Rapid aneuploidy detection in prenatal diagnosis : the clinical use of multiplex ligation-dependent probe amplication

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Chapter 1

General introduction

Prenatal diagnosis using karyotyping is routinely offered to all pregnant women in developed countries who have an increased risk of carrying a child with a chromosomal abnormality. The aim of prenatal diagnosis is to determine the presence or absence of chromosomal abnormalities to allow parents an informed choice on the course of pregnancy[1]. Prenatal diagnosis is ultimately a patient's choice. Prenatal diagnosis starts with counseling of the patient; explaining the intervention, the chromosomal abnormalities that can be detected and the consequences of these abnormalities. In case an abnormality is detected, prenatal diagnosis implies decision making on the continuation of pregnancy, timely medical treatment and emotional and psychological care.

REFERRAL INDICATIONS

Prenatal diagnosis is offered to pregnant women with a higher than reference risk for fetal chromosomal abnormalities. The assessment of risk for fetal chromosomal abnormalities is based on several risk indicators; a family history of chromosomal abnormalities, the presence of ultrasound abnormalities, advanced maternal age, and an increased risk following prenatal screening tests.

Presence of a parental chromosomal abnormality or a previous pregnancy with a chromosomal abnormality leads to an increased risk of chromosomal abnormalities in (the subsequent) pregnancy[2]. For example, if one of the parents is a carrier of balanced translocation between chromosome 13 and 14, the risk on having offspring with a trisomy 13 is 1%[3].

Single and especially multiple fetal abnormalities seen on ultrasound scan are associated with the presence of chromosomal abnormalities[4, 5]. These ultrasound abnormalities can be detected early in pregnancy. Since its introduction in 1990, first trimester nuchal translucency (NT) measurement has been implemented as a screening test for fetal chromosomal abnormalities. NT thickness is increased in fetus with trisomies 13, 18, and 21, and it is also associated with cardiac defects[6] and genetic syndromes[7]. The 20-week anomaly scan was initially developed for the detection of neural tube defects but is now part of the national prenatal screening programme and carried out to detect or rule out the presence of structural abnormalities. Occasionally, soft markers are identified, e.g. echogenic bowel, mild ventriculomegaly, and echogenic focus in the heart. These soft markers may be related to fetal chromosomal abnormalities[8]. Subsequently, advanced ultrasound screening is done at a prenatal diagnostic centre. If the findings are confirmed, invasive prenatal diagnosis is offered[9].

The most common indications for prenatal diagnosis are 1) advanced maternal age, 2) an increased risk of Down syndrome following prenatal screening, and 3) abnormalities detected at ultrasound scan. For these indications, Down syndrome is the most commonly detected abnormality.

Advanced maternal age is defined either as a maternal age of 35 years or 36 years during the 18th gestational week. In the Netherlands, the most common indication is advanced maternal age (66%)[10, 11]. Women of at least 36 years of age in the 18th gestational week are eligible for prenatal diagnosis in the Netherlands. The risk of carrying a child with Down syndrome at term increases from 1: 940 at 30 years of age to 1:353 at 35 years of age and 1:85 at 40 years of age. The combined risk for other common chromosomal abnormalities (Patau syndrome, Edward syndrome and sex chromosomal abnormalities) is also age-dependent, rising from 1:384 (30 years), to 1:178 (35 years), to 1:62 (40 years). On balance, when amniocentesis is performed, 43.5% of the chromosomal abnormalities detected are Down syndrome (trisomy 21), 10.3% are Edward syndrome (trisomy 18), 1.6% are Patau syndrome (trisomy 13) and 13% are sex chromosomal abnormalities, 15.4% are balanced structural rearrangements, 9.6% are mosaics, and the final 6.6% consist of unbalanced structural rearrangements, marker chromosomes or polyploidies[2].

Thirty years ago, prenatal screening using maternal serum markers became available to estimate the risk of carrying a child with Down syndrome. First trimester screening based on maternal age, serum markers, and nuchal translucency measurement is regarded upon as an effective screening test with a detection rate of 75.9-90.0% and a 3.3-5.0% false positive rate[12-16]. If an increased risk of carrying a baby with Down syndrome is present, prenatal diagnosis is offered. In the Netherlands, this is the second most common referral indication for prenatal diagnosis (10%). A cut-off risk level of 1 in 200 at the time of testing, comparable with a risk of 1 in 280 at term[17], is used in the Netherlands[18]. The number of pregnant women participating in prenatal screening increased due to a change in government policy in 2003, making screening for Down's syndrome available to all pregnant women, regardless their age. In 2006, 45,000 tests were performed[18], leading to an uptake of approximately 27%.

Psychological indicators are not formally part of the selection criteria for prenatal diagnosis. However, in clinical practice, parental distress or anxiety is considered an admissible criterium to undergo invasive prenatal diagnosis. It is used in 1% as a reason to undergo invasive prenatal diagnosis in our country. In the Netherlands, for all the above mentioned indications, except parental anxiety invasive prenatal diagnosis is fully covered by the insurance companies.

PRENATAL DIAGNOSIS

If at least one of the above mentioned risk indicators is present, prenatal diagnostic care is offered and parents can decide to undergo an invasive diagnostic test; i.e. prenatal diagnosis. Prenatal diagnosis is performed on amniotic fluid cells obtained by amniocentesis or chorionic villi obtained by chorionic villus sampling (CVS).

Amniocentesis is the most commonly used invasive prenatal diagnostic procedure worldwide[19] and is performed in one in 30 pregnancies in the Netherlands[10]. Amniocentesis usually is performed at 15 to 20 weeks' gestational age. Amniocentesis (figure 1) has a miscarriage risk of 0.06-1.4%[20-22]. The amniotic fluid cells are cultured for karyotyping and the result is known in 2-3 weeks. Once a chromosomal abnormality has been detected and parents decide to terminate the pregnancy, delivery is induced.

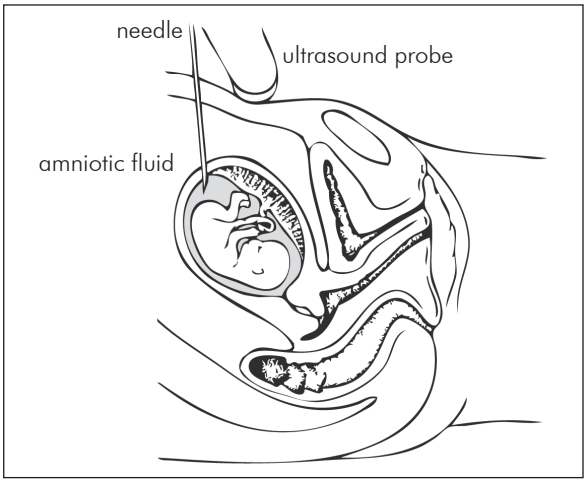


Figure 1: Amniocentesis: amniotic fluid cells are aspirated transabdominally using ultrasound guidance.

CVS (figure 2) usually is done at 10 to 13 weeks of pregnancy either transabdominally or transcervically. CVS has a miscarriage risk of 1.3%-2%[21, 22]. Specimens yielded are cultured which takes 8-10days to give a result. Once a chromosomal abnormality has been detected a dilatation and evacuation can be performed to terminate pregnancy.

Karyotyping

After withdrawal of fetal material either by amniocentesis or CVS, karyotyping (figure 3) is performed. It has been used for almost 50 years to determine if fetal chromosomal aberrations are present. It is a robust technique that is able to detect a range of numerical and structural chromosomal abnormalities with high accuracy (99.4-99.9%)[23-25]. Karyotyping requires culture of fetal cells in order to obtain cells at the metaphase stage. The cells may be grown

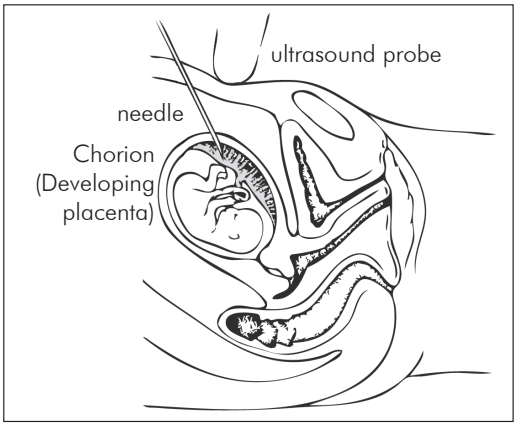


Figure 2: Chorionic villus sampling: chorionoc villi are aspirated transabdominally or transcervically using ultrasound guidance.

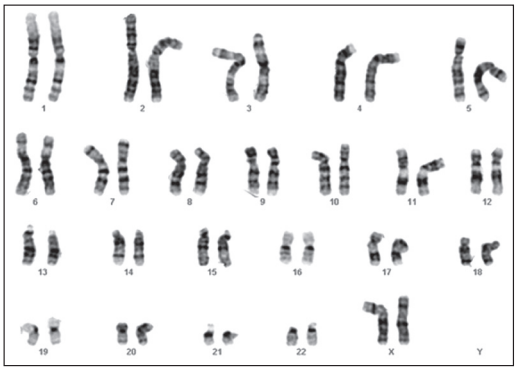


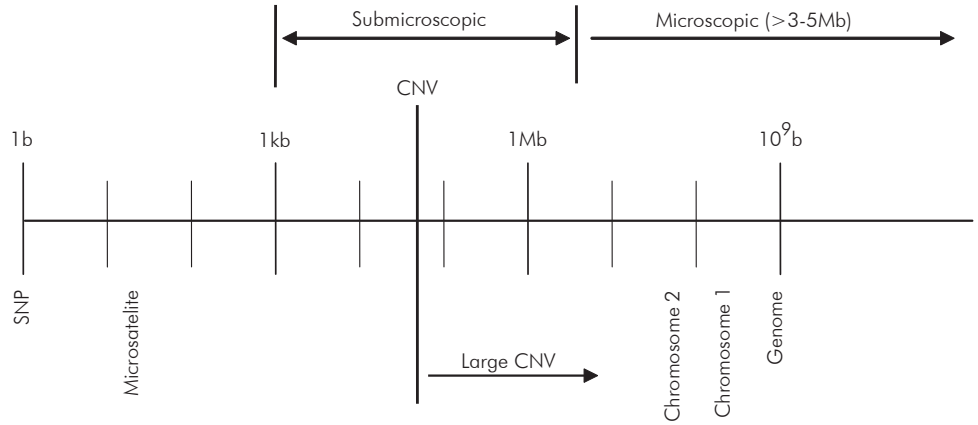
Figure 3: Normal female karyotype

in tissue flask (the flask method), in which the cells have to be enzymatically removed prior to harvest or with an in situ method, in which cells are analysed without subculture. Failure to culture the fetal cells obtained occurs in a small number of cases; the average rate reported in the UK in 1999 was 0.3% of cases[26]. In the last five years, the failure rate for the culture of amniocytes was less than 0.01% in the Netherlands. On average 10 metaphases from 10 different colonies are examined and analysed[27].

Cell culture takes on average 10 to 14 days before slides are stained for chromosomal banding. Parents have to wait two to three weeks for the test results, which generally leads to parental anxiety[28]. Karyotyping is considered time consuming and labour-intensive, both leading to high costs.

Karyotyping is able to detect any microscopic chromosomal abnormality of 3 to 5 Mega base (Figure 4)[29], including chromosomal abnormalities with unclear or mild clinical relevance. The latter findings may cause difficult counselling issues, patient anxiety, and emotional dilemmas concerning the continuation of pregnancy in situations in which the outcome is uncertain or the phenotype predicted to be relatively mild[30].

Figure 4: Arrangement and size of submicroscopic and microscopic abnormalities. Mb= Megabase, b= base, Kb= kilobase. SNP= single nucleotide polymorphism, CNV= copy number variant



RAPID ANEUPLOIDY DETECTION

Due to technical progress in molecular biology, new molecular techniques have become available which have also been applied in prenatal diagnosis. These techniques, commonly referred to as rapid aneuploidy detection (RAD) techniques, do not need cultured cells and are therefore able to deliver quick results. Currently, there are three RAD techniques; fluorescent in situ hybridisation (FISH), quantitative fluorescent polymerase chain reaction (QF-PCR) and multiplex ligation-dependent probe amplification (MLPA). These techniques share several characteristics. They only use a small part of a chromosome and are able to detect only a few chromosomes within one test. Moreover, these tests are designed to detect only the most common fetal chromosomal abnormalities; i.e. aneuploidies of chromosomes 13, 18, 21, X and Y. RAD is therefore a targeted test on chromosomes 13, 18, 21, X and Y which also implies that other chromosomal abnormalities will remain undetected. Compared to karyotyping, several advantages of RAD have been put forward; the shortening of the waiting time, the procedure is considerably less labour intensive since cultured cells are avoided, the test requires less amniotic fluid and it is suitable for high throughput testing. These factors all add to the assumed higher efficiency of RAD compared to karyotyping. Below we discuss the three RAD techniques in more detail.

Fluorescent in situ hybridisation (FISH)

FISH is a type of hybridization that uses a labelled complementary DNA or RNA strand (i.e., probe) to localize a specific DNA or RNA sequence in a portion or section of tissue in the interphase nucleus. The probe hybridizes to the target sequence at elevated temperature, and then the excess probe is washed away. Then, the probe that is labelled with fluorescent-labelled bases is localized and quantified in the tissue using fluorescence microscopy. FISH is a powerful general technique and has also become an integral part of a comprehensive cytogenetic evaluation of structurally abnormal chromosomes, mosaicism and marker chromosomes in prenatal diagnosis[31]. A variety of probe types can be employed to detect chromosome rearrangements and aneuploidy. For RAD, probes are used for chromosomes 13, 18, 21, X and Y only. Although FISH produces results in 1 to 2 days, the process still is still labour intensive requiring much expertise.

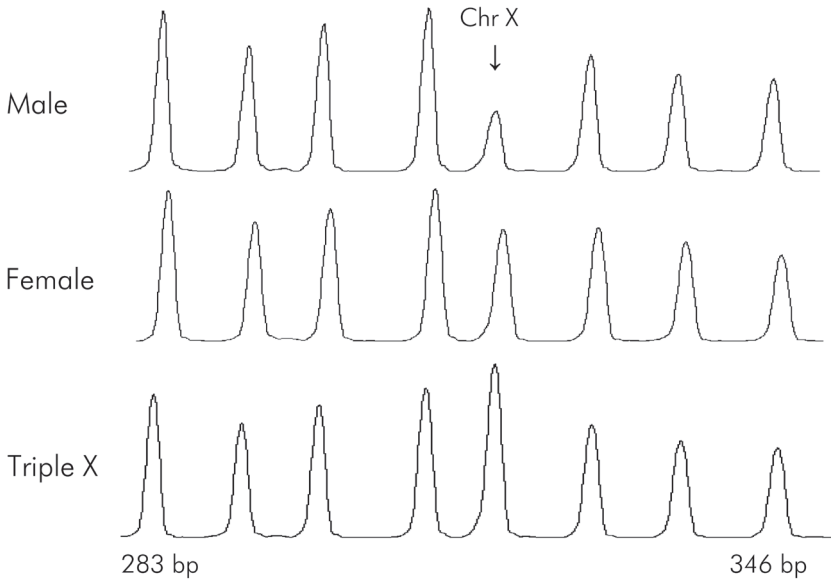
Quantitative fluorescent polymerase chain reaction (QF-PCR)

In QF-PCR, highly polymorphic short tandem repeats (STRs) on chromosome 13, 18, 21, X and Y are amplified using fluorescence primers and PCR in a multiplex assay, followed by the automated analysis of fluorescence intensity of the alleles in a genetic analyser[32]. Generally, a minimum of 3-4 STRs for each chromosome tested is used to reduce the number of uninformative results. In normal cases at least two informative marker results for each investigated chromosome consistent with a normal diallelic (heterozygous) pattern with two peaks in a 1:1 ratio are required, a monoallelic (homozygous) pattern with one peak being uninformative. In trisomic cases three alleles are evident by three peaks in a 1:1:1 ratio (triallelic trisomy pattern) or two alleles in 2:1 or 1:2 ratios (diallelic trisomy pattern). Peak height, peak area or both can be used to calculate allele ratios. QF-PCR can be performed at highly automated protocols. At the start of our clinical study, no commercially available kits were available and non-informativeness of the polymorphic markers occurred regularly.

Multiplex Ligation-dependent Probe Amplification (MLPA)

The third RAD technique is Multiplex Ligation-dependent Probe Amplification (MLPA, MRC Holland). The commercially available kit SALSA P095 is designed to detect trisomies 13, 18, 21, X and Y. More than 40 loci per multiplex can be tested in one reaction. For each genomic target, a set of 2 probes is designed to hybridize immediately adjacent to each other on the same target strand. Once hybridized, the two probes are joined by a ligase and the probe can then be amplified by PCR. All ligated probes have identical end sequences, permitting simultaneous PCR amplification by only one primer pair (a universal primer). The different length products are separated on an automated capillary sequencer. The relative

Figure 5: Detection of chromosome X (Chr X): the ratio for the male sample, containing one X chromosome, is 0.5; the ratio for the normal female sample, containing two X chromosomes, is 1.0; the ratio for the Triple X sample, containing three X chromosomes, is 1.5.



quantity of each of the PCR products is proportional to the number of copies of target sequence. Results are given as allele copy numbers as compared to normal controls: a ratio of about 1 is obtained if both alleles are present, a ratio of about 0.5 when one allele is absent and a ratio of about 1.5 if one allele is duplicated (figure 5). MLPA is not expected to detect low grade chromosomal mosaicism[33, 34].

In 2003, a preclinical study of Slater et al. showed MLPA to be robust in detecting aneuploidies of chromosome 13, 18, 21 and non-mosaic sex chromosome abnormalities using amniotic fluid: highly automated protocols provided a test result within a few days[33]. This preclinical study, however, did not reveal if the favourable performance of MLPA could also be achieved in routine clinical care, nor the impact of MLPA on patient's health related quality of life, patient's and physician's preferences and its costs-effectiveness.

THE CLINICAL PROBLEM

In prenatal diagnosis, there is neither agreement on the specific chromosomal abnormalities that should and should not be detected, nor on the degree of certainty required for a result to be negative or positive. Initially, only karyotyping was available and its 'broad' detection capacity and its high diagnostic accuracy made karyotyping to be accepted as gold standard.

Nowadays, due to technical progress, other prenatal diagnostic tests have become available next to karyotyping. The decision problem which test to use, and under what circumstances, indirectly raises the question what to test for in prenatal diagnosis. Which test strategy is considered optimal, depends on evaluative data from comparative clinical studies, with support from psychological, and decision analytic studies.

In this thesis, MLPA is our RAD technique of choice, since a preclinical study showed MLPA to be a good test with high diagnostic accuracy at highly automated protocols[33, 35]. At study onset the commercially available SALSA P095 kit had been validated on amniotic fluid in the eight genetic centres in the Netherlands in contrast to other RAD tests. MLPA by design cannot detect chromosomal abnormalities other than aneuploidies of chromosome 13, 18, 21, X and Y. Therefore, we evaluated if the diagnostic accuracy of MLPA was non-inferior to karyotyping when applied in a routine prenatal diagnosis setting and we assessed patient outcomes as well as the preferences of pregnant women and physicians for various tests and test strategies. We also estimated cost-effectiveness of MPLA compared to standard karyotyping.

STUDY AIM

The aim of the study was to assess the diagnostic accuracy, impact on patient's quality of life and preferences, and cost-effectiveness of MLPA in comparison to karyotyping as the reference diagnostic test, in clinical practice for women undergoing amniocentesis on behalf of their age, increased Down syndrome risk following first trimester prenatal screening, or parental anxiety. Should MLPA be implemented in prenatal diagnostic care and if yes, what is its optimal test strategy?

The specific research questions were:

Is diagnostic accuracy of MLPA to detect trisomies 13, 18, 21 and sex chromosomal aneuploidies in routine clinical practice comparable (non-inferior) to karyotyping?

Do anxiety and quality of life differ between a combined strategy (MLPA followed by karyotyping) and karyotyping? And if MLPA has comparable diagnostic accuracy, is quality of life influenced by offering individual choice between standalone karyotyping and standalone RAD?

Is MLPA cost effective compared to karyotyping, taking into account short term and long-term effects?

Which test and which test characteristics do patients value most? Which type of test is preferred by physicians involved in prenatal diagnosis?

Since karyotyping and MLPA have different detection capacities, which chromosomal abnormalities should be detected in prenatal diagnosis according to experts?

OUTLINE OF THE THESIS

Part 1: Clinical evaluation

Chapter 2 describes the diagnostic accuracy and failure rate of MLPA compared to karyotyping as reference test on 4585 amniotic fluid samples. Undetected chromosomal abnormalities are described (research question 1).

In Chapter 3 we analyse different aspects of health related quality of life, using validated questionnaires between women who receive both MLPA as well as a karyotype result and women who only receive karyotyping results (research question 2).

In Chapter 4 we assess the motives and reasons to choose either karyotyping or RAD and evaluate different aspects of health related quality of life, using validated questionnaires of women who are offered individual choice between standalone RAD and karyotyping (research question 2).

In Chapter 5 we present a detailed cost-effectiveness analysis. The analysis includes short term costs, i.e. time frame from amniocentesis until the decision to continue or terminate pregnancy, and long term costs, i.e. time from decision to continue or terminate pregnancy (research question 3).

Part 2: Patients 'and physicians' preferences

Chapter 6 describes the differences in preferences for prenatal testing between physicians (obstetricians, clinical geneticists, clinical cytogeneticists, midwives, general practitioners) involved in prenatal diagnosis and pregnant women undergoing amniocentesis (research question 4).

In Chapter 7 we investigate patient's preferences for karyotyping or RAD and assess the value women place on test specific characteristics by using discrete choice experimentation. (research question 4)

In Chapter 8 we present the consensus and dissensus opinions of an expert panel of professionals on broad versus targeted testing by evaluating which chromosomal abnormalities should be detected and which should not be detected (research question 5).

Part 3: General discussion, conclusion and summary

In Chapter 9 we discuss the results and give clinical implications and implications for future research. Finally our conclusions are postulated.

In Chapter 10 and 11 we summarize the results presented in this thesis in English and Dutch.

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