal skin, mucosae, uro-genital and gastro-intestinal tracts and amniotic membranes. The non-corpuscolar part is often used to measure the levels of alphafetoprotein (AFP) and other biochemical markers, while amniocytes are primarily used for cytogenetic analysis and, eventually, molecular and biochemical testing both directly or on cultured cells.

Chorionic villi sampling is an invasive technique used to obtain an aliquot of chorionic villi (>25.000 samples per year in Italy) through an ultrasonographic-guided transabdominal puncture, ideally around 10 -12 weeks of amennorhea. The risk of abortion related to the invasiveness of this technique is estimated at around 2-3% but it varies widely in relation to the operator's experience. The sample obtained can be used for cytogenetic analysis directly on cytotrophoblastic cells or on cultures (villus mesenchymal cells). The combined use of these two techniques provides information on cell populations of different embryonal origin, allowing to solve a possible discrepancy between placentar and fetal karyotype (reported in about 2% of samples), resulting from postzygotic mutation. Chorionic villus sampling allows to acquire a relatively large amount of biological material and, therefore, it represents the cornerstone technique for molecular diagnosis of disease-causing genes and for biochemical analyses. The benefit resulting from the earliness of the technique, compared to amniocentesis, is counterbalanced by its invasiveness and by acquisition of placentar and not fetal tissue.

Cordocentesis is a technique of fetal blood sampling carried out through a transabdominal puncture at around 17 weeks of amenorrhea. The risk of abortion related to the invasiveness of the technique is about 2%, but it varies widely in relation to the operator's experience. This technique is extremely disused (<350 samples per year in Italy) being especially performed to

monitor some infectious disorders and, sometimes, to interpret uninformative cytogenetic results obtained by amniocytes' analysis.

Non-invasive prenatal testing has been developed over the last 30 years and it essentially relies on analysis of biochemical markers in maternal blood, combined with US investigations. The prototype for this analysis was AFP assay, which was initially used as a marker for neural tube defects (increased levels) and, then, for Down syndrome (DS; decreased levels). As time passed by, this screening test based on association of several markers has been further developed to determine the probability of fetal aneuploidy, especially in mothers belonging to an age group with low risk of fetal chromosomal disorders and thus not subject to invasive monitoring during pregnancy. Triple test or triple screen allowed to predict about 65% of DSs, with a false positive rate ranging from 5 to 10%, and it was based on second trimester AFP, chorionic gonadotropin and unconiugated estriol assay, combined with maternal age and US-assessed gestational age. Many other protocols were added over the years, based on differenct combinations of several markers and on anticipating the screening from second to first trimester. At the same time, biochemical markers were integrated with US findings, in particular when assessing fetal nuchal skin fold thickness (nuchal translucency – NT), which is non-patognomic for DS but diagnostic for about 75% of cases between weeks 11 to 14 of amenorrhea, with a false positive rate of 5%. Double test has emerged in recent years, consisting in the measurement of the free fraction of beta-chorionic gonadotropin and PAPP-A (Pregnancy Associated Plasma Protein A), a high-molecular-weight glycoprotein, in maternal blood samples taken approximately during week 11. This analysis, integrated with the assessment of NT and maternal age can predict about 80% of DSs, with a false positive rate of about 6% (see Attachment 1). In this context, contingent screening should also considered (NT + biochemical markers at weeks 11-13; US markers at weeks 12-13 or biochemical markers at weeks 14-16 in groups with intermediate risk), allowing to improve the specificity of the test (Nicolaides et al 2014).

2. Cell-free fetal DNA in maternal blood

Lo (1997) first described the presence of Y chromosome in the plasma of women with male fetus, using the analysis of cell-free DNA (cfDNA) in maternal circulation.

It is proved that, starting from the first trimester of pregnancy, cell-free DNA of fetal origin (cell-free fetal DNA; cffDNA) is present in the maternal bloodstream, from which it can be sampled in a non-invasive way and used for studying some fetal diseases.

cfDNA originates from maternal and placental cell lysis. Starting from week 5 of amenorrhea, the placental cytotrophoblast anchors to the uterine decidua parietalis, decidual spiral arteries supply blood into lacunaes between decidua and placenta, and the cytotrophoblast invades, covers and remodels the walls of the uterine spiral arteries. Cytokine-mediated replacement of trophoblast cells, covering spiral-shaped arteries walls, releases DNA. Degraded fetal DNA fragments contain approximately 180 base pairs (bps) and are suspended in the arterial plasma.

cffDNA can be early isolated starting from week 10, when its amount is sufficient for a potential clinical use. Its percentage can vary between <4%, which is not useful for diagnosis, and 40%, with an average of 10%, at week 12, when about 90% of plasma circulating cell-free DNA fragments originate from apoptosis in the maternal epithelium, creating a mix of cffDNA and maternal cfDNA. The amount of cffDNA is called "fetal fraction" (FF). A few hours after birth cffDNA can no longer be found in the maternal circulation and it is probably eliminated by renal excretion.

2.1 General information on NIPT and fetal fraction

Regardless of the technique used, protocols for Non-Invasive Prenatal Testing (NIPT) are based on comparisons. For chromosome 21 (CR21), the technique compares the number of CR21 fragments in the tested pregnancy, with the number of fragments derived from another chromosome in the same sample (internal comparison), under a condition of disomy (two copies of a given chromosome, for example chromosome 1 or 10 or their combination), or with those of a pool of reference disomic pregnancies (two CR21). If the sample obtained from the tested pregnancy contains two pairs of CR21 (two for the mother and two for the fetus), the relationship among assays (number of CR21 fragments for the test/number of fragments for the reference disomic samples) is roughly equal to 1.

In case of a fetus with trisomy 21 (T21), the FF increases due to the presence of additional circulating fragments released from the supernumerary fetal CR21. The increase extent depends on the amount of total FF and the bp number in CR21, in relation to bps in the whole fetal genome.

Maternal plasma presents variable FF values, which differ from a sample to another. FF generally corresponds to about 10% of cfDNA, ranging between <4% and 40%, at approximately week 12. Accuracy of chromosome analysis can vary in relation to the total FF values of the sample and to their increase in the case of trisomy.

With a circulating FF of 10%, for example, the increase in FF with T21 is equal to approximately 5% of the total amount, and the ratio (R) between the number of CR21 fragments in the tested sample and the number of reference disomic fragments increases from 1 to about 1.05. With a FF of 20%, the

increase in the total FF related to fetal T21 is about 10%, with a consequent increase in the value of R from 1 to about 1.10. With a FF of 4%, thee increase in the FF related to fetaal T21 is about 2% and the R value increases from 1 to about 1.02. Finally, if the FF is less than the threshold value of 4%, R is <1.02, a value that can not be statistically differentiated from 1, predicting disomy of CR21, that is, if the fetus is normal. This explains why the ≥4% threshold is critical to avoid false negative results (FNR), with no/low FF.

It is therefore appropriate to measure the FF in tested samples, using protocols providing, before or during NIPT, another test that is usually based on the analysis of single nucleotide polymorphic sites (the so-called SNPs - Single Nucleotide Polymorphisms).

However, not all currently available NIPT protocols provide this analysis. Some NIPT tests enter the FF value into the algorithm to assess the risk of trisomy, while others use predetermined normalization factors that can still achieve high levels of reliability (Dan et al, 2012; Zhang et al, 2015).

2.2 Unused samples

In approximately 2% of samples taken at the end of the first trimester of amenorrhea, the FF does not exceed the 4% threshold. A preliminary study suggests that the rate of chromosomal disorders in these samples is significantly higher compared to that of the samples with a FF ≥4% (13.8% compared to 2.4%). With a second sampling, about half of these cases confirms that the FF is <4%. If confirmed, these data would suggest that samples not adequate for the predictive screening due to the low FF could indirectly suggest an increased risk of fetal chromosomal disorders. Consequently, assessment pf the FF value in the sample could guide clinical management of pregnancy (Turocy et al, 2015).

3. Techniques for cfDNA analysis

The currently used techniques analyze total cfDNA, without differentiating its fetal or maternal origin. Since **NIPT** is based on a mixture of maternal and placental DNA, it **must not be regarded as a diagnostic but rather as a screening test**. As in the traditional tests, the use of dedicated algorithms allows to define the post-test probability for a fetus being affected by one of the major autosomal trisomies (trisomy 21 [T21], trisomy 18 [T18], trisomy 13 [T13]) or by a sex chromosome aneuploidy (X, XXX, XXY, XYY), by analyzing the number of cffDNA fragments of the selectively tested chromosomes.

For the analysis of aneuploidy by NIPT, three main techniques based on second-generation sequencing (Next Generation Sequencing - NGS) are used: whole genome NGS; NGS of specific regions; Single Nucleotide Polymorphisms (SNP). NIPT based on array technology is currently proposed and, according to the first validation data, it would guarantee the same, or even an higher, performance than NGS techniques (Juneau et al, 2014).

Whole-genome NGS technique relies on cffDNA sequencing in the maternal plasma. It generates millions of short sequences of the whole genome, which are then mapped on a reference sequence, in order to determine their origin and establish the number of fragments originating from the chromosome of interest to be compared with the number of fragments obtained from the other chromosomes (Fan et al, 2008). Several algorithms determine whether the number of fragments in the sample is either increased or decreased, in relation to a threshold value suggestive of aneuploidy (Fan et al, 2008; Lo et al, 2014; Sehenert et al, 2011). For example, if a fetus is affected by T21, more CR21 fragments will be found in the maternal plasma compared to the expected number unaffected controls.

An alternative NGS technique amplifies in a selective way specific genomic loci on the chromosome of interest, which are then sequenced. The technique is less expensive due to the reduced number of sequenced regions, but analysis relies on the study of only a few pre-selected regions of interest. Following the first validation assessment of this technique for T21 and T18 in some high-risk populations, some general population have shown that the test provides reliable results, even for pregnancies with *a priori* low-risk (Lau et al, 2014; McCullough et al, 2014; Norton et al, 2014; Norton et al, 2015; Pergament et al, 2014).

The third technique is a variation of the previous one and it is based on the amplification of several polymorphic loci (SNPs) on the chromosome of interest (Nicolaides et al, 2013; Zimmermann et al, 2012).

Regardless of the instruments for sequencing and the bioinformatics algorithm used, bot sensitivity and specificity for major aneuploidies are high for all these three techniques (Boon et al, 2013; Gil et al, 2014).

4. Experiences with the use of cffDNA in prenatal screening

The NIPT has been promoted and implemented as a screening test by a few companies, that have started its marketing with clinical purposes since 2012: *Sequenom* (*MaterniT21*), *Verinata* (*Verifi*), *Ariosa* (*Harmony*), *Natera* (*Panorama*), *BGI* (*Nifty*).

A number of learned societies (ACOG, ACMG, ASHG, ESHG, ISPD, ISUOG, NSGS, SIEOG, SIGU) have since agreed on clinical validity and limitations, ethical and economic issues, meaning and role of NIPT (Dondorp et al, 2015).

4.1 Autosomal aneuploidies

4.1.a Sensitivity and specificity of T13, T18, T21 screening in single pregrancies

A recent meta-analysis of 37 studies (Gil et al, 2015) referring to the three major autosomal aneuploidies in single pregnancies, reported the following sensitivity (detection rate - DR) and specificity (false positive rate - FPR) of NIPT:

- T21 - DR 99.2% (95% CL, 98.5-99.6%); FPR 0.09% (95% CL, 0.05-0.14%);
- T18 - DR 96.3% (95% CL, 94.3-97.9%); FPR 0.13% (95% CL, 0.07-0.20%);
- T13 - DR 91.0% (95% CL, 85.0-95.6%); FPR 0.13% (95% CL, 0.05-0.26%).

Several factors explain these discrepancies, including *vanishing twin*, maternal metastatic disease and chromosomal mosaicism, and no/low FF, even when the main cause is feto-placental mosaicism. In fact, cffDNA in maternal plasma originates from placental cytotrophoblast, which can display a karyotype is discordant with the fetal one. Chromosome analysis in trophoblast samples has identified different percentages of mosaicisms.

Specificity for cffDNA testing in studies enrolling more than 10,000 samples has shown a FPR <1/1,000, in agreement with the percentage of feto-placental discrepancies derected by cytotrophoblast chromosome analysis.

4.1.b Sensitivity and specificity of T13, T18, T21 screening in twin pregnancies

Analysis of cffDNA can be performed also in bigeminal pregnancy, even after gamete donation. The analysis is limited to the screening of major autosomal trisomies and and its results express a probability distributed between the two fetuses.

With a limited number of cases, studies have quantified FF values by SNPs, and allowed to distinguish and measure the contribution of each of the two twins in dizygotic (DZ) pregnancies. In DZ pregnancies, the smaller twin, which provides a lower amount of DNA, produces a statistically lower FF than the average amount present in single pregnancy (8.7%, range 4.1-30%, compared to 11.7%, range 4-38.9%; $p<0.001$) (Bevilacqua et al, 2015).

These data suggest that, in these pregnancies, the contribution to FF by the two placentas is dishomogeneous and it could be even possible that one of the two is not sufficiently represented (FF <4%), increasing the risk of FNRs due to absent/insufficient contribution to the FF by one of the two placentas.

A meta-analysis of five publications has reported a sensitivity of 95% for T21; 86% for T18; 100% for T13 (numerical data for T13 and T18 are still too limited to be regarded as a plausible value of sensitivity). No FPR was found for any of the three trisomies (Gil et al, 2014; Huang et al, 2014; Bevilacqua et al, 2015). However, following a positive result, this test cannot predictd which of the two fetuses is affected.

4.2 Sex chromosome aneuploidies

Sensitivity and specificity for X and Y chromosome aneuploidies in single pregnancies

The specificity of NIPT in the screening of sex chromosome aneuploidies is lower than in autosomal trisomies.

A meta-analysis on 37 studies has reported a sensitivity (DR) of 90.3% (95% CL, 85.7-94.2%) and a specificity (FPR) of 0.23% (95% CL, 0.14-0.34%) for X monosomy. For all other sex chromosome aneuploidies (SCA), sensitivity was 93.0% (95% CI, 85.8 to 97.8%) and specificity 0.14% (95% CL, 0.06-0.24%) (Gil et al, 2015).

As far as X monosomy is concerned, cytogenetic analysis of trophoblast discloses a FPR for a mosaicism confined to the cytotrophoblast, and a FNR for a mosaicism confined to the fetus, with figures of about 1/1,421 (95% CL: 1,031-1,958) and 1/14 (95% CI: 8-26), respectively. Thids risk is higher, compared to that reported for autosomes, as X monosomy is the more frequent aneuploidy in feto-placental mosaicisms (Grati, 2014). As a result, NIPT shows for X monosomy (and in general for all SCAs) a reduced specificity, with a cumulative FPR >1%, due not only to confined to the placenta mosaicisms, but also to constitutional maternaò mosaicism, found in about 8.6% of NIPTs positive for a SCA (Nielsen et al. 1991; Thompson and Thompson, 2001; Wang et al, 2014).

4.3 Microdeletions

cfDNA can be ideally used to search also for specific deletions in the DNA sequence. Panels analyzing microdeletions associated with clinically-recognizable disorders (including 1p36 deletion, 5p deletion, 15q deletion, 22q deletion) have been created with the aim of developing whole-genome analysis techniques. However, preliminary results show a low sensitivity (62-95%) (Sequenom Presentations, NSGC, 2014), and a high FPR. The only published study to date used SNP genotyping and investigated four microdeletions (22q11.2, 1p36, 5pter, 15q11.2 associated with Prader Willi and Angelman syndromes; Wapner et al, 2014). The validation process showed some critical points. In particular, the majority of cases used for validation were not based on antenatal screening samples (plasma of pregnant women), but samples created in the laboratory, which simulated a disease (PlasmArt™). Therefore, sensitivity and specificity reported were not representative of the actual performance of the test in the clinical settings. Considering the sensitivity and specificity of a “promising” test (assuming a prevalence of 1/1000 of 22q11

deletion), positive predictive value of the test (i.e. the probability of being true of the microdeletion identified by the protocol followed) did not exceed 7%.

4.4 Mendelian disorders and other indications

The first clinical application of cffDNA concerned fetal sex determination (Lo et al, 1997), in relation to the presence/absence of sequences of SRY and DYS14 from Y chromosome in maternal plasma. In 2000, the same technique was used in Italy to rule out the paternal segregation of myotonic dystrophy (Amicucci et al, 2000). This technique is currently used in some countries to monitor pregnancies at risk of some X-linked diseases, such as Duchenne muscular dystrophy, in order to offer targeted molecular analysis only for pregnancies with male fetuses (Hill et al, 2011; Pan et al, 2014).

Another potential application is early non-invasive detection of fetal gender in pregnancies at risk of congenital adrenal hyperplasia (adrenal-genital syndrome), where identification of a male fetus justifies stopping treatment with steroids (Tardy- Guidollet et al, 2014). This analysis can also be useful in the management of fetuses with ambiguous genitalia detected by US (Everett and Chitty, 2014).

Another clinical application of cffDNA concerns the Rh phenotype prediction for fetuses conceived from RhD-negative mothers. A positive result in the maternal plasma is due to the paternal transmission of RhD-positive genotype. In these positive cases, the test allows a prompt prophylaxis with anti-RhD immunoglobulin (Finning et al, 2008).

Fetal or neonatal alloimmune thrombocytopenia is caused by maternal alloantibody response to paternally inherited antigens in fetal platelets. In 20% of cases, this disease results in intracranial hemorrhage, which can produce long-term sequelae in children. Studies of cffDNA can early analyze the disease-related gene, *HPA-1*, and then identify affected fetuses (Le Toriellec et al, 2013).

The most important challenge for development and research on clinical use of cffdNA is that of Mendelian disorders. There are currently some studies on this subject, such as those on fetal diagnosis of achondroplasia, either originated from de novo mutation or inherited from an affected father (Chitty et al, 2011; Lench et al, 2013), on thanatophoric dwarfism (Chitty et al, 2013), and Apert syndrome (Everett and Chitty, 2014).

In case of autosomal recessive disorders, where parents are heterozygous for different mutations, exclusion or presence of the father's allele can be used to assess the risk of an affected fetus; this risk is excluded in absence of the paternal mutation, while it increases when it is present. In the latter case, fetal genotyping should be performed with an invasive technique. This approach has been tested in pregnancies at risk of thalassemia (Papasavva et al, 2013; Saijun Liu et al, 2014) and cystic fibrosis (Twiss et al, 2014).

Most protocols using cffDNA to monitor pregnancies at risk of monogenic diseases are still under development; however, some of them have been approved recently for clinical use in the United Kingdom (Everett and Chitty, 2014).

5. Sensitivity, specificity and ethical issues

Data emerging from cytogenetic analysis of amniocytes show that T21, T18, T13 represent 50 and 75% of all chromosomal disorders, according to maternal age.

Sensitivity and specificity of cfDNA tests for T21, T18, T13 are high and consistent with results obtained from chromosomal analysis in trophoblast samples.

Biological limits

In addition to feto-placental discrepancies, which are shared by all investigations using fetal DNA in the first trimester and which can generate FPR and FNR, cfDNA tests can be affected by other factors, including:

1. constitutional chromosomal mosaicism in the mother: since the test is performed on a mix of maternal and fetal plasma DNA, the presence of anomalies involving the maternal chromosomes, like an abnormal cell line not necessarily resulting in an abnormal clinical phenotype, cam jeopardize the test results;
2. chromosome anomalies of iatrogenic origin in the mother, and therefore not constitutional: the test can be compromised by plasmatic fragments of maternal DNA mutated because of clastogenic agents (pharmacological, physical and viral agents that can damage DNA);
3. v*anishing twin* associated with a terminated pregnancy: in the first trimester of pregnancy, an important cause of discrepancy in genetic tests based on placental DNA is the presence of DNA fragments originating from the placenta of an early miscarriage.

Ethical profiles

The introduction of NIPT in clinical practice has opened a debate on pros and cons of its use as a prenatal screening procedure:

- NIPT is a non-invasive screening with a higher sensitivity than current screening tests, combinig biochemical analysis and nuchal translucency, which may precede or not the invasive diagnostic tests;

- NIPT dramatically decreases the use of invasive diagnostic procedures, reducing the number of abortions related to fetal tissues sampling and potential rare complications for pregnant women;

- Easy access to NIPT through blood sampling is not an incentive to the inappropriate use of prenatal tests to the detriment of current practice; however, this screening is currently targeted to the three major autosomal trisomies for which a high proportion of women generally ask to be informed about. Therefore, with regard to these trisomies, NIPT reduces the inappropriate use of genetic testing allowing to quiet expectant mothers and relieve their anxiety.

On the other hand:

- The higher sensitivity of this screening test in identifying the three major autosomal aneuploidies can send out the message that, with a simple blood test, it is easier to identify diseased fetuses putting the mother in a position to terminate them, although this technique limits the number of abortions of unaffected fetuses related to the invasiveness of traditional prenatal diagnosis techniques.

NIPT aims at providing correct information to couples who ask for it, so that their subsequent choices and decisions, whatever they are, can be based on an early knowledge as accurate as possible following protocols that do not jeopardize pregnancy.

Prenatal counseling is an integral part of the NIPT screening. Genetic counseling explains pros and cons of the screening, provides information on informations that can be obtained, on risks and benefits, on potential consequences related to perception and acceptance by parents of the information received and to decisions to be taken, on services of healthcare available, and on the path to follow after and abnormal result.

Centers offering the test must have expertise in US and prenatal diagnosis, they should be able to provide pre-test and post-test counseling, be connected with a medical genetics service and with the laboratory performing the test, which must be certified, participate in national and international quality controls, and be staffed with experts in NGS techniques.

These techniques raise new problems as the number of detected genetic diseases does increase. The availability of a large amount of information through a simple blood test changes the overall view of prenatal investigation, sometimes amplifying existing problems, such as the opportunity to offer NIPT to all pregnant women or rather only to those at higher risk of having affected children**.**

6. Socio-economic impact

At present, in Italy, NIPT is offered by some private clinics and laboratories mostly connected with commercial companies, which take charge of financially supporting tests, whose results are validated by documents produced by several scientific societies. Some national laboratories are starting to perform the test in an almost independent way and others are gearing up. It is estimated that now a day, in our country, potential users of this service account for about 50,000 mothers/year. The tests are charged to the user, with costs varying between 350 and 900 Euros.

Some geneticists and obstetricians have reported different realities in Italy. On one hand, some structures apply an information protocol of pre-test and post-test counseling. On the other hand, many candidates for the test complain of having sometimes experienced a biased use of scientific data, approximation in reporting the test limits, lack of pre-test and post-test counseling, and inadequacy of the informed consent process.

Cost-benefit evaluation of NIPT is not easy. In fact, any implementation model of the test should take into account a number of variable features, including actual acceptance of the test by pregnant women, false positive rate when the test is extended to several aneuploidies, acceptance of invasive diagnosis with a positive test, rate of termination of pregnancy after a fetal disease is confirmed. It should also be noted that other methods, first of all combined screening, give additional and broader information than the only set of chromosomes (eg. heart malformations), so it would be incorrect to consider NIPT as a replacement test for them.

With respect to T21, Beulen et al. (2014) have assessed the economic impact of NIPT introduction in the Dutch national health program and have suggested that the most effective choice would be NIPT application as the primary method of screening. Indeed, they have calculated that, using the test alone, detection rate for T21 would increase by more than 50%. However, if it used as an optional secondary screening test supporting combined screening of the first trimester, dection rate for T21 would increase by only 36%. Large-scale application of NIPT allow in any case to significantly reduce invasive prenatal diagnostic tests and, hence, risk of miscarriage. On the other hand, NIPT not only is the most effective method of non-invasive screening, but at the moment it is also the most expensive; its use as a primary screening method would increase costs by 157% for the Dutch national screening program, while the use of NIPT as an optional secondary test would increase costs by only 21%. The use of NIPT as a primary screening test is therefore bound to a significant reduction of costs of the technique.

Clinical utility and cost of NIPT based on cfDNA analysis was the object of another study in the United States, where it was compared with Combined Test (CT, including NT, βHCG, PAPP-A), and with INTegrated Test (TINT, including CT + AFP, estriol, hCG, inhibin A) (Song et al, 2013). At a base price of $795, NIPT was more clinically effective and less expensive than CT and TINT. NIPT T21 identified 4,823 T21 out of 5,330 invasive techniques; CT 3,364 T21 out of 108.364 investigations; TINT 3,760 T21 out of 108.760 investigations. Therefore, NIPT identified 28% more T21 than TINT and 43% more than CT, and reduced by more than 95% invasive techniques and by more than 99% abortions of euploid fetuses. The total costs were $3,786 millions for CT, $3,919 millions for TINT and $3,403 for NIPT. Therefore, compared to other non-invasive techniques, NIPT has better diagnostic capabilities, reduced fetal losses and reduced costs to the health system.

In order to provide NIPT screening for major autosomal aneuploidies in Italy, it is essential to centralize the screening in a limited number of laboratories, collecting samples from from different Regions. This would make it possible to lower the costs of analysis, which would become competitive compared to those currently covered by programs of invasive prenatal diagnosis (Kagan et al, 2015). At present, the NHS reimburses about €922.89 per cytogenetic investigation carried out using chorionic villus sampling and €595.11 per each cytogenetic investigation performed by amniocentesis (the costs include counseling, US, sampling and karyotype). It should be noted that NIPT could become an automatable laboratory method that may require complex management and fine-tuning but, once completed this initial stage, analysis of a high number of samples should not cause any particular complexity. Besides, its precocity and its scheduled execution do not imply critical and emergency situations that can be frequent in classical invasive diagnosis (e.g. ultrasound detection of anomalies, resulting in the need for urgent cytogenetic diagnosis by invasive procedure). Critical and emergency situations can and need to be managed, with an inevitable impact on operating costs of the laboratory (available staff, reagents, instruments).

It is evident that centralization should concern execution of the test, while its management in terms of acceptance (pre-test counseling, informed consent) and result communication (post-test counseling) should be planned according to the different situations in the Italian territory. This network should be connected to fetal medicine centers/services and genetics laboratories for the management of positive cases.

7. Quality and accreditation

Tests based on NGS techniques and platforms require a greater collaboration of the different professionals involved in prenatal diagnosis (primarily geneticists and obstetricians) than that required by traditional genetic testing. This in order to optimize the definition of scope and aims of the investigation, and to establish, on a case-by-case basis, results that require to be analyzed thoroughly.

In relation to the technology available, laboratories that are about to implement and offer these tests need to have the competencies (even in bioinformatics) required to characterize variants of potential clinical relevance, particularly through the use of up-to-date databases and tools, and they must be certified (ISO 15189 or similar; Laboratory accreditation and certification; http://www.eurogentest.org/).

8. Recommendations for the use of cffDNA testing

Tests based on cfDNA, involving DNA analysis, are genetic tests. Results obtained and data on sensitivity and specificity must be interpreted according to the results derived from cytotrophoblast and amniocyte cytogenetic analyses.

NIPT must be associated with and preceded by accurate US after week 11, and carried out by accredited operators for pregnancy monitoring during weeks 11-14. When US data suggest an increased risk of chromosomal disorders in the fetus, it must be consider the possibility to directly execute an invasive prenatal diagnosis for fetal karyotype analysis, and other techniques when necessary (eg. array-CGH, to increase the diagnostic power in detecting pathogenic submicroscopic imbalances, which is estimated in about 6%) (Novelli et al, 2012; Wapner et al, 2012).

It is mandatory for women willing to undergo NIPT to obtain in advance, through an interview and, if appropriate, genetic counseling, information necessary to understand the characteristics of the test and its limits, even in relation to the other prenatal diagnostic techniques available, and to sign the informed consent document, before the test.

A Centre offering the test should be able to offer post-test genetic counseling and comprehensive support to the patients throughout the entire prenatal diagnostic procedure.

A Centre should also mange the pregnancy follow-up, taking into account the mother's residence.

9. Conclusions

1. DNA-based Non-Invasive Prenatal Testing (NIPT) is not a diagnostic test. NIPT investigates the probability of a fetus being affected by the most common aneuploidies, with specificity and sensitivity that are significantly higher than in combined non-invasive testing (NT + PAPP-A / βHCG). NIPT defines the presence of the specific fetal disease investigated, on a probability basis. **Therefore, any positive result must be confirmed by a traditional invasive technique (chorionic villus sampling/amniocentesis).**

2. Testing should be preceded by ultrasound and pre-test counseling, which aims at explaining the meaning of the test and all the alternative options available for pregnancy monitoring. Before testing, pregnant women must give their consent, in particular to the use of residual biological samples (Attachments 10.3 and 10.4).

3. In 2% of cases at least, samples acquired cannot be reported. Results are reliable if obtained from a percentage of free fetal DNA that is not less than 4% of total free DNA present in the maternal plasma.

4. The investigation is currently targeted and validated for major autosomal aneuploidies (T21, T18, T13). The chromosomal anomalies investigated concern only a portion, although relevant (50-70%), of the chromosome aberrations that might be present in the fetus. In relation to the technique used, it will possible in the future to get more extensive information on other aneuploidies (of sex chromosomes, for example), micro-rearrangements and Mendelian disorders. As a result, the rationale of the test should be reconsidered. **NIPT can be performed in twin pregnancies, even after gamete donation.**

5. Generally, results indicative of a "low risk of trisomy" should be considered reassuring for the mother, due to the high specificity of the test and its high negative predictive value. However, the results of screening could refer to genetic characteristics of the cytotrophoblast (placenta) that, in rare cases, may be inconsistent with those of the fetus (feto-placental discrepancy).

6. NIPT is not a substitute for other tests and, therefore, it does not avoid pregnant women undergoing other clinical, laboratory and instrumental investigations that are integral part of pregnancy monitoring.

7. Centers providing testing must:

a) have expertise in ultrasound diagnosis;

b) have expertise in prenatal diagnosis;

c) be able to offer pre-test and post-test counseling;

d) be connected to the laboratory referred to in paragraph 8.

8. Laboratories performing testing should:

a) be certified;

b) participate in national and international quality controls

c) be staffed with experts in NGS techniques.

When laboratories develop new protocols, it is necessary that bioinformatics techniques and procedures are free access and available for scientific validation.

9. Since NIPT represents the most sensitive non-invasive test for prenatal diagnosis, it is necessary that its introduction - as first or second-choice test for the detection of major autosomal aneuploidies - must be set up at central (Ministry of Health, NHS) and regional (SSR) level.

10. The characteristics of the test require its execution in a small number of laboratories at national level; therefore, interregional planning and agreement are desirable.

11. It is necessary to provide information campaigns to the public and training to professionals, to ensure equity in access to the test.

12. The progressive clinical impact of lastest-generation genetic technology, combined with a reduction of their costs, recommends a proactive debate among health professionals and learned societies on future purposes of prenatal screening for fetal defects. Currently, NIPT-based screening has no reason to be extended to diseases other than T21, T18, T13 (Attachment 10.1).

10. Attachments

Attachment 10.1

Sensitivity (SENS), specificity (SPEC), prevalence (PREV), positive predictive value (PPV) and negative predictive value (NPV) of screening protocols

The term "screening" defines an investigation strategy (protocol) used to identify, within a population, individuals at risk of a disease that represents a major problem of public health in terms of prevalence (high) and clinical symptoms (severe).

According to Wilson and Jungner (1968), the main characteristics of screening include the following:

- The disease under investigation is an important public health problem;
- Appropriate treatment is available for patients;
- Facilities for diagnosis and treatment of the disease under screening are available;
- The disease has a latent symptomatic or recognizable pre-clinical phase;
- A test or an examination is available, which is appropriate for identifying at-risk persons;
- The test is considered acceptable by the population;
- Natural history of the disease, starting from a latent to overt phase, is sufficiently well known;
- A shared treatment protocol is available;
- From an economic point of view, the costs for cases' identification (including patients' diagnosis and treatment) is balanced in respect to the overall cost of medical care;
- Screening is an ongoing (surveillance) process and not a sporadic event.

Screening tests detecting at-risk individuals must be valid and reliable, and produce acceptable results in terms of positive predictive value (PPV) and negative predictive value (NPV).

Screening for fetal aneuploidies has considerably evolved in recent years, leading to a general improvement in detection rate (sensitivity, SENS) but, above all, to a reduction of false positive rate (FPR 5%), considering the percentage of women undergoing an invasive test after a FPR from screening. Recent developments of screening tests based on cffDNA have further reduced the FPR (<0.1% for T13,18,21).

SENS, SPEC, PPV AND NPV

Sensitivity (SENS), specificity (SPEC), positive predictive value (PPV) and negative predictive value (NPV) are indicators of the validity of a screening test.

SENS and SPEC define "efficiency" as meaning that they measure the frequency for a test result to be confirmed by a diagnostic test that can differentiate affected from unaffected fetuses. In particular, "sensitivity" (SENS) defines the ability of a test to classify as “positive” people with the disease, while "specificity" (SPEC) defines the ability to classify as “negative” those without the disease. SENS measures false negative rate (1-SENS), while SPEC measures false positive rate (1-SPEC). SENS and SPEC are considered stable characteristics of the test, but they can vary in the different test settings (characteristics of the population study, quality and type of the sample analyzed, instrument calibration, correct sample handling, laboratory skills, quality assessment of internal controls, etc.).

SENS and SPEC must be assessed in relation to each other. A test with a high SENS can be useless, if a sensitivity of 100% is reached using the simple strategy of always producing a positive result: anyone, with or without the disease, would give a positive result and SPEC would be zero, making the test useless for clinical decision-making. An ideal screening test is one where SENS is 100% and SPEC 100% and it is then possible to separate the two populations (healthy and affected), without classifying individuals erroneously (a healthy individual classified as affected, or a false positive [FP]; an affected individual classified as healthy, or false negative [FN]). It is likely that such condition does not exist, since screening always reports SENS and/or SPEC <100%. It follows that screening always classifyies a proportion of patients erroneously. Therefore, the definition of an arbitrary cut-off is an integral part of the test settings, since it determines the percentage of "acceptable" cases of FP and FN, in relation to the characteristics of the disease under screening.

Positive predictive value (PPV) and negative predictive value (PPN) define the "performance" of a screening test. Performance provides a measure of the (overt or latent) disease previously undiagnosed, which becomes diagnosed and treated after the screening. In the first place, performance depends on the prevalence (PREV) of the disease in the population (which, in some cases, represents the pre-test probability of the disease). Higher performances are obtained when screening investigates a common disorder in a given population.

A further important factor that influences performance is the test efficiency. If the test has a low SENS in identifying the condition under screening, performance is consequently low. Therefore, PPV and NPV correlate SENS and SPEC with PREV of the disease.

In the case of T21 screening, PPV refers to the probability for a pregnancy with a positive screening test (high probability) for T21 to be affected by T21 (probability that a positive test actually reflects the presence of T21). NPV concerns the probability for a pregnancy with a negative screening test (low probability) for T21 to be unaffected by T21 (probability that a negative test actually reflects the absence of T21).

Attachment 10.2

Analysis techniques for cffDNA

The sequencing lane is the operating unit of the test; each lane can read 100 DNA Gbases (100 million reads).

DNA extraction: genome sequencing.
MPSS technique perfoms whole-genome sequencing without distinguishing specific chromosomes of interest; it performs 4-5 tests per lane simultaneously; it is sensitive to density differences in GC bases, which can impair the result. For this reason, it uses a normalization factor (CNV), which makes blocks of sequence reads comparable each other. In order to identify T12, 25 Gb (25 millions) of sequence reads are required, in a lane that reads 100 millions of them. If smaller blocks are analyzed, the assay of fragments of a trisomic sample can not be differentiated from that of a disomic one.

Fetal DNA extraction: sequencing of 21,18, 15, X and Y chromosomes.
DANSR technique performs a selective (*high multiplexed*) sequencing of DNA fragments limited to the chromosomes being tested. A million sequence blocks is required to identify T21. If smaller blocks are analyzed, the assay of the fragments of a trisomic sample can not be differentiated from that of a disomic one. Fragments are selected by hybridation of fluorescent probes with 576 SNP markers (which are not polymorphic for chromosomes 21, 18, 13), to detect trisomies, and with 192 SNP markers (which are polymorphic for chromosomes 1-12), to determine fetal fraction for each sample. Only fragments that anchor to fluorescent probes at the end of a locus-specific ligation reaction are analyzed for assay and probability assessment. DANSR performs 96 tests for sequencing lane.

SNP-targeted PCR analysis of cfDNA.
From isolated cfDNA 19,488 polymorphic SNP are amplified in a single reaction, showing a high level of heterozygosity; then, the product of this amplification is sequenced by NGS. Besides, it is necessary to obtain a blood sample from the father for measuring fetal fraction and analyzing data through a specific algorithm. This technique can also identify triploidy.

Risk calculation for trisomy
The application of specific algorithms allows the analysis of cfDNA fragments anticipating risk of the most common fetal aneuploidies. Using different methods for different extraction techniques, the test calculates likelihood odds ratio related to the probability that a sample is disomic or trisomic.

For MPSS technique, the algorithm defining the threshold value for trisomy risk is based on *one sample set*. Calculation for a single sample is necessary when the number of tests for sequencing lane is low:

1) binary hypothesis (positive-negative) with t-Student (*z-score*) and *Likelyhood Odds Ratio*;
2) normalization factor for CNV sequences;

3) rate variations among different sequencing lanes corrected with a z-score algorithm*;*
4) definition of a threshold value for trisomy (*z-score* value between 3 and 4).
The algorithm does not consider the fetal fraction. Therefore, with low FF, positive and negative score values are very close, increasing the risk of FPR and FNR.

For SNP-targeted DANSR technique, the algorithm defining the threshold value for trisomy risk is based on a *multiple sample set*. Calculation for multiple samples is possibile when blocks of sequence reads (1 million reads) are small, allowing to analyze several samples in a sequencing lane and to compare them.
1) percentage of hypothesis with *likelihood odds ratio* related to disomic/trisomic models (normal distribution curves);
2) calculation of fetal fraction;
3) integration of maternal and gestational age in the calculation algorithm.
The algorithm considers fetal fraction; therefore, score values with low FF are normalized, in order to assess risk, regardless of the amount of cffDNA.

Attachment 10.3

Informed consent form for single pregnancies

Dear Madam,

Non-Invasive Prenatal Testing (NIPT) are performed both in natural pregnancies and in pregnancies initiated with medically assisted procreation. In this latter case, you are requested to specify the technique applied.

NIPT requires sampling of 10-20 ml of maternal blood.

In 2% of cases, the amount of fetal DNA (FF) in maternal plasma is not sufficient for analysis.
The test is aimed at diagnosis of some numerical chromosomal anomalies and it has been validated by several international studies enrolling large numbers of pregnancies.

Detection of a trisomy (presence of an extra chromosome) by the test is based on the analysis of free DNA in maternal plasma (cfDNA), which contains a portion of DNA of maternal origin and a portion of fetal DNA originating from placenta (cffDNA). The test can also provide information on the fetus' sex (presence/absence of Y chromosome). If the test detects a chromosome abnormality, interpretation of the result is left to genetic counseling and eventually to additional diagnostic tests based on fetal samples acquired by an invasive techniques (chorionic villi sampling, amniocentesis), for which specifc information will be provided for consent.

Currently, prenatal tests based on the analysis of fetal DNA in maternal plasma allows to carry out:

1) A TEST FOR THE MAIN AUTOSOMAL TRISOMIES.

This test assesses the possibility to identify fetuses with trisomy of chromosomes 21, 18, 13 (T21, T18, T13), starting from week 10; these trisomies account for 50-70% of all autosomal aneuploidies. The term "trisomy" identifies a chromosomal abnormality consisting in the presence of three, rather than two, copies of a chromosome.

- ➢ Trisomy 21 (T21) is the more common aneuploidy (abnormal number of chromosomes), characterized by an extra copy of chromosome 21, and it is associated with Down syndrome.
- ➢ Trisomy 18 (T18) consists of an extra copy of chromosome 18, and it is associated with Edwards syndrome.
- ➢ Trisomy 13 (T13) consists of an extra copy of chromosome 13, and it is associated with Patau syndrome.

The test directly analyzes free DNA in maternal blood by integrating in the results fetal fraction DNA (cffDNA), maternal age (or donor age in case of egg donation), gestational age, based on data provided through the request form for the test.

The test has been validated for single and bigeminal pregnancies from week 10. The test is not valid for twin pregnancies with more than two fetuses, and it does not predict mosaicism, partial chromosomal aneuploidies, translocations, maternal aneuploidies, or other abnormalities that may be associated with genetic malformations and/or disability of the unborn.

It is estimated that about 50% of chromosomal abnormalities found with amniocentesis concern T21, T18, T13, which are the primary target of NIPT. Full analysis of fetal karyotype is only possible by using an invasive technique (chorionic villi sampling or amniocentesis).

NIPT is a screening test and, therefore, it measures the probability that a fetus has a genetic abnormality, but it is not designed for a conclusive diagnosis. The test must be interpreted by a medical doctor within the overall pregnancy.

Despite its sensitivity, the technique does not identify all aneuploidy fetuses.

International validation studies on tests for fetal DNA in maternal plasma for T21, T18, T13 have shown a specificity >99% and a sensitivity of 92-99%. Other studies on pregnancies at high and low risk, with an average maternal age of 30 years, have reported a specificity of 99.9% and a sensitivity of 99%.

Probability of a false negative result (i.e. of not detecting the genetic anomaly) is less than 1%. However, it must be considered that some pregnancies with fetal trisomy can give a result of "low probability" and, therefore, trisomy may remain undetected. These cases can be diagnosed only by direct analysis of karyotype with chorionic villi sampling or amniocentesis.

Probability of a false positive result (i.e. of suspecting a genetic abnormality in a unaffexcted fetus) is less than 0.1%. Therefore, some pregnancies without fetal trisomy can rarely provide a result of "high probability". In these cases, the result of NIPT can be confirmed only with an invasive diagnostic procedure (chorionic villus sampling or amniocentesis).

2) A TEST FOR AUTOSOMAL TRISOMIES AND FETAL SEX DETERMINATION.
The test assesses the probability that a fetus is affected by trisomy of chromosomes 21, 18, 13 and searches for the presence of Y chromosome.

3) A TEST FOR AUTOSOMAL TRISOMIES, X AND Y CHROMOSOME ANEUPLOIDY AND FETAL SEX DETERMINATION.

The test assesses fetal sex, the risk of trisomy of chromosomes 21, 18, 13, and of aneuploidy of X and Y chromosomes (4,XYY; 47,XXX; 47,XXY; monosomy X), with an efficiency in detecting aneuploidy of X and Y chromosomes ranging from 60 to 99%.

NIPT occasionally does not provide a result for different reasons, as problems related to the samples' transport, absence of fetal DNA in the maternal blood sample, or other causes.

The blood sample will be sent to [*indicate the center where to send the sample, particularly if abroad*]_____, which will take charge of performing the test and communicating the result to_____

I, the undersigned, declare that I understand the above, in particular that:

- NIPT does not provide a diagnosis, but it measures the probability that the fetus will be affected by trisomy;
- It is possible that fetal karyotype DOES NOT match the result provided by the test;

- Full fetal karyotype analysis demands an invasive procedure (amniocentesis or chorionic villus sampling);

- In order to perform the test, the sample may be sent to a country where data protection law could be different from the Italian legislation;

- The sample acquired for NIPT will not be used for any other investigation without my consent and it will be *kept for…. [indicate the time period or destroyed immediately after the execution of the test*].

Signature of the woman who has requested NIPT _____

My signature on this form means that I have read, or that someone have read and explained to me the information given above, and that I have fully understood it. Accordingly, I authorize the execution of the:

- ☐ TEST FOR AUTOSOMAL TRISOMIES: T21, T18, T13
- ☐ TEST FOR AUTOSOMAL TRISOMIES: T21, T18, T13 AND FETAL SEX DETERMINATION
- ☐ TEST FOR AUTOSOMAL TRISOMIES, ANEUPLODY OF X AND Y CHROMOSOMES, AND FETAL SEX DETERMINATION

I also had the opportunity to ask any questions that I felt needed and the doctor explained to me the purpose, implications and possible risks of the test. I understand that, upon request, I can ask for genetic counseling, before signing this consent.

I am aware that, in approximately 2% of cases, the test is not able to provide a result, due to an insufficient amount of fetal DNA. In this case, I can ask for the test to be repeated or for reimbursement.

In accordance with Decree No. 196/03, concerning protection of personal data, art. 32 of the Italian Constitution and Law 145/01, personal identification and health data will be incorporated into a personal details record owned by_____and they will be only used to communicate with the patient, to provide the healthcare services requested and to bill them.

Place _____ Date _____

Signature of the woman who has requested NIPT _____

Residence/Contact details _____

Signature of the professional who gave information to the patient
and received her consent_____

Attachment 10.4

Informed consent form for (disygotic) twin pregnancies

Dear Madam,

Non-Invasive Prenatal Testing (NIPT) can be performed in bigeminal (two twins) pregnancies, both in natural pregnancies and in pregnancies initiated with medically assisted procreation. In the second case, you should specify the technique applied.

Experience on non-invasive prenatal diagnosis in twin pregnancies is significantly more limited than the one available for single pregnancies.

NIPT requires sampling of 10-20 ml of maternal blood.

In 2% of cases, the amount of fetal DNA (FF) in maternal plasma is not sufficient for analysis. Detection rate of aneuploidies, i.e. numerical chromosomal anomalies, is similar to that obtained in single pregnancies, but clinical data for test validation in terms of sensitivity and specificity are still limited in dyzigotic pregnancies.

Detection of a trisomy (presence of an extra chromosome) by the test is based on the analysis of free DNA in maternal plasma (cfDNA), which contains a portion of DNA of maternal origin and a portion of DNA originating from the placentas of the two fetuses (cffDNA). The test DOES NOT distinguish which fetus may have a higher risk of chromosomal abnormality. The test DOES NOT provide information on the fetuses' sex. If the test detects an increased risk of chromosomal abnormality, interpretation of the result is left to genetic counseling and to other possible diagnostic tests using invasive diagnostic techniques (chorionic villi sampling, amniocentesis), for which specifc information will be provided for consent.

NIPT analyzes free fetal DNA in maternal plasma and detects trisomy of chromosomes 21,18, 13 (T21, T18, T13) starting from week 10 of pregnancy.

The test analyzes free DNA in maternal blood, which contains a portion of fetal DNA. The results are integrated with data concerning maternal age (or donor age, in case of egg donation), and gestational age, based on data provided through the request form for the test. Tests has been validated for single and bigeminal pregnancies from week 10. Test are not used and valid for twin pregnancies with more than two fetuses, and they do not predict mosaicism, partial chromosomal aneuploidies, translocations, maternal aneuploidies, or other abnormalities that may be associated with genetic malformations and/or disability of the unborn.

It is estimated that about 50% of chromosomal abnormalities found with amniocentesis concern T21, T18, T13. Full analysis of fetal karyotype is only possible by using an invasive technique (chorionic villi sampling or amniocentesis).

- Trisomy 21 (T21) is the more common aneuploidy, characterized by an extra copy of chromosome 21, and it is associated with Down syndrome.

- ➢ Trisomy 18 (T18), characterized by an extra copy of chromosome 18, is associated with Edwards syndrome.
- ➢ Trisomy 13 (T13), characterized by an extra copy of chromosome 13, is associated with Patau syndrome.

NIPT is a screening test and, therefore, it measures the probablity that a fetus may have a genetic abnormality, but it is not designed for a conclusive diagnosis. The test should be interpreted by a medical doctor, in the context of the overall clinical picture of pregnancy.

Despite its sensitivity, the technique does not identify all aneuploidy fetuses.

Validation studies of NIPT for T21, T18, T13 have shown a specificity >99% and a sensitivity of 92-99%. Other studies on pregnancies at high and low risk, with average maternal age of 30 years, have reported a specificity of 99.9% and a sensitivity of 99%.

Probability of a false negative result (i.e. of not detecting the genetic anomaly) is less than 1%. However, some pregnancies with fetal trisomy can give a result of "low probability" and, therefore, trisomy can remain undetected. These cases can be diagnosed only by the direct analysis of karyotype by chorionic villi sampling or amniocentesis.

Probability of a false positive result (i.e. of suspecting a genetic abnormality in an unaffected fetus) is less than 0.1%. Therefore, some pregnancies without fetal trisomy can provide at times a result of "high probability". In these cases, the NIPT result can only be confirmed with an invasive diagnostic procedure (chorionic villi sampling or amniocentesis).

It is possible that the test does not provide a result for different reasons, as problems related to the samples' transport, absence of fetal DNA in the maternal blood, or other causes.

The blood sample will be sent to [*indicate the center where to send the sample, particularly if abroad*]_____, which will take charge of performing the test and communicating the result to_____

I, the undersigned, declare that I understand the above, in particular that:

1. NIPT does not provide a diagnosis, but it measures the probability that the fetus is affected by trisomy;
2. It is possible that chromosomal sets DO NOT match the result provided by the test;
3. Full fetal karyotype analysis can be performed only by using an invasive procedure (amniocentesis or chorionic villi sampling);
4. In order to perform the test, the sample may be sent to a country where data protection law is different from the Italian legislation;
5. The sample acquired for NIPT will not be used for any other investigation without my consent and it will be kept for…. *[indicate the time period; destroyed immediately after the execution of the test*].

Signature of the woman who has requested NIPT _____

My signature on this form means that I have read, or that someone have read and explained to me the information given above, and that I have fully understood it. Accordingly, I authorize

☐ cffDNA testing (T13,T18,T21).

I also had the opportunity to ask any questions that I felt needed and the doctor explained to me the purpose, implications and possible risks of the test. I understand that, upon request, I can ask for genetic counseling, before signing this consent.

I am aware that, in approximately 2% of cases, the test is not able to provide a result due to an insufficient amount of fetal DNA. In this case, I can ask for the test to be repeated or for reimbursement.

In accordance with Decree No. 196/03, concerning protection of personal data, art. 32 of the Italian Constitution and Law 145/01, personal identification and health data will be incorporated into a personal details record owned by_____and they will be only used to communicate with the patient, to provide the healthcare services requested and to bill them.

Place _____ Date _____

Signature of the woman who has requested NIPT _____

Residence/Contact details _____

Signature of the professional who gave information to the patient
and received her consent_____

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