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Current status of non-invasive prenatal testing (NIPT): genetic counseling, dominant methods and overall performance

Derzeitiger Stand von Nicht-invasiver Pränataltestung (NIPT): genetische Beratung, dominierende Methoden und Leistungsdaten

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Abstract: The introduction of non-invasive prenatal testing (NIPT) into prenatal care represents a paradigm shift. With the absence of any intervention risk in contrast to invasive diagnostic procedures, NIPT has been widely adopted for the detection of fetal trisomy 13, 18 and 21. Additionally, fetal sex chromosome aneuploidy testing and sex determination are available, but can be compromised by both, medical and legal factors. Available validation studies were predominantly based on patients with a high *a priori* aneuploidy risk, determined by trimester screening or invasive diagnostics. In this review, we discuss the interpretation of NIPT results in context of patient specific risk constellations, the available performance data and dominant methodical approaches of NIPT including necessary content of genetic counseling.

Keywords: fetal fraction; negative predictive value; non-invasive prenatal testing; positive predictive value; test failure; trisomy.

Zusammenfassung: Die Einführung von nicht-invasiven Pränataltests in die pränatale Patientenversorgung stellt einen Paradigmenwechsel dar. Die Abwesenheit eines eingriffsbedingten Risikos wie bei invasiven Verfahren resultierte in einer breiten Anwendung des Testverfahrens zur Erkennung der fetalen Trisomien 13, 18 und 21. Zusätzlich können auch geschlechtschromosomale Aneuploidien

und das fetale Geschlecht festgestellt werden, jedoch müssen dabei einschränkende medizinische, als auch gesetzliche Faktoren mitberücksichtigt werden. Verfügbare Validierungsstudien wurden hauptsächlich an Patienten mit einem hohen *a priori* Aneuploidierisiko durchgeführt, welches mittels Trimester Screening- oder invasiven Verfahren festgestellt wurde. In diesem Übersichtsartikel diskutieren wir die Interpretation von NIPT-(nicht-invasive Pränataltestung) Befunden in Patienten-spezifischen Risikokonstellationen, die verfügbaren Leistungsdaten und dominierende, methodische Herangehensweisen der NIPT inklusive der notwendigen Inhalte einer genetischen Beratung.

Schlüsselwörter: fetale Fraktion; negativ prädiktiver Wert; nicht-invasive Pränataltestung; positiv prädiktiver Wert; Testversagen; Trisomie.

Introduction

Advanced maternal age is associated with an increased risk for fetal aneuploidies [1]. Especially fetuses with trisomy 13, 18 and 21 can survive up to the third trimester or birth exhibiting specific ultrasound- and biochemical markers prenatally. These markers are assessed during first- or second-trimester screening, which result in a patient specific risk assessment for the mentioned chromosomes. Complementary to such screening results, invasive prenatal diagnostic procedures like amniocentesis (AC) and chorionic villi sampling (CVS) are able to provide a full fetal karyotype. Both methods, CVS and AC, have a low but significant intervention risk, like e.g. infection, amniotic leakage or rhesus disease, which potentially depends on the experience of the operator [2]. This intervention risk promoted the search for other non-invasive

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prenatal procedures. In 1959, fetal cells were discovered in the maternal blood stream during pregnancy and seemed to be an ideal candidate source for a fetal karyotype obtained non-invasively. After decades of labor intensive research, this approach proved to be unsuitable for clinical use due to the low number of available fetal cells and lack of specific enrichment methods [3]. In 1997, cell-free fetal DNA (cffDNA) was detected and re-inspired the idea of a non-invasive fetal genome assessment [4]. This kind of DNA floats in the maternal blood stream packaged in microparticles, which avoid DNA degradation by plasma nucleases. CffDNA represents approximately 10% of the total cell free DNA (cfDNA) circulating in the maternal blood and can be utilized for non-invasive prenatal detection of fetal aneuploidies of all chromosomes. The concept behind trisomy testing with cffDNA is based on the observation, that a fetus with trisomy 21 contributes a higher proportion of chromosome 21 specific cffDNA fragments into the total cell-free DNA pool than a non trisomic fetus. By using next generation sequencing (NGS), this marginal difference in cfDNA amount can be reliably detected, which led to the commercial introduction of non-invasive trisomy 21 testing in the U.S. in 2011 [5]. Since that time, the number of commercial non-invasive prenatal testing (NIPT) providers and NIPT validation studies increased enormously. This article reviews the performance of NIPT in high- and low risk patients, the dominant technical approaches including their strengths and limitations and finally details the necessary content of genetic counseling.

Genetic counseling for NIPT

The basic principles of genetic counseling are that it is undertaken voluntarily, with the right to an individual decision, the right not to know and non-directivity. The counseling should provide information on the medical aspects of the planned investigation or disease, information on the significance and the limits of the diagnosis, as well as ethical and legal aspects of a planned investigation and hence the consequences of the investigation. The goal of genetic counseling is that those seeking advice can make an individual, informed decision. The Genetic Diagnostics Act (GenDG) which came into force in 2010 in Germany, stipulates genetic counseling should occur before and after a prenatal investigation. Additionally, before any genetic test is carried out, information about the planned investigation must be provided and written consent given by those seeking advice. The doctor providing the information thus becomes the “responsible medical person” in accordance

with GenDG, and also receives the results. Subject to having the appropriate qualifications, this person can also perform the (subject specific) genetic counseling. Genetic counseling in pregnancy, specifically before prenatal diagnostics, will initially seek to define and clarify individual risks e.g. through chromosome analysis of both partners in the case of recurrent miscarriage, chromosome analysis in the case of Down syndrome in one family or unclear mental retardation in the family. To this end, a personal and family medical history is taken, the latter covering at least three generations. Furthermore, the possible consequences of a significant finding must be addressed, and where appropriate due to pathological findings, the possibility of a termination of pregnancy with conditions and procedures. The possibility of NIPT has brought about a change in genetic counseling. Until now, (invasive) prenatal diagnosis was indicated as a result of an assessment of the pregnant woman’s individual risk compared to the risk of performing a diagnostic puncture (AC, chorionic villus sampling, umbilical blood sampling). With NIPT, this risk assessment and with it the classical medical indication – primarily maternal age over 35 years – are removed. NIPT as a screening method that does not require consideration of the intervention risk, is thus in principle, suitable for every pregnant woman. With regard to NIPT, it must be clarified during genetic counseling whether there are any genetic risks which cannot be detected by NIPT (e.g. chromosomal translocation involving chromosomes other than those detected with NIPT, or gonosomal mosaicism in the mother which can lead to false NIPT results, or monogenic diseases in the family). The significance and the limitations must be declared. For screening methods such as NIPT, this includes the false-positive rate, false-negative rate, positive predictive value (PPV) and the limitations of the investigation. For NIPT, limitations include the origin of fetal DNA from cytotrophoblasts; the informative value can never be better than a CVS direct preparation [6], the fetal fraction of cell-free DNA and its dependence on exogenous factors, limitations in twin pregnancies and in particular the so-called vanishing twin, which can lead to false results. Equally, it should be pointed out that the PPV is dependent on the disease prevalence, therefore, in very young pregnant women with a low aneuploidy risk, a low significance must be assumed. It should also be noted that it is possible that a test may not have a result [7]. In summary, the basic principles of genetic counseling must also be applied to NIPT. In the course of the counseling, individual risks should be identified and clarified, to enable the decision regarding type of investigation, or a decision for or against diagnosis to be made. The consultation also includes what is investigated. This means that in

the course of counseling before prenatal diagnosis several common chromosomal abnormalities and their effects must be explained. In NIPT, only specific chromosomes are examined and this must also be addressed. Additionally, how and with which sensitivity the individual investigation methods work, and whether it is diagnostics or a screening method, must also be discussed. Once a specific investigation has been chosen, further information can be provided in the explanatory meeting. Although NIPT is an available investigation method with a high diagnostic confidence and without procedural risks, and therefore in principle, suitable for every pregnant woman, due to the bioinformatic evaluation and the current reduction to a few common aneuploidies, it cannot be compared with classical invasive methods, and this must be communicated in the context of genetic counseling.

Methods of NIPT

There are two dominant technical approaches for NIPT: random massively parallel sequencing (MPS) and MPS following targeted enrichment of DNA. MPS is the most straight forward approach since almost all available cfDNA fragments present in the blood sample can be quantitatively analyzed [8]. The sensitivity of such a MPS based test can be upscaled easily to a desired precision, e.g. in case of low fetal fraction, by using a higher

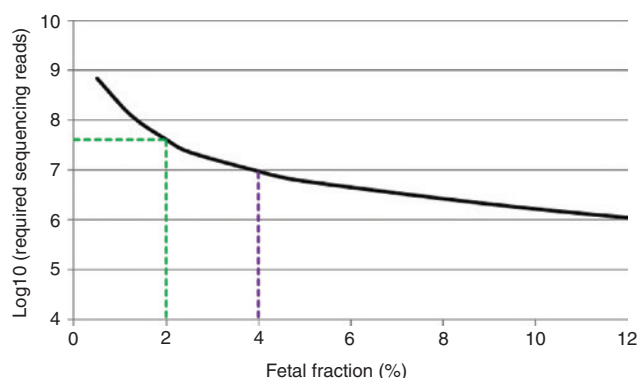


Figure 1: Sequencing depth as a function of fetal fraction (modified from Fan and Quake [9]).

As fetal fraction decreases, the confident call of a trisomy 21 requires a higher sequencing depth. By upscaling sequencing depth, the sensitivity can be increased to arbitrary precision, e.g. FF = 2% [which is a cutoff of some MPS based NIPTs (green dashed line)]. Several targeted enrichment based NIPTs have established a FF-cutoff of 4% (violet dashed line), which is responsible in part for the higher assay failure rate compared to MPS based methods. The number of samples with FF between 2% and 4% can comprise up to 7% of a mixed risk test population [10].

sequencing depth [9] (Figure 1). Targeted MPS instead relies on PCR based amplification of chromosome specific target sequences, which need to possess two or more defined and intact oligonucleotide binding sites [11, 12]. The preselected target sites can then be sequenced with far lower sequencing depth representing the most cost effective approach. One sophisticated targeted MPS approach uses single nucleotide polymorphisms (SNP) markers as target sites, which allow, next to counting reads per chromosomal target site, the qualitative determination of the most likely parental origin of each chromosome [12]. Taken together, both NIPT approaches have proven to detect trisomy 21 with high sensitivity and specificity. The analytical differences of both approaches mentioned come into play, when chromosomes different from chromosome 21 need to be analyzed or the starting material is suboptimal.

CfDNA originating from different chromosomes can be sequenced with a varying degree of reproducibility. The number of reads e.g. for chromosome 13 varies significantly from sequencing run to sequencing run and also shows a detectable intra-run variation [13, 14]. One major cause for these intra- and inter-run variabilities is fragment size- and guanosine-cytosine (GC) – bias, defined as the preferential amplification and sequencing of shorter and GC-rich templates [9, 15]. This kind of bias affects all NIPT tests which rely on NGS, since the latter mostly includes the generation of clonal sequencing fragment amplification on the sequencer's flow cell (so called cluster generation) and library enrichment by PCR. A targeted enrichment approach of chromosomal target sites before NGS in contrast to untargeted MPS presents an additional source of bias: phenomena like preferential amplification, allelic drop out and total amplification failure can compromise the sequencing data and require thorough bioinformatic bias correction. These obstacles are more pronounced when the starting material is suboptimal, like the presence of amplification inhibitors or low fetal fraction: it has been shown that patients medicated with low molecular weight heparin have smaller total cfDNA fragments with an unusual high GC content [16]. Also, a fetal fraction below 4% is mainly responsible for test failures of targeted enrichment based NIPT approaches [7]. To overcome these biases, it is necessary to make targeted enrichment approaches very redundant, e.g. by amplification of several hundred to thousand loci per chromosome and using a very strict cutoff of fetal fraction. The same is true, although to a lesser extent, for untargeted MPS, as it also relies on thorough bioinformatic bias correction to avoid false positive and false negative NIPT results.

Fetal fraction has been promoted as the most important validity predictor of a NIPT result. But it is pivotal to know by which method fetal fraction is measured and how precise the measured value is. To date, there are four concepts to determine fetal fraction: quantitation of Y-chromosomal sequences [17], copy number differences [18], differential methylation [19] and polymorphic SNP allele dosage [20]. Quantifying Y-chromosomal cfDNA is only feasible in pregnancies with a male fetus and constitutively underestimates fetal fraction in comparison to a SNP allele dosage method [21, 22]. One explanation would relate to the small size of the Y-chromosome, which would translate into significant variation of total Y-chromosomal read number. Copy number variant- and methylation based approaches are fetal sex-independent but limited by the available number of informative marker loci and their variable nature, e.g. variable copy numbers or variable degrees of hypo- and hyper-methylation [18, 23]. Additionally, these assays quantify fetal fraction in the cfDNA isolate, not in the sequencing library used for fetal aneuploidy calling. SNP allele counting is considered the gold standard for fetal fraction measurement: by using MPS based quantitation of several hundreds to thousands of SNP-markers, the fetal fraction in the sequencing library is deduced by statistical methods from the fetal-specific allele quantitation data. However, it is important to consider that also this highly sophisticated approach can only be performed with acceptable accuracy if the number of used SNP-markers is high (preferably ≥ 1000) and the sequencing depth is ≥ 230 [24]. It is of note, that most targeted enrichment based NIPT methods have to put in place a strict cutoff for fetal fraction = 4%. This is mostly due to the method inherent extra bias introduced by the target enrichment PCR on top of the constitutive MPS bias. In contrast, untargeted MPS methods can deliver reliable results with a far lower fetal fraction (e.g. 2%) [10] if a high sequencing depth is used; using plenty of reads per chromosome reduces statistical variation between affected versus unaffected samples [25]. In conclusion, fetal fraction *per se* is one important factor for NIPT result validity, but also the method of measurement and its inherent imprecision should be considered. Next to fetal fraction, the sequencing depth of NIPT plays a pivotal role as it can compensate for a low fetal fraction in the sample (Figure 1).

The performance of NIPT

The performance metrics of NIPT are outlined in Figure 2. These numbers were assessed in a meta-analysis of 37

studies (performed in 2011–2015) and report the pooled and weighted sensitivity and specificity of all available NIPT approaches [26]. Eligible patients for these studies mostly had a high risk for aneuploidies and did not belong to a general clinical population (32 studies with high risk patients, five studies with low risk patients). NIPT results were reported in concert with invasive testing results or clinical outcome (e.g. newborn physical examination). Apparently, the sensitivity for trisomy 18, trisomy 13 and monosomy X is lower compared to trisomy 21. This is in line with previous reports showing a strong correlation of non-uniform chromosomal representation after sequencing and chromosomal GC content [27, 28].

The high sensitivity and specificity values are impressive, but not true for all patients tested with NIPT. It is important to consider the patient specific *a priori* risk for trisomy based on e.g. maternal age or first trimester screening results. This *a priori* risk affects the chance of a positive NIPT result to be true positive in comparison to be false positive. The latter is defined as the PPV. *Vice versa*, the negative predictive value (NPV) answers the question: “What is the probability that a prospective mother, who does not have a trisomic fetus, receives a negative NIPT result?”. Both values are dependent on the prevalence of trisomy in a test population, hence a high risk test population receives less false positive NIPT results than a low risk population. The PPV values for trisomy 13, 18, 21, X and other gonosomal aneuploidies in Figure 2 are calculated for the test population of the meta-analysis, which predominantly has a high risk for aneuploidy. In a low risk cohort, e.g. patients of young age, the PPV is expected to be significantly lower as the prevalence of trisomic pregnancies is lower. However, the false-positive rate, which is inherent to the testing procedure, remains constant. It is of note, that some studies report a reduced PPV in a low risk population [29, 30], others do not [31–33], although the outlined correlation between PPV and prevalence represents a statistical imperative.

The same correlation is true for the NPV: a low prevalence of non-trisomic pregnancies, e.g. in a high risk cohort, would result in a lower NPV. There is little information available to date regarding false negative NIPT results except a few case reports [34, 35]. This can be explained by the mere laborious and questionable requirement of follow-up testing of every negative NIPT result by pre- or neonatal karyotyping as a means to identify the false negative rate. Nevertheless, one could estimate a false negative frequency by determining the proportion of false negative results after testing short term cultured chorionic villi (STC-villi) [6]. The latter tissue represents the cytotrophoblastic origin of cfDNA in the

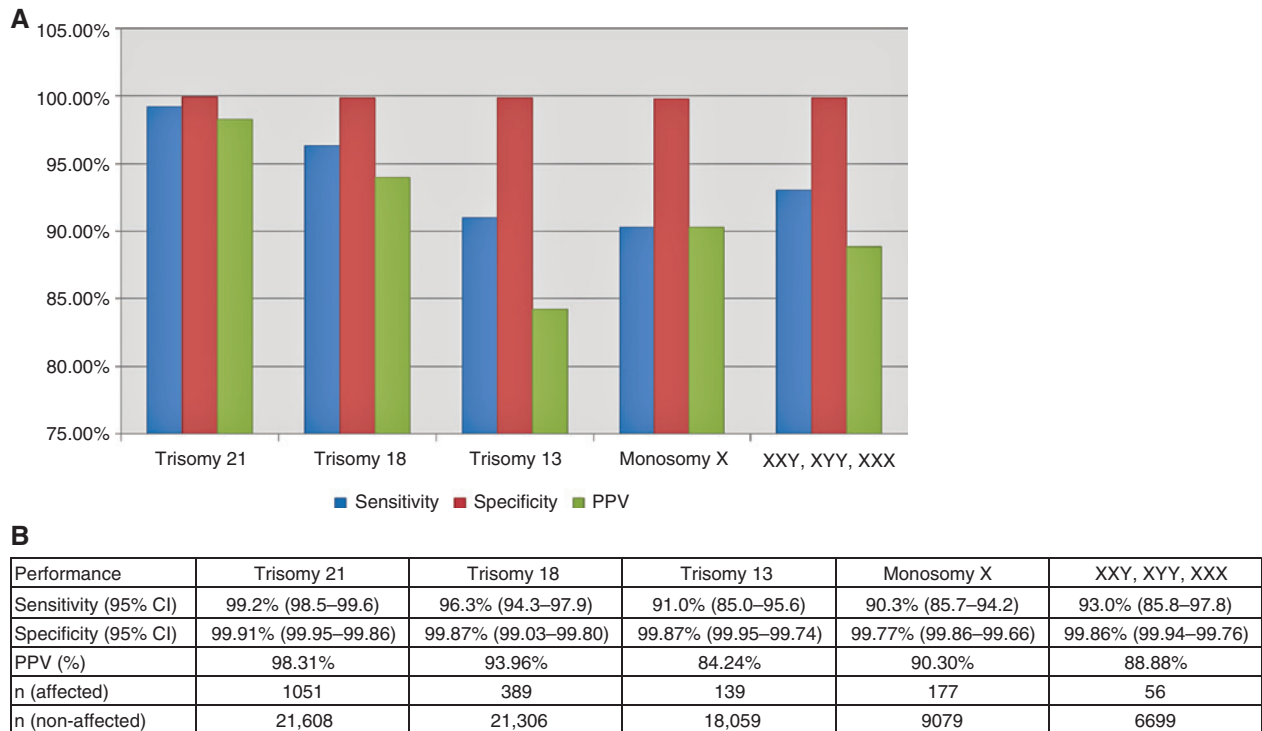


Figure 2: Meta-analysis based performance metrics of NIPT (modified from Gil et al. [26]).

Weighted pooled sensitivities and specificities for each chromosome were calculated from 37 relevant studies. The overall number of affected samples in a population of unaffected samples with a predominant high aneuploidy risk is stated in each column of the table. PPVs were calculated by dividing the number of affected samples n (affected) by n (affected) + n (False positive) (data not shown, refer to Gil et al. [26]). The histogram (A) is based on the Table (B).

maternal plasma and is analyzed routinely during CVS. Instead, long term cultured villi (LTC-villi) originate from the extra-embryonic mesoderm and are closer related to the embryo/fetus than STC-villi. By comparing STC-villi results with LTC-villi, it is possible to identify discordant results from both tissues: for trisomy 21 and 18, around 2% and 7.3% of true trisomic cases, respectively would have resulted in false-negative NIPT reports [6]. In particular, the high number of possible false negative trisomy 18 cases is supported by another study [36]. Therefore, a negative NIPT report should always be evaluated in concert with the patient specific *a priori* risk: if ultrasound evaluation indicates an increased nuchal translucency, which automatically categorizes the patient as having a high trisomy risk, the possibility that the negative NIPT report is a false-negative is higher. In conclusion, one reasonable way of integrating NIPT clinically would be its application in an intermediate risk patient cohort, which would profit most from a NIPT result with acceptable false-negative and false-positive results [37].

A performance metric, which influences NIPT sensitivity and PPV directly, is the test specific failure rate. Meta-analytical NIPT evaluation revealed that the failure

rate of analyzed samples ranges from 0% to 12.2% [26]. Especially low fetal fraction is responsible for test failures in 0.5%–6.1% of all cases. Another parameter for the impossibility of obtaining a NIPT result was suggested to be the methodical approach: in a recent study, targeted enrichment based approaches seemed to exhibit higher failure rates than MPS based approaches (~3%–6% versus ~2%) [7]. It is of note, that two MPS based NIPT studies were excluded from failure rate calculation, as unclassified results were excluded from their test statistic calculation [8, 38]. Another bias was introduced to [7] by ignoring the test specific differences in terms of fetal fraction measurement: not all NIPT providers measure fetal fraction, which renders their failure rates as almost incomparable. However, by examining all “No call” samples reported, a 2.5–6.7 times higher prevalence of fetal aneuploidy was detected among cases analyzed with targeted enrichment based NIPT approaches [29, 39], compared to MPS based NIPT (MPS: not significantly different from the overall aneuploidy rate, [7, 40–42]). In line with the PPV- prevalence correlation, this can have direct implications on how one proceeds with these “No call” samples: “No call” results, obtained by targeted enrichment based NIPTs

have a higher probability to be aneuploid, therefore invasive prenatal diagnostics as a follow-up test might be a justified option. In contrast, for MPS based “No call” results, invasive prenatal diagnostics together with test repetition or intensive clinical follow-up monitoring should be assessed equivalently. In conclusion, the test specific NIPT performance can potentially be overestimated by exclusion of failed cases, although differences in the “No call” group have direct clinical follow-up implications.

NIPT for fetal sexing and detection of sex chromosome aneuploidies (SCA) should be considered very carefully, e.g. during genetic counseling. Several factors render this NIPT test option sensitive. First, SCAs result only in mild phenotypes, if clinically detectable at all. A singular exception might be monosomy X, which can be detected prenatally by the presence of cystic hygroma and results in prenatal or perinatal fetal demise in up to 99% of all cases [43]. Second, fetoplacental and maternal mosaicism is more frequent for SCAs: it has been demonstrated, that with advanced maternal age, the mosaic loss of a single maternal X-chromosome is more likely [44, 45]. Additionally, placental mosaicism is observed in around 60% of Turner syndrome neonates [46, 47]. Third, prenatally obtained fetal sex information can favor unethical sex selection, if not prohibited by legal obligations. Fourth, NIPT based fetal sex determination can potentially be discordant to ultrasound- or karyotype- based gender determination due to congenital adrenal hyperplasia [48], androgen insensitivity [49] or gonosomal translocations [50]. Apart from these confounding factors, several technical challenges with gonosomal sequencing result in suboptimal SCA- or fetal sex- detection. Among these are gonosomal GC bias, sequence mapping issues due to sequence homology and low Y-chromosomal read number related to the small sized Y-chromosome. Taken together, SCA testing by NIPT exhibits suboptimal performance metrics, comparable to trisomy 13 testing and is not fully recommended by some professional societies [51, 52]. Therefore, genetic counseling should include the aforementioned limitations which come along with this particular test option.

Invasive prenatal diagnostics in a NIPT context

The intervention risk of invasive prenatal diagnostic procedures has been aggressively emphasized by commercial NIPT providers. Upon detailed investigation in a meta-analysis and another large scale study, the

miscarriage risk after CVS or AC before 24 weeks of gestation is not significantly different from patients not receiving these invasive procedures [53, 54]. The estimated abortions induced by these procedures range from 0.1% to 0.2%. Despite this negligible intervention risk, the introduction of NIPT suggests a reduction of invasive prenatal diagnostic procedures by ~40%–98% [55, 56]. However, if malformations or a pathologic nuchal translucency are strikingly detectable in patients, direct invasive testing is advisable: first, CVS and AC can detect other genetic causes of malformations than trisomy 13, 18 and 21. This is especially true for monogenic disorders and mosaic or non-mosaic microdeletion and –duplications, which cannot be detected currently with acceptable reliability by available NIPTs [57]. Second, invasive procedures deliver diagnostic results in short time with the highest sensitivity and specificity possible and do not require confirmatory testing [58]. Third, CVS and AC are exclusive methods for the detection of fetal conditions and allow non-disclosure testing for inherited or acquired diseases of the prospective mother, e.g. Huntington disease or maternal malignancies [59]. Incidental maternal findings can be avoided completely if a maternal contamination is excluded in the sampled material. Finally, CVS or AC complements NIPT, especially if NIPT was unable to provide a result for indefinite reasons.

Conclusions

A reasonable application of NIPT requires genetic counseling including careful consideration of test- and patient specific factors. A patient specific risk adjustment by trimester screening, performed *a priori*, can reduce the probability of false-positive and –negative NIPT results. NIPT results indicative of a trisomy should always be confirmed by invasive methods.

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