NIPT: Non-Invasive Prenatal Testing

This presentation will guide you through the different steps of a non-invasive prenatal test and will discuss its accuracy, indications and limitations.

Detection of cell-free DNA (slide 3-4)

During pregnancy, apoptotic fetal trophoblast cells are shed continuously, resulting in micro-particles of fragmented fetal DNA ending up in the maternal bloodstream. These pieces of DNA have a short half life and are rapidly cleared (within 2 hours). As the pregnancy progresses, the amount of fetal DNA present in the bloodstream of the mother increases and reaches levels which can be reliably detected by week 11 and 12 onwards. A study by Lun *et al.* revealed that the median prevalence of fetal DNA during the first and second trimester is underestimated when measured by RT-PCR (3%) as compared to digital PCR (10%).

It is thanks to the presence of this fetal DNA in the maternal bloodstream that non-invasive methods of prenatal testing (by venipuncture) are possible.

Overview of the NIPT technique (as described by Lo *et al.*) (slide 5-6)

Underneath the different steps of the NIPT technique as described by Lo *et al.* are elaborated on. These different steps are:

1. Phlebotomy
2. Plasma isolation
3. Cell-free DNA extraction
4. Library preparation
5. Cluster generation
6. Sequencing
7. Data analysis
8. Reporting

1. **Phlebotomy** (slides 6-8)

Maternal blood is collected through phlebotomy from 12 weeks gestation, when concentrations of fetal DNA are high enough for reliable detection. Blood is collected in special tubes (named "Streck" tubes in the presentation) which contain stabilizing agents, inhibiting both:

(a) the release of genomic DNA from nucleated (maternal) cells: to avoid large amounts of maternal DNA being released into the plasma fraction, which would effectively lower the percentage of fetal DNA (versus total DNA) in the plasma.
(b) nuclease activity: which would otherwise break down DNA present in the blood sample.

The difference in stability of (fetal) DNA in these special tubes versus regular EDTA blood collection tubes (bct) is clearly visualized in figures 1A and 1B of slide 7, with a rapid and steady decrease of DNA concentrations over time in the latter, and stable DNA concentrations over several days in the former. E.g. 72 hours after blood collection (the maximum amount of time it usually takes for blood

to arrive at the laboratory and be processed) the amount of DNA in the special tubes has not decreased at all, while DNA in EDTA bct's has decreased dramatically, making sensitive and reliable testing impossible.

In the same way, figures 1A and 1B of slide 8 illustrate the large amounts of genomic DNA which are released from nucleated, maternal cells over time in normal EDTA bct's, where no such release is detected in the special bct's, thereby ensuring maximal relative fetal DNA concentration and consequently maximal sensitivity of subsequent tests.

2. **Plasma isolation** (slides 9-10)

After arrival of the blood at the laboratory, plasma (containing both maternal as well as fetal cell-free DNA) is separated from the buffy coat (containing white blood cells and platelets) and the red blood cells. This is done in two steps: (a) a centrifugation at low centrifugal forces to separate cells from the plasma without inducing lysis of the nucleated cells, which would cause the release of maternal genomic DNA in the plasma fraction, (b) a centrifugation step at high centrifugal forces to remove cell debris and nuclei which could interfere with further downstream processing of the plasma (such as clogging the filter during DNA extraction).

3. **Cell-free DNA extraction** (slide 11-12)

In this step, cell-free DNA is extracted from the blood plasma. This can be done in a variety of ways. As an example, slide 12 illustrates the 'Qiagen QIAamp Circulating Nucleic Acid procedure', which is based on four steps: (a) lysis in denaturing conditions to disrupt the capsules and liposomes surrounding the DNA, (b) binding/adsorption of DNA to a silica filter/membrane, (c) washing of DNA to get rid of the lysate containing proteins and other contaminants, (d) elution of the DNA from the silica membrane. Another frequently used method to extract DNA follows a similar process but uses silica beads instead of a silica membrane.

It is important to remember that this extracted DNA is from maternal origin for the most part; only about 10 to 15% is fetal.

4. **Library preparation** (slide 13-15)

Now that pure, cell-free DNA has been obtained, it has to be prepared for sequencing in a step called 'library preparation'. Here, the individual DNA fragments are modified in such a way that a sequencing instrument will be able to read its sequence. The following modifications are performed:

- End repair: the DNA fragments have a mix of blunt ends, 3' overhangs and 5' overhangs. These need to be repaired and all converted into blunt ends.
- Adenylation: an adenine is added to the 3' end of each blunt fragment, which will allow for adapter ligation in the next step.
- Adapter ligation: adapters are ligated to each fragment, containing a series of elements necessary for the sequencing reaction and identification of the sample (PCR primer binding sites, flow cell recognition sites, sequencing primer binding sites, bar codes for sample identification).
- Library purification: Correct library fragments are purified by filtering out adapter dimers (erroneous products of the adapter ligation step).

- PCR amplification: In this step, the DNA (library) fragments are amplified in order to reach concentrations sufficiently high to allow for a successful sequencing reaction.
- Library purification: Erroneous PCR products are removed, resulting in a pure library of amplified DNA fragments ready for sequencing.
- Library validation: CfDNA fragments have an average length of 150 to 170 base pairs. During library preparation, adapters are added, resulting in fragments with an average length of 300 base pairs. The length of these fragments is verified by capillary electrophoresis. If the majority of the fragments are found to have the correct length, the library is validated and declared ready for sequencing.

Note that during library preparation, a unique bar code is added per patient sample to every fragment. This allows for the (equimolar) pooling of the libraries of different patients so that they can be sequenced all together in the same mix. Thanks to the bar codes, each read of the sequencing reaction can be assigned to the correct patient.

5. **Cluster generation** (slide 16-19)

The pooled library sample has to be attached to a glass slide (a flow cell) so that every unique fragment binds to a random but distinct zone on the slide. Library fragments contain adapters which will bind on complementary adapters present on the slide, thereby effectively attaching them.

After isothermal amplification, each distinct zone on the slide will contain multiple copies of a unique library fragment. This multitude of identical copies of a fragment is called a cluster. The amplification of each fragment is needed to overcome current technical limits, as it is not possible to detect or genotype a single DNA fragment.

6. **Sequencing** (slide 20-22)

First, all double stranded fragments are converted into single stranded fragments to serve as a template for the sequencing reaction. Next, a sequencing primer anneals to the sequencing primer binding region which was added to the fragment during the library preparation. From there on, a 'sequencing by synthesis' process starts, with iterations of DNA polymerization (one base per cycle), laser scanning (to identify the incorporated base) and decapping.

7. **Data analysis** (slide 23-30)

In terms of resolution of detection (the detail with which DNA sequences can be analyzed), it is interesting to know that the coverage is the determining factor (the number of reads per sample). The higher the number of reads, the better the resolution will be. This comes at a cost however: the higher the number of reads (per sample), the higher the sequencing cost per sample becomes. Currently implemented number of reads for detecting aneuploidies range from 2×10^6 to 25×10^6 reads. Some commercial providers use even lower numbers of reads.

As a first step of the data analysis, raw sequencing data needs to be corrected for GC content bias. Raw data contains information on how many times each fragment has been detected during sequencing. As this fragment count depends on its GC content however, this bias needs to be corrected for. After correction, reliable statistics are obtained of the frequency of detection per fragment.

Next, Z-scores are calculated. In order to do so, the data is first normalized by dividing the number of fragments per chromosome by the total number of fragments. Subsequently, the number of standard deviations is determined from the mean. This mean is an average obtained from a large number of control samples (the reference, healthy individuals). In short, if the count of chromosome 21 of a particular patient sample differs too much from the reference (too many standard deviations from the mean), this gives us a strong suspicion that chromosome 21 is under or overrepresented due to an aneuploidy.

Slides 29 and 30 give examples of trisomy's (21, 18 and 13) in a typical NIPT result sheet. On the X-axis of each graph the chromosomal position is given. The Y-axis represents the number of standard deviations from the mean. Clearly, in case of trisomy, Z-scores are much higher than in normal samples, making the sensitive detection of such aneuploidies reliable. The last example shows the detection of a microdeletion (22q11 deletion, the cause of DiGeorge syndrome). Instead of an entire chromosome which is over or under represented, the deletion of a region of several megabases is detected here, which is still large enough to be detected with the currently achieved resolution of the test.

Claimed accuracy (slides 31-36)

The sensitivity and specificity of the test vary per chromosome and have been optimized for chromosomes 21, 18 and 13. In the table on slide 32, obtained accuracy is summarized from five different studies, slide 33 shows the results from a meta-analysis, showing sensitivities (and specificities) close to 100%.

The way data analysis is performed (with bio-informatics) also has an influence on accuracy. The table on slide 34 illustrates how different ways of calculating Z-scores and correcting for GC content bias affect sensitivity, specificity as well as positive and negative predictive values (PPV and NPV) of detecting aneuploidies.

The PPV of a test is the proportion of positive test results which are actually truly positive (i.e. correct diagnoses). As can be seen from the formula on slide 35, PPV is (strongly) dependent on the prevalence of the condition. The lower the prevalence, the lower the PPV becomes. PPV is also dependent on specificity and to a lesser extent sensitivity. In the table on slide 36, PPV is seen to be relatively low, as can be expected from a condition with very low prevalence (Down and Edwards syndrome). Nevertheless, it is also shown that PPV is significantly higher with cfDNA testing as compared to standard testing.

Indications and limitations (slides 37-39)

As of the 1st of July 2017 NIPT is reimbursed in Belgium for all pregnancies. In order for a NIPT test to be performed, three conditions need to be fulfilled:

1. Informed consent needs to be obtained.
2. The first semester ultra sound needs to be done.
3. A blood sample is required which is not taken before 12 weeks of gestation, as counted from the last menstrual period.

There are, however, a few cases which are not eligible for a NIPT, namely in a pregnancy with more than 2 fetuses, or when the mother has undergone a blood transfusion, stem cell therapy, immunotherapy or a transplantation within the last 3 months.

NIPT can also be more challenging in the following cases:

- Heparin therapy: as heparin interferes with PCR (see 'library preparation')
- High maternal weight: often negatively influences the fetal DNA fraction
- Vanishing twins: continue to shed fetal DNA into the bloodstream, thereby possibly obscuring results of the healthy twin
- Anomalies in parental genetic material

Due to the nature of the test (counting of reads and comparing relative frequencies), NIPT will fail to detect:

- Balanced translocations
- Polyploidy, such as triploidy

In the following situations, NIPT might or might not fail, depending on the kind, size or degree of:

- Unbalanced translocations
- Sub chromosomal aberrations
- Maternal chromosomal abnormalities
- (Placental) mosaicism

Due to the limited sensitivity of the NIPT, it might also not detect:

- Micro-deletions or -duplications
- Mosaic chromosomal aberrations

NIPT is also not able to detect monogenic abnormalities, such as cystic fibrosis or fragile X syndrome.