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Innovation of prenatal genetic diagnostics in relation to improvement of care

Angelique Kooper
Innovation of prenatal genetic diagnostics in relation to improvement of care

The studies presented in this thesis were performed at the Department of Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands.

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**Abbreviations**

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>AChE</td>
<td>Acetyl Cholin Esterase</td>
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<tr>
<td>AF</td>
<td>Amniotic Fluid</td>
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<td>AFAFP</td>
<td>Amniotic Fluid Alpha (α) Foeto Protein</td>
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<td>AFP</td>
<td>Alpha (α) Foeto Protein</td>
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<td>AS</td>
<td>Angelman Syndrome</td>
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<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>cffDNA</td>
<td>cell free foetal Desoxyribo Nucleic Acid</td>
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<td>cffRNA</td>
<td>cell free foetal Nucleic Acid</td>
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<td>cffRNA</td>
<td>cell free foetal Ribo Nucleic Acid</td>
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<tr>
<td>CMT</td>
<td>Charcot-Marie-Tooth</td>
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<tr>
<td>CI</td>
<td>Confidence Interval</td>
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<tr>
<td>CNAG</td>
<td>Copy Number Analyser for GeneChip</td>
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<tr>
<td>CNV</td>
<td>Copy Number Variant (Variation)</td>
</tr>
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<td>CPM</td>
<td>Confined Placental Mosaicism</td>
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<td>CVS</td>
<td>Chorionic Villus Sample</td>
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<td>DECIPHER</td>
<td>DatabasE of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources</td>
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<td>DNA</td>
<td>Desoxyribo Nucleic Acid</td>
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<td>DS</td>
<td>Down Syndrome</td>
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<td>DSCR</td>
<td>Down Syndrome Critical Region</td>
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<tr>
<td>ECARUCA</td>
<td>European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations</td>
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<td>FISH</td>
<td>Fluorescence In Situ Hybridization</td>
</tr>
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<td>FTS</td>
<td>First Trimester Screening</td>
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<tr>
<td>fβ-hCG</td>
<td>free βèta (ß) subunit of human Chorion Gonadotrophin</td>
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<tr>
<td>HF</td>
<td>Hydrops Foetalis</td>
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<tr>
<td>HMM</td>
<td>Hidden Markov Model</td>
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<tr>
<td>ICSI</td>
<td>Intra-Cytoplasmic Sperm Injection</td>
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<td>I-FISH</td>
<td>Interphase-Fluorescence In Situ Hybridization</td>
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<td>LCR</td>
<td>Low-Copy Repeat</td>
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<tr>
<td>LSD</td>
<td>Lysosomal Storage Disease</td>
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<td>LTC</td>
<td>Long-Term Culture</td>
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<tr>
<td>M.A.K.E.</td>
<td>MLPA And Karyotyping, an Evaluation</td>
</tr>
<tr>
<td>Mb</td>
<td>Megabase</td>
</tr>
<tr>
<td>MCA</td>
<td>Multi Congenital Anomalies</td>
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<td>MCC</td>
<td>Maternal Cell Contamination</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MIM</td>
<td>Mendelian Inheritance in Man</td>
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<td>MLPA</td>
<td>Multiplex Ligation-dependent Probe Amplification</td>
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<td>MPS</td>
<td>Muco Poly Saccharides</td>
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<td>MR</td>
<td>Mental Retardation</td>
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<td>NIHF</td>
<td>Non-Immunological Hydrops Foetalis</td>
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<td>NIPD</td>
<td>Non-Invasive Prenatal Diagnosis</td>
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<td>NPNDN</td>
<td>Network Prenatal Diagnostics Nijmegen</td>
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<tr>
<td>NT</td>
<td>Nuchal Translucency</td>
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<tr>
<td>NTD</td>
<td>Neural Tube Defect</td>
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<td>OMIM</td>
<td>Online Mendelian Inheritance in Man</td>
</tr>
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<td>PAPP-A</td>
<td>Pregnancy-Associated Plasma Protein A</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PWS</td>
<td>Prader-Willy Syndrome</td>
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<tr>
<td>QF-PCR</td>
<td>Quantitative Fluorescent Polymerase Chain Reaction</td>
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<td>RAD</td>
<td>Rapid Aneuploidy Detection</td>
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<tr>
<td>RNA</td>
<td>Ribo Nucleic Acid</td>
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<tr>
<td>SCA</td>
<td>Sex Chromosomal Aneuploidy</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>sSMC</td>
<td>small Supernumerary Marker Chromosome</td>
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<tr>
<td>STC</td>
<td>Short-Term Culture</td>
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<tr>
<td>TFM</td>
<td>True Foetal Mosaicism</td>
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<tr>
<td>TK</td>
<td>Traditional Karyotyping</td>
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<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
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<tr>
<td>TS</td>
<td>Turner syndrome</td>
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<tr>
<td>UPD</td>
<td>Uni Parental Disomy</td>
</tr>
<tr>
<td>US</td>
<td>Ultrasound Scan</td>
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<tr>
<td>WHS</td>
<td>Wolfs-Hirschhorn Syndrome</td>
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</tbody>
</table>
Chapter 1

General introduction and outline of this thesis

1.1 Invasive testing and traditional karyotyping
1.2 Measurement of biomarkers in amniotic fluid
1.3 Advances in ultrasound examination
1.4 Implementation of a national prenatal screening program
1.5 Clinical aspects of chromosomal imbalances
1.6 Application of molecular tests
1.7 Outline of this thesis
Prenatal diagnostic genetic tests are performed to assess individual pregnancies at risk for the presence of a genetic disorder due to an advanced maternal age, a positive family history, a positive screening test and/or the presence of a foetal abnormality detected by ultrasonography. The major aim of these tests is to reveal the presence of a chromosome abnormality that may severely affect the foetus, thus enabling future parents to decide in a well-informed manner about the course of the pregnancy (van Zwieten et al., 2005). During almost 50 years, chromosomal abnormalities have been identified through traditional karyotyping (TK), a microscopic method that allows the detection of gains, losses and/or rearrangements of chromosomal material in dividing cells. In recent years, however, new prenatal diagnostic tests have become available at an increasing rate, driven by rapid developments in molecular technologies, prenatal screening tests and ultrasound methodologies. These new tests may differ from the existing ones in various ways, i.e., they may be more accurate, less labour-intensive and/or less uncomfortable for pregnant women (Bossuyt et al., 2006). In addition, they may result in shorter reporting times and/or provide targeted test results that may be easier to interpret. The ongoing developments in high-resolution genomic profiling technologies, however, may also yield interpretation problems. The work described in this thesis aims at assessing the efficacy of the various prenatal diagnostic tests that are currently available and/or those that are anticipated to become available in the future to assure that pregnant women receive appropriate prenatal care.

1.1 Invasive testing and traditional karyotyping

In the past, amniocentesis and chorionic villus sampling have been widely used to collect material for prenatal karyotyping. In 1966, Steele and Breg reported for the first time that cells cultured from amniotic fluid (AF) can be used to determine the chromosomal constitution of the foetus (Steele and Breg, 1966). Since then, this method has been used for establishing the foetal karyotype by TK. Usually, TK is combined with a measurement of the α-foetoprotein (AFP) level in AF, which may serve as a biomarker for an open neural tube defect. Amniocentesis during the second trimester is relatively safe (Marthin et al., 1997), i.e., 15-20 ml AF is aspirated transabdominally and the procedure-related risk for foetal loss is estimated to be 0.6% (Mujezinovic and Alfrevic, 2007). At present, amniocentesis is still used for a wide variety of purposes.

The chorion represents the moiety of the foetal membrane that eventually develops into the foetal part of the placenta. As such, chorionic villi can be used to assess the genetic makeup of the foetus. Since chorionic villus sampling is typically performed during the first trimester of a pregnancy, diagnostic test results can be provided earlier
than those obtained after amniocentesis. A chorionic villus sample (CVS) can be retrieved via two alternative ways, i.e., transcervical or transabdominal. Both are performed in the 10th-11th week of gestation under careful ultrasound guidance to prevent adverse effects. In comparison with amniocentesis, a slightly higher risk of pregnancy loss (0.7%) has been reported for CVS (Mujezinovic and Alfirevic, 2007). In 1973, the first foetal karyotype from a transcervical placental biopsy was reported (Kullander and Sandahl, 1973). During the past decades, twice as many pregnant women have undergone amniocentesis as compared to chorionic villus sampling in the Dutch population (WPDT, 2005), which conforms to the international situation (Martin et al., 2002).

**Traditional karyotyping of amniotic fluid cells**

For TK, amniotic fluid (~20 ml) samples are cultured to obtain mitotic cells. After 6-8 days, an average number of nine colonies (representing different clones) per culture chamber is obtained. Subsequently, the cultures are harvested and processed for TK, resulting in a final reporting time of 14-21 days. To minimise the risk of contamination, or culture loss due to incubator failure, duplicate cultures are handled separately and two separate cell culture media are used. Examination of 10 metaphases from 10 different colonies results in an exclusion of a chromosomal mosaicism (see below) of 26% at a 95% confidence interval or more (Hook, 1977).

**Traditional karyotyping of chorionic villus samples**

Before a chorionic villus sample can be cultured for prenatal diagnosis, the maternal decidua must be removed in order to prevent maternal cell contamination (MCC). The diagnostic accuracy obtained from this extra-embryonic tissue may be hampered by MCC and/or the presence of a genetic mosaicism in the placenta. TK is routinely performed on short-term culture (STC) and long-term culture (LTC) villi preparations to reduce the incidence of false-positive and false-negative findings, respectively. Together, these procedures result in a final reporting time of less than 10 days. In about 1-2% of the CVS, a chromosomal mosaicism is encountered. This may be due to a postzygotic nondisjunction event generating a trisomic cell line in an initially normal conceptus (mitotic origin) or to a postzygotic loss of one chromosome in an initially trisomic conceptus (meiotic origin and trisomy rescue). Depending on the distribution of the abnormal cell line, the mosaicism may either be confined to the placenta (CPM) or generalised to the foetus (TFM, true foetal mosaicism) with a possible risk of foetal uniparental disomy (UPO) (Hahnenmann and Vejerslev, 1997).

Traditional karyotyping of AF or CVS has been the ‘gold standard’ for invasive prenatal diagnostic testing for nearly half a century. Both invasive test procedures allow the detection of all numerical chromosome aberrations, as well as all major structural abnormalities (i.e., deletions, duplications, inversions, and translocations) larger than ~5 megabase (Mb) in size. Next to routine Giemsa-trypsin-Giemsa (GTG) staining for obtaining chromosome-specific banding patterns, additional methods such as C-banding can be used for further cytogenetic characterization. Through this latter procedure centromeric regions and regions containing constitutive heterochromatin can specifically be identified. The resolution of these procedures, however, is limited by that of light microscopy. In addition, they require tissue culture, which is laborious and time consuming. Examples of GTG-based TK and C-banding are shown in Figure 1.

### 1.2 Measurement of biomarkers in amniotic fluid

The amniotic fluid surrounds the foetus and consists for 98% of water and metabolic products (i.e., proteins, salts, glucose, uric acid) that are required for, and by-products of, reproductive biological processes. The AF is in direct contact with the foetal oropharynx, lungs, gastrointestinal tract, skin, and urinary system and is the only body fluid that derives from multiple tissues (Slonim et al., 2009). The chemical composition of its substances varies with gestational age. Within certain limits, AF mirrors the metabolic status of the foeto-placental unit and, for that reason, measurement of its components and their respective variations in different weeks of pregnancy can provide useful information on the status of the foetus (Modena and Fieni, 2004).

In 1972, a relationship between abnormally high α-foetoprotein (AFP) levels in AF and open neural tube defects (NTDs) was established (Brock and Sutcliff, 1972). AFP is a protein specific to foetal life and is synthesized in both the foetal liver and the yolk sac. Due to foetal immaturity, AFP is filtered through the glomeruli and, subsequently, shed into the foetal urine. From here, AFP reaches the AF in which it is present in milligrams per litre. A peak in AFP level is found around 12 weeks of gestation, after which it steadily decreases by an average of 10% per week during the second trimester. Even though AFP was considered to be the ‘gold standard’ biomarker for NTDs, elevated AFP levels in AF can also be encountered in several other AFP-related foetal disorders. Due to this limited specificity, for several years a biochemical diagnosis of NTD has been based on the additional assessment of AF-derived cholinesterases (Wald et al., 1989).

In recent years, it has become clear that deficiencies of intermediary metabolites can affect the overall activity of a specific biochemical pathway, and that this may result in an altered transport of metabolites within or outside the cell. Lysosomal storage disorders are known to result from such an inborn error of metabolism and culture and uncultured AF cells have been used to detect deficiencies in the activity of e.g. lysosomal enzymes (Nadler, 1968).
1.3 Advances in ultrasound examination

During the last few decades, the use of ultrasonography for the detection of foetal abnormalities has become widespread in many industrialised countries. This has resulted in a shift in timing of the diagnosis of congenital abnormalities in infants from the neonatal period to the prenatal period. As equipment is improving, and as experience is growing, more precise diagnoses are obtained. These novel possibilities to examine the foetus and to concomitantly detect foetal anomalies has changed the daily practice of obstetrics and neonatology.

First trimester nuchal translucency measurement

The role of first trimester nuchal translucency (NT) measurement as a screening test for Down syndrome (DS) was first discovered in 1990 (Szabo and Gellen, 1990). Subsequently, NT measurement by ultrasound scan (US) at 10 to 14 weeks of gestation has been established as a screening test for the putative presence of foetal chromosomal anomalies in many prenatal centres (Snijders et al., 1998). The aim of this screening test is to preselect a high-risk group for invasive testing. Increased NT thickness, which is caused by the subcutaneous accumulation of fluid in the neck of the foetus, is a characteristic US finding in cases with trisomy 21, 18 and 13 and/or certain other chromosome abnormalities. However, even in the absence of aneuploidy, NT thickening is clinically relevant because it is associated with an increase in adverse perinatal outcome, which may be caused by a variety of malformations, dysplasias, deformations, disruptions and/or genetic syndromes (Souka et al., 2005). In addition, extensive studies have revealed strong associations between cardiac defects and increased NT thickness (Hyett et al., 1996; Bilardo et al., 1998).

Second trimester ultrasound scan

The routine second trimester US, also named the 20-week anomaly scan or the foetal anomaly scan, was initially developed for the detection of NTDs and for ruling out other structural abnormalities. Nowadays, however, the second trimester scan has been implemented in national screening programs and has become an integral part of prenatal care. The optimal period for this scan lies between 18 and 21 weeks. When a foetal anomaly is detected, there are several options for subsequent obstetric management. In case an invasive procedure is initiated, laboratory test results should become rapidly available for clinical and parental decision-making (the Dutch law prohibits termination of a pregnancy at a gestational age of 24 weeks or beyond). A side-effect of obstetric ultrasound scanning is the detection of so called soft markers. These soft markers are of interest since they may be related to foetal congenital abnormality.
anomalies, in particular those associated with aneuploidy. The detection of only one soft marker is not considered to be diagnostic. When two or more such markers are detected, however, TK should be offered (Loughna, 2009). Soft markers may create uncertainties for pregnant women and their care providers (Grijseels et al., 2008). Upon testing women's perception and knowledge on the 20-week anomaly scan, it was found that 95% correctly thought that its purpose was to check for structural abnormalities in the foetus. However, a concomitant awareness on the existence of soft markers was low, i.e., 92% of the women indicated that they had never heard of it (Basama et al., 2004).

1.4 Implementation of a national prenatal screening program

From January 2007 on, all pregnant women in the Netherlands are offered information about first trimester screening (FTS) for DS by a combination test and a second trimester foetal US, originally designed for the detection of NTDs (see above). This FTS has the aim to inform pregnant women and their partners on the possible presence of DS in their unborn child. The combination test for DS is composed of three elements: i) assessment of the serum concentration of pregnancy-associated plasma protein A (PAPP-A) and the free ß subunit of human chorion gonadotrophin (ß-hCG) between 8 and 14 weeks of gestation; ii) ultrasound NT measurement between 10 and 14 weeks of gestation, and iii) maternal age (Spencer et al., 1999; Wapner et al., 2003). In eight international studies, including a total of 77,000 women and 433 DS cases, an overall detection rate of 91% and a false-positive rate of 6.7% were reported, respectively (Cuckle, 2006). In the Netherlands, a screening-derived risk estimate of ≥1 in 200 (i.e., odds for an infant with trisomy 21 born alive at term) is used as an objective criterion for classifying women as 'high-risk'. Subsequently, invasive testing for foetal karyotyping is offered. In case the foetus is diagnosed with trisomy 21, the prospective parents have the possibility to choose for the birth of a child with DS or for a termination of the pregnancy. The implementation of DS screening results in fewer invasive procedures and, hence, in a decrease in the number of iatrogenic miscarriages. Therefore, it is a more effective method to identify women at risk for carrying a DS foetus than maternal age alone (Bornstein et al., 2008).

Figure 2 provides an overview of the participation of pregnant women for FTS in relation to the number of invasive prenatal tests in the Netherlands. This overview shows an impressive increase in the number of women applying for the FTS test from 2,251 in 2002 to 41,699 (23% of all pregnant women) in 2007 (Wortelboer et al., 2009), the year of national implementation. The number of pregnancies in which invasive prenatal diagnoses were carried out was 9,552 in 2005 (WPDT, 2005) of which 76% had an increased risk for DS.

1.5 Clinical aspects of chromosomal imbalances

Aneuploidy reflects a change in chromosome number which, concomitantly, results in an alteration of the normal amount of genes within a cell. An estimated 10-30% of fertilized human eggs exhibits aneuploidy, most of them being either trisomic or monosomic (Hassold and Hunt, 2001). Recently, a study of Vanneste et al. showed chromosomal aberrations in 90% of cleavage stage embryos by genome-wide microarray analysis of a single cell blastomere obtained from embryos for pre-implementation genetic diagnosis combined with aneuploidy screening. Compared to in vivo fertilized human eggs, these aberrations appeared to be mitotic of origin and, therefore, not representative for the genome of all cells of the embryo (Vanneste et al., 2009).

Approximately one-third of all miscarriages are aneuploid, which makes it the leading cause of pregnancy loss. Among the conceptions that survive to term, aneuploidy is the leading cause of developmental disability and mental retardation. Foetal aneuploidies arise mostly as a result of chromosomal nondisjunction during oogenesis or spermatogenesis during the first meiotic division. In the oocyte (>70% of all cases) this process is responsible for 93% of trisomies 18, 95% of trisomies 21 and 100% of trisomies.
1.5.1 Most common chromosomal aneuploidies

Down syndrome (trisomy 21)

Down syndrome (DS) is associated with the most common form of aneuploidy and involves the presence of a complete or partial extra copy of chromosome 21. It is named after John Langdon Down, who first described the syndrome in 1866. Subsequently, the underlying genetic cause was resolved through the identification of a chromosome 21 trisomy by Jérôme Lejeune in 1959. The first prenatal diagnosis of DS by means of amniocentesis was reported in 1968 (Valenti et al., 1968). The presence of three free copies of chromosome 21 is encountered in approximately 95% of DS cases. In 4% of the cases a chromosome 21 translocation, and in 1% of the cases a chromosome 21 mosaicism, is observed (Mutton et al., 1996). Several studies have provided evidence that in 72% of the cases chromosomal nondisjunction occurred during maternal meiosis I and in 21% of the cases during maternal meiosis II, i.e., around conception (Peterson et al., 1992; Yoon et al., 1996). In addition, Antonarakis disclosed that in 2.7% of the cases nondisjunction occurred during paternal meiosis I and in 4.3% of the cases during paternal meiosis II (Antonarakis, 1998).

Worldwide, the overall prevalence of DS is 10 in 10,000. In countries in which abortion is illegal, such as Ireland (Dolk et al., 2005) and the United Arab Emirates (Murthy et al., 2007) prevalence is higher, varying from 17 to 31 per 10,000. Conversely, the prevalence in France is relatively low (7.5 per 10,000), which is probably due to a high percentage (77%) of DS pregnancy terminations. A recent Dutch national study reported a DS live birth prevalence of 16 in 10,000 (Weijerman et al., 2008).

The life expectancy of children with DS continues to improve. The current five year survival rate is 90% (Halliday et al., 2009) and the overall life expectancy is ~60 years (Morad et al., 2009). Due to this improved life expectancy and the stable or even slightly increasing world-wide prevalence of DS, its total population is expected to grow substantially (Weijerman et al., 2008).

Although DS is mostly associated with an impairment of cognitive abilities and physical growth, as well as with a typical facial appearance, it is estimated to be associated with approximately 80 distinct phenotypes (Váčk et al., 2005). These phenotypes include, next to cognitive impairment, craniofacial dysmorphologies, congenital heart defects, gastrointestinal tract abnormalities, acute megakaryoblastic leukemia, immunologic defects, endocrine abnormalities, neuropathology leading to dementia, and dysmorphic physical features. Although preliminary attempts have been made to genetically assign these phenotypes, their exact susceptibility still remains to be explored.

Edwards syndrome (trisomy 18)

Edwards syndrome is a genetic disorder caused by the presence of an extra chromosome 18 or a part thereof. It is named after John H. Edwards, who first described the syndrome in 1960 (Edwards et al., 1960). It is the most common autosomal trisomy after DS that carries to term and affects 1 in 6,000 live born infants. An estimated 60% of trisomy 18 cases is associated with maternal meiosis II nondisjunction events (Buge et al., 1998).

The overall survival rate of Edwards syndrome is low. About 95% of the infants die in utero. Of the resulting live born infants, survival statistics indicate that the first year mortality rates range from 90% to 100%. The vast majority of these infants die during the first neonatal month (Rasmussen et al., 2003). Those who do live beyond the first year experience shortened lives marked by severe neurological and physical impairments. The major causes of death include respiratory arrest and heart abnormalities. It is impossible to predict the exact prognosis of an Edwards syndrome child during pregnancy and/or its neonatal period. Because major medical interventions are routinely withheld from these children, it remains difficult to assess what the survival rate or prognosis would have been with aggressive medical treatment. One percent of children born with this syndrome lives to an age of ten years, typically in cases with a less severe mosaic pattern.

In Edwards syndrome two non-contiguous regions encompassing 18q12.3-q22.1 are thought to be associated with severe mental retardation (Boghosian-Sell et al., 1994). In addition, it has been suggested that a decreased cholesterol synthesis may act as an aetiological factor in the occurrence of Edwards syndrome-associated malformations (Lam et al., 2003).

Patau syndrome (trisomy 13)

Patau syndrome is a genetic disorder caused by the presence of an extra chromosome 13 or a part thereof. This syndrome and its cause were first described by Klaus Patau in 1960 (Patau et al., 1960). The presence of trisomy 13 results in developmental anomalies which are characteristic of Patau syndrome. In approximately 90% of the cases the extra
chromosome 13 is maternal in origin, with an almost equal distribution between causative meiosis I and II nondisjunctional events (Bugge et al., 1998). Patau syndrome affects approximately 1 in 5,000 live births. Like all nondisjunction-related disorders, its risk increases with maternal age. Most embryos with trisomy 13 die in utero thus resulting in spontaneous abortions. After live birth, 85% of the infants die during the first month and 90% do not survive longer than one year. Severe malformations of the central nervous system are the major cause of this high mortality rate. Infants that survive over one year often suffer from long term neurological disabilities, feeding difficulties and respiratory infections such as pneumonia. As yet, little is known about putative genotype-phenotype correlations in Patau syndrome.

1.5.2 Sex chromosomal aneuploidies

In the past, the prenatal detection of sex chromosome aneuploidies (SCA) has been increasing due to the widespread use of amniocentesis and CVS (Brun et al., 2004). Such a finding is usually the unexpected by-product of a test carried out for another purpose, especially for advanced maternal age or an increased estimated risk resulting from a screening test. Most SCA cases are compatible with a normal life expectancy and often go undiagnosed (Abramsky and Chapple, 1997). It has been estimated that SCA may be present in about 0.26% of live births (Jacobs, 1979). These SCAs include 45,X (Turner syndrome), 47,XXY (Klinefelter syndrome), 47,XXX, 47,XYY, and mosaics and/or structural variants of the sex chromosomes. Genetic counselling of parents faced with a SCA diagnosis is often challenging because of i) the genotypic variability within these syndromes and ii) the uncertain prognosis due to phenotypic variability (Verp et al., 1988; Sagi et al., 2001).

Klinefelter syndrome

Klinefelter syndrome was first described in 1942 as an endocrine disorder characterised by small firm testes, gynaecomastia, hypogonadism, and higher than normal concentrations of follicle-stimulating hormone (FSH) (Klinefelter et al., 1942). With a reported prevalence of 0.1 to 0.2% in the general population and of up to 3.1% in the infertile male population (Ekerhovd and Westlander, 2002), the syndrome is the most common form of male hypogonadism and concomitant chromosome aneuploidy in human beings. Approximately 64% of the patients with Klinefelter syndrome remain undiagnosed, whereas 10% is diagnosed prenatally, and 26% is diagnosed during childhood or adult life (Abramsky and Chapple, 1997). The commonest clinical indication for a male to be karyotyped suspect for Klinefelter syndrome is the presence of hypogonadism, gynaecomastia or infertility (Lanfranco et al., 2004). In infancy, 47,XXY males may undergo a chromosomal evaluation due to the presence of hypospadias, a small phallus, cryptorchidism and/or developmental delay. School-aged 47,XXY children may present with language delay, learning disabilities or behavioural problems. Androgen replacement therapy should begin at puberty, around the age of 12 years, in increasing dosage sufficient to maintain age-related serum concentrations of testosterone, estradiol, follicle stimulating hormone and luteinizing hormone (Visootsak and Graham, Jr., 2006).

The information that parents across Europe receive after a prenatal diagnosis of Klinefelter syndrome has been made varies with the specialty and country-of-origin of the health professional consulted and his/her perception of a quality of life with that condition. This variation seems to reflect both personal, cultural and professional differences (Hall et al., 2001).

Triple X syndrome

About 1 in 1,000 females has an extra X chromosome, a condition referred to as triple X syndrome (Jacobs, 1979). In 1959, Jacobs described the first 47,XXX case (Jacobs et al., 1959). Triple X syndrome often remains undiagnosed. Affected girls tend to be tall with particularly long legs. The behavioural phenotype often includes abnormal auditory processing, distorted language development and problems in forming stable interpersonal relationships. Also psychiatric disorders seem to be relatively common in triple X syndrome patients (Otter et al., 2009). Prenatal cases of triple X syndrome do not show ultrasound abnormalities and, thus, are mostly diagnosed incidentally through cytogenetic analysis for another reason.

XYY syndrome

Males with an additional Y chromosome (47,XYY) exhibit physical and behavioural features that may result from an increased Y chromosomal gene dosage (Park et al., 2008). About 1 in 1,000 males is born with one or more extra Y chromosomes. Affected males are sometimes taller than average, but show a normal sexual development and, usually, a normal fertility. Most of them have a normal intelligence, though some may have learning disabilities, speech/language problems and/or behavioural problems. As with triple X females, most affected males are unaware of the presence of a chromosomal abnormality unless it was incidentally diagnosed during e.g. prenatal testing. The clinical information presented to prospective parents has changed considerably over time. The first information, introduced in the 1960s, was based on case reports and studies performed in mental and penal institutions. In 1968 the 47,XXY condition was linked to criminal behaviour (Jacobs et al., 1968). This link, which was based on an
ascertainment bias, resulted in the stereotype ‘supermales’ with a tendency to criminal behaviour (Bender et al., 1987). The ascertainment bias of these early investigations was avoided in subsequent longitudinal studies. As a consequence, more positive and accurate information has become available to prospective parents faced with a prenatal diagnosis of 47,XY,YY. 

**Turner syndrome**

Turner syndrome (TS) is caused by the absence of all or part of one X chromosome. It is a common cause of early pregnancy loss and accounts for about 7% of spontaneous abortions. Based on a live born frequency of 1-2 in 10,000, it is estimated that less than 1% of 45,X conceptuses survive to term (Chu et al., 1995).

About 30% of all individuals with TS are mosaics, with both a 45,X cell line and either a 46,XX cell line or a cell line containing a rearranged X chromosome (Hook and Warburton, 1983). Mosaic individuals (observed in AF or peripheral blood) will on average have a milder phenotype than those with a 100% 45,X constitution (Baena et al., 2004). TK revealed the presence of a Y chromosome mosaicism in about 5% of individuals with TS. Such a mosaicism represents a risk factor for the development of gonadoblastoma. Therefore, molecular screening for the presence of cryptic Y chromosomal sequences in TS individuals, who are negative for a Y chromosome by TK, is recommended in order to estimate the future risk for developing gonadoblastoma (Sallai et al., 2009). Molecular studies have shown that the remaining X chromosome is of maternal origin in 60 to 80% of the cases (Uematsu et al., 2002).

The US, this latter method does not allow a reliable distinction between cases with a 69,XXY karyotype and, therefore, requires further investigation. In spite of the fact that in almost all clinically recognized cases significant developmental abnormalities have been detected using US, this latter method does not allow a reliable distinction between cases with a 69,XXY or 69,XXX karyotype (McWeeney et al., 2009).

Triploidy commonly occurs during human gestation and is observed in 2-3% of pregnancies. This anomaly often culminates in an early spontaneous abortion, but occasionally it persists throughout the foetal period, resulting in the birth of an affected infant. Gestations with a 69,XY,YY karyotype are less frequently encountered than those with a 69,XXX or 69,XXY karyotype. This latter phenomenon is as yet not well understood and, therefore, requires further investigation. In spite of the fact that in almost all clinically recognized cases significant developmental abnormalities have been detected using US, this latter method does not allow a reliable distinction between cases with a 69,XXY or 69,XXX karyotype (McFadden et al., 2009).

Triploidy may be the result of either digyny (extra haploid set from mother) or diandry (extra haploid set from father). Diganic triploidy predominates in foetuses, whereas diandry accounts for 50-60% of early triploid spontaneous abortions (McFadden et al., 1993; Redline et al., 1998; McFadden and Langlois, 2000). Two distinct phenotypes observed in triploid foetuses have been shown to be associated with this parental origin: i) the diandry phenotype is characterized by a normally sized or mildly growth retarded foetus with normal adrenal glands, and is associated with an abnormally large cystic placenta with histological features known as partial hydatidiform mole, ii) the digyncic phenotype is characterized by an asymmetric intrauterine growth restriction, marked
adrenal hypoplasia, and a very small non-molar placenta (McFadden et al., 1993; McFadden and Langlois, 2000).

While triploid foetuses may exhibit a wide variety of congenital anomalies such as complete syndactyly of the third and fourth fingers, syndactyly of the toes, abnormal genitals and cardiac, urinary tract and brain anomalies, these features do not appear to differ between the digynic and diandric foetuses. Also, there does not appear to be any difference in growth between the diandric and digynic embryos, suggesting that these differences develop later during gestation (McFadden and Robinson, 2006). Early prenatal recognition of triploid pregnancies, particularly those with placental findings of partial hydatidiform mole, is important since these pregnant women are at risk of pre-eclampsia and persistent trophoblastic disease (Ngan et al., 2006).

Chromosomal mosaicism

A mosaicism is denoted as the occurrence of more than one genetically distinct cell line within an individual. In case of cytogenetic abnormalities, such a mosaicism usually reflects one normal and one abnormal cell line. Less frequently, multiple cell lines may be present and/or the occurrence of a normal cell line may not be apparent. A mosaicism arises either due to a clonal somatic (mitotic) error in a normal conception or, alternatively, due to a clonal somatic correction event in an abnormal conception. The former somatic error occurs most commonly and results in mosaics encompassing a wide range of numerical and structural abnormalities. The latter somatic error is predominantly related to the correction of a wide range of trisomies (zygote rescue). This zygote rescue mechanism may lead to uniparental disomy (UPD) in case both remaining foetal chromosomes are of either paternal or maternal origin (Kalousek and Vekemans, 2000). UPD may result in distinct abnormal phenotypes in case imprinted genes (i.e., genes whose expression depends on whether they are inherited from the mother or the father) are located on the chromosomes involved. Theoretically one third of the zygote rescue cases will result in UPD, but this number may very well be biased (Kalousek and Vekemans, 1996).

The occurrence of mosaicsm for chromosomal anomalies, usually aneuploidies, has important implications for prenatal genetic counselling. The key to predicting the clinical significance of such mosaicsm is to understand how these error/repairing events affect early embryonic development. In early cleavage stage embryos all cells are totipotent. Approximately 5-7 days post-fertilization, however, the first major cell differentiation steps have taken place. At this (blastocyst) stage, the embryo has become a hollow sphere of trophoblast cells with a clump of cells, the inner cell mass, located inside this sphere. The trophoblast cells will eventually form the trophoblast layers of the chorionic villi. A sub-population of the inner cell mass cells will form the foetus proper, whereas the remaining inner cell mass cells will form all other extra-embryonic cell lineages (Norwitz et al., 2001). The simplest scenario for the occurrence of a mosaicism is a normal conception with an abnormal cell line that arises after blastocyst formation. If it arises in the ‘foetal cells’ the foetus will be affected. If it arises in the ‘extra-embryonic’ cells the foetus will not be affected.

When after traditional karyotyping of a chorionic villus sample (CVS) a low-level of mosaicism is observed, the questions arises as to whether it represents the foetal or the extra-embryonic tissue (Wallerstein et al., 2000). Several studies have indicated the strength of CVS analysis when a combination of ‘semi-direct’ preparations (short-term cultured villi; STC-villi) and long-term cultured preparations (LTC-villi) are used to reduce the incidence of false-negative findings for the detection of chromosomal aberrations (Pittalis et al., 1994; Hahnenmann and Vejerslev, 1997; van den Berg et al., 2000; van den Berg et al., 2006). Grati et al. reported 273 cases of mosaicism in a consecutive series of 15,109 STC and LTC analyses (Grati et al., 2006). They found that the mosaics were confined to the placenta in 87.2% of the cases: 39.9% type I (confined to the trophoblast), 40.4% type II (confined to the mesenchyme) and 6.9% type III (mosaicsms in both tissues). In the remaining 12.8% the presence of the chromosomal abnormality was also encountered in foetal amniosites. The phenotypic effect of a mosaicism is thought to depend on the nature of the anomaly and the cell type in which it occurs, and inversely correlates with the percentage of cells that are euploid (Youssoufian and Pyeritz, 2002).

Chromosomal mosaicsms frequently occur in human foetuses and are known to underlie 25% of spontaneous abortions (Vorsanova et al., 2005). Although chromosomal mosaicsms are thought to be confined to specific tissues, it has recently been found that almost all somatic tissues, if thoroughly analyzed, may contain aneuploid cells (Iourov et al., 2008). The exact relevance of these aneuploid cells is as yet unclear and requires further investigations. Such investigations are expected to lead to new insights into the pathobiology of human diseases and the role of intercellular genomic variation therein.

1.5.4 Structural chromosomal abnormalities

Structural chromosomal abnormalities are considered balanced if there is no gain or loss of chromosomal material, and unbalanced if there is gain or loss. In general, balanced abnormalities do not elicit phenotypic effects. There are, however, important exceptions: a gene may be disrupted or a chromosomal break may affect the expression of a neighbouring gene by separating it from a control element and/or by inducing local chromatin alterations.

General introduction and outline of this thesis

Chapter 1
Robertsonian translocations, which involve acrocentric chromosomes (i.e., 13, 14, 15, 21 and 22) are among the most common structural chromosomal rearrangements found in humans, with an incidence of 12 in 10,000 live births (Nielsen and Wohletz, 1991). Since the breaks occur in the proximal short arms of the chromosomes involved, the resulting translocation chromosomes are dicentric. Due to the fact that the two centromeres are very close to each other they actually function as one, and the translocation chromosomes segregate normally. The acentic distal parts of the two short arms, containing stretches of repeated ribosomal RNA genes, are lost. Since this has no phenotypic consequences, Robertsonian translocations are considered as balanced. Unbalanced abnormalities can arise directly through deletion or duplication, or indirectly through mis-segregation of chromosomes during meiosis in a carrier of a balanced abnormality. The parental origin of chromosomal abnormalities seems to depend on its type: while autosomal aneuploidies are mainly maternal in origin (Hassold et al., 1993), de novo structural aberrations are mainly paternal in origin (Hill et al., 2003). In a study of 115 de novo unbalanced structural chromosome abnormalities (i.e., 39 terminal deletions, 35 interstitial deletions, 8 ring chromosomes, 12 duplications and 21 unbalanced translocations) the majority was of paternal origin, varying from 84% for interstitial deletions and rings to 58% for duplications (Thomas et al., 2006).

### 1.5.5 Sub-microscopic aberrations

Sub-microscopic aberrations may result from small hemizigous losses (microdeletions) or gains (microduplications) of DNA, ranging in size from 1 to 5 Mb. These losses and gains cannot be detected by TK, of which the best resolution does not surpass ~5 Mb. These sub-microscopic aberrations may result in specific syndromes, such as microdeletion syndromes, which are estimated to be among the major causes of mental retardation, next to Down syndrome and Fragile X syndrome (Zahir and Friedman, 2007). The most common microdeletion and microduplication syndromes are briefly discussed below and the loci and genes involved are listed in Table 1.

Prader-Willi syndrome (PWS) is associated with a deletion of the paternal copies of the imprinted SNRPN gene, the necdin gene and, possibly, other genes within chromosomal region 15q11-q13. PWS affects approximately 1 in 10,000-15,000 newborns and is characterized by hypotonia, short stature, polyphagia, obesity, small hands and feet, hypogonadism, and mild mental retardation (Cassidy and Driscoll, 2008).

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Locus</th>
<th>MIM</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prader-Willi syndrome</td>
<td>del 15q11-q13</td>
<td>#176270</td>
<td>SNRPN gene (182279)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Necdin gene (602117)</td>
</tr>
<tr>
<td>Angelman syndrome</td>
<td>del 15q11-q13,</td>
<td>#105830</td>
<td>UBE3A gene (601623)</td>
</tr>
<tr>
<td></td>
<td>Xq28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miller-Dieker syndrome</td>
<td>del 17p13.3</td>
<td>#247200</td>
<td>Several genes on 17p</td>
</tr>
<tr>
<td>22q11 microdeletion</td>
<td>del 22q11.2</td>
<td>#188400</td>
<td>TBX1 gene (602054)</td>
</tr>
<tr>
<td>syndrome</td>
<td></td>
<td></td>
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<tr>
<td>Cri-du-Chat syndrome</td>
<td>del 5p15.2</td>
<td>#123450</td>
<td>TERT gene (187270)</td>
</tr>
<tr>
<td>Smith-Magenis syndrome</td>
<td>del 17p11.2</td>
<td>#182290</td>
<td>RAI1 gene (607642)</td>
</tr>
<tr>
<td>Williams-Beuren syndrome</td>
<td>del 7q11.2</td>
<td>#194050</td>
<td>Several genes on 7q11.24</td>
</tr>
<tr>
<td>Wolf-Hirschhorn syndrome</td>
<td>del 4p16.3</td>
<td>#194190</td>
<td>Several genes on 4p</td>
</tr>
<tr>
<td>17p11.2 microduplication</td>
<td>dup 17p11.2</td>
<td>#116200</td>
<td>MPZ2 gene (159440)</td>
</tr>
<tr>
<td>syndrome</td>
<td></td>
<td></td>
<td>PMP2 gene (601097)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CX32 gene (304040)</td>
</tr>
</tbody>
</table>

Angelman syndrome (AS) is usually caused by deletion or inactivation of genes on the maternally inherited chromosome 15 (Cassidy and Schwartz, 1998). About 70% of AS cases result from de novo maternal deletions of 15q11-q13, about 2% from paternal uniparental disomy of 15q11-q13, and another 2-3% from imprinting defects. A subset of the remaining ~25% is caused by mutations in the gene encoding the ubiquitin-protein ligase E3A (Kishino et al., 1997). AS is diagnosed with a frequency of approximately 1 in 10,000-15,000 newborns and is characterized by intellectual and developmental delay, sleep disturbance, seizures, jerky movements especially hand-flapping, and frequent laughter or smiling (Clayton-Smith and Laan, 2003).

Miller-Dieker syndrome is associated with deletions of chromosome region 17p13.3 (Dobyns et al., 1993) and is characterized by type I lissencephaly caused by incomplete neuronal migration. It occurs in about 1 in 100,000 newborns (Verloes et al., 2007). Phenotypic features include a characteristic facial appearance, delayed growth and
Distal 22q microdeletion syndrome, also named 22q13.3 microdeletion syndrome or Phelan-McDermid syndrome, is characterized by neonatal hypotonia, global developmental delay, normal to accelerated growth, absent to severely delayed speech, autistic behavior, and minor dysmorphic features (Precht et al., 1998). Monosomy of SHANK3, one of the genes included in the minimum critical region is possibly responsible for the neurologic deficits, i.e., developmental delay and delayed/absent speech, in this syndrome (Bonaglia et al., 2006). The prevalence of 22q13.3 microdeletion syndrome is unknown (Phelan, 2008). Paucity of significant dysmorphic features and the clinical variability observed may lead to under-recognition of this syndrome in the newborn period. Because of the absence of multiple major and/or minor anomalies, a chromosomal abnormality would usually not be suspected (Phelan et al., 2001). As more cases are reported and the phenotype becomes better delineated, associated structural anomalies that will aid in an improved recognition of the syndrome may become apparent (Manning et al., 2004).

Wolf-Hirschhorn syndrome (WHS) is characterized by severe growth retardation and mental retardation, microcephaly, ‘Greek helmet’ facies, and closure defects (cleft lip or palate, coloboma of the eye, and cardiac septal defects) (Hirschhorn et al., 1965; Wolf et al., 1965). The incidence is estimated to be 1 in 20,000-50,000 births. The disorder is caused by a partial deletion of the short arm of chromosome 4 (4p16.3), and is generally considered to be a contiguous gene syndrome. Indeed, gene(s) causing prenatal and/or postnatal growth retardation and microcephaly are located in the 4p16.3 region. Despite the broad spectrum of clinical manifestations, however, it is also thought that a single gene may underlie the distinct phenotypic features (Maas et al., 2008).

17p11.2 microduplication syndrome results from a mutation in the gene encoding myelin protein zero, first identified in Charcot-Marie-Tooth (CMT) families, and underlies one of the most frequent hereditary neuromuscular disorders affecting approximately 1 in 2,500 newborns (Skre, 1974). Most cases of CMT are associated with a 1.5 Mb tandem duplication in 17p11.2-p12, including the PMP22 gene. Other forms of CMT are associated with mutations in the MPZ (CMT1B) and Cx32 (CMTX) genes. Thus, alterations in different genes within 17p11.2-12 can result in similar phenotypes (Murakami et al., 1996).

**1.6 Application of molecular tests**

Fluorescence in situ hybridization

The implementation in the mid-1990s of rapid aneuploidy detection (RAD) in interphases of uncultured AF cells by fluorescence in situ hybridization (FISH) has enabled the
Quantitative fluorescent PCR

A main limitation of FISH is its unsuitability for automation. In addition, the technique is labour-intensive. During the past 10 years, quantitative fluorescent PCR (QF-PCR) has been introduced as an alternative for RAD of the chromosomes 13, 18, 21, X and Y (Adinolfi et al., 1997; Pertl et al., 1999). The clinical utility of this assay has repeatedly been confirmed together with its high sensitivity and specificity (Schmidt et al., 2000; Cirigliano et al., 2001; Mann et al., 2001). One of the main advantages of QF-PCR over FISH is the possibility to automate part of the procedure, which allows a high throughput of samples at a low cost (Cirigliano et al., 2001; Hulten et al., 2003). An example of QF-PCR is shown in Figure 4.

QF-PCR analysis involves the amplification, detection and analysis of highly polymorphic chromosome-specific short tandem repeats (STRs). Fluorescently labelled marker-specific primers are used for PCR amplification and the resulting copy numbers of the markers reflect the original copy number of the corresponding chromosome. The PCR products can be analyzed and quantified using an automated genetic analyzer. STRs may vary in...
length between individual chromosomes and subjects, depending on the number of repeated sequences present. The relative copy number of each allele is determined by calculating the ratio of the peak areas or peak heights measured for each marker. A normal diploid sample has two alleles of a chromosome-specific marker, detected as two peaks in a 1:1 ratio when the marker is heterozygous, and as one peak when the marker is homozygous. The detection of an additional allele as three peaks in a 1:1:1 ratio or as two peaks in a 2:1 or a 1:2 ratio is indicative for the presence of an additional copy of the corresponding chromosome, as in case of a trisomy.

An additional advantage of this genotyping technique is the ability to detect maternal cell contamination (MCC) through the presence of extra allele peaks or inconsistent dosage ratios for each chromosome. It should be noted, however, that such a pattern may also be indicative for the presence of a twin or a chimera. Using QF-PCR, MCC rates of 3.1% for CVS (Craig et al., 1989; Antoniadi et al., 2002) and 0.7% for AF have been reported (Liao et al., 2009). Another advantage of the QF-PCR technique is its capacity to distinguish trisomy errors originating from nondisjunction events in meiosis I or meiosis II, or to identify the parental origin of an allele. This is for example relevant for the diagnosis of hydatidiform mole pregnancies. A recently diagnosed sample of a mole pregnancy in our clinic e.g. revealed a 46,XX karyotype. QF-PCR analysis revealed homozygosity for all markers tested. Subsequent QF-PCR analysis of parental blood cells showed that the mole was of complete paternal origin. Therefore, the mole appeared to be derived from an anuclear empty ovum that was fertilized by a haploid (23,X) sperm, which then replicated its own chromosomes (Kooper et al., unpublished results).

**Multiplex Ligation-dependent Probe Amplification**

An alternative test for RAD is Multiplex Ligation-dependent Probe Amplification (MLPA), a PCR-based technique that enables robust quantification of up to 40 sequences in a single test (Schouten et al., 2002) locus one pair of probes is used, designed in such a way that they hybridize adjacent to each other on one DNA target sequence. MLPA kit P095 contains probes for eight target sequences on each of the chromosomes 13, 18, 21 and X and four for the Y chromosome. These target-specific probes also contain a universal forward or reverse PCR primer-binding site. In between these, one of the probes contains a so-called stuffer sequence, varying in length from 130 to 490 base pairs (bp). This length is specific for each target sequence. After hybridization, the probes are ligated and PCR is performed using a universal fluorescent-labeled primer pair. The relative amount of each PCR product is proportional to the amount of the target sequence present in the test sample. The differently sized amplification products are separated by capillary electrophoresis. An MLPA example is shown in Figure 5.

---

**Figure 5.**

Detection of trisomy 18 by MLPA. Capillary electrophoresis patterns from a normal female sample (upper panel) and a female sample with a trisomy 18 (lower panel) analysed with kit P095 are shown. The P095 probe-mix contains 36 different markers with amplification products ranging in size from 136 to 454 bp. Four of the probes will only generate a signal on male DNA samples (i.e., Y chromosome-specific). Every set of four peaks represents markers on chromosome 21, 18, 13 and X, respectively. The arrows mark the alterations: an increase of the fluorescent signals for 18m1-18m8 in the trisomy 18 sample relative to the same markers in the normal sample.

**Microarray-based genomic profiling**

Methods such as FISH, QF-PCR and MLPA are difficult to scale up to a genome-wide level. In contrast, recently developed microarray-based methods such as array CGH allow a genome-wide assessment of genomic copy number anomalies, i.e., microdeletions and/or microduplications. Microarray analysis has the potential to be used for prenatal diagnosis and may address many of the limitations of both TK and the above mentioned molecular cytogenetic methods. The question, however, has been raised whether to use
a targeted or a whole-genome array. Targeted arrays contain clones from genomic regions of known clinical significance, for example all known microdeletion syndrome regions and all sub-telomeric regions, which are known to be frequently affected in patients with mental retardation (Bejjani et al., 2005; Cheung et al., 2005; Wong et al., 2005). However, the current diagnostic use of targeted microarrays will likely be surpassed by the availability of affordable whole-genome arrays combined with rapid increases in our knowledge on the clinical interpretation of these microarrays (Veltman and de Vries, 2006). Current whole-genome arrays can detect the most important types of genomic variants, i.e., i) single nucleotide polymorphisms (SNPs) and ii) copy number variations (CNVs) and enables the genotyping of almost 2 million markers (about 1 million SNPs and one million CNVs) dispersed throughout the human genome (Genome-wide Human SNP Array 6.0) (Bugert, 2009).

Microarray analysis has the potential to enhance or replace current approaches to prenatal diagnosis (Rickman et al., 2005). With its application on uncultured samples, the time required to report results back to patients could be significantly reduced. In addition, its enhanced resolution will enable the simultaneous detection of common aneuploidies and sub-microscopic aberrations, which may be considered of major benefit in prenatal diagnosis. A microarray example is shown in Figure 6.

1.7 Outline of this thesis

Since the introduction of prenatal genetic diagnosis about 50 years ago, chromosomal abnormalities have been identified through traditional karyotyping (TK). In recent years, however, new prenatal diagnostic tests have become available at an increasing rate, driven by rapid developments in molecular technologies, prenatal screening tests and ultrasound methodologies. These new tests may be more accurate and/or less labour-intensive than TK. In addition, they may result in shorter reporting times and/or provide targeted test results that may be easier to interpret. The rapid and accurate diagnosis of the foetal condition has become an essential part of clinical management and has changed routine prenatal care. However, also major difficulties may arise by introducing these innovations into daily practice. The main aim of this thesis was to assess the effects of laboratory innovations in a routine prenatal setting with the goal to improve prenatal care. To this end, the following question was formulated: ‘what prenatal diagnostic tests should be offered now and in the future to assure that pregnant women receive appropriate prenatal care’?

In Chapter 2, we re-evaluate the diagnostic value of routine measurement of AFP in amniotic fluid for the detection of neural tube defects in light of the introduction of the second trimester foetal anomaly scan. In Chapter 3 we describe a targeted strategy to diagnose lysosomal storage diseases in hydrops foetalis pregnancies. In Chapters 4 and 5 we describe the implementation and performance of a novel MLPA test for rapid aneuploidy detection in amniotic fluid cells and in Chapter 6 we describe the application of this test in chorionic villus samples. The prenatal detection of sub-microscopic aberrations through microarray-based analyses and its implications for prenatal use are discussed in Chapter 7. A general discussion and future prospects on prenatal testing are provided in Chapter 8.
Chapter 2

Foetal anomaly scan potentially will replace routine AFAFP assays for the detection of neural tube defects

Kooper AJA, de Bruijn D, van Ravenwaaij-Arts CMA, Faas BHW, Creemers JWT, Thomas CMG, Smits APT

Prenatal Diagnosis. 2007;27:29-33.
**Abstract**

**Objectives**
Introduction of the second trimester foetal anomaly scan and the decision to offer this scan to every woman in the 18th-22nd week of pregnancy necessitates a re-evaluation of the diagnostic value of the measurement of α-fetoprotein (AFP) concentrations in the amniotic fluid (AF) for the detection of neural tube defects (NTDs).

**Methods**
In this study of 6,501 women who underwent amniocentesis, amniotic fluid AFP (AFAFP) concentrations were measured. The women were divided into three categories: group I, without any increased risk of foetal NTD (N=6,188); group II, with an increased risk of foetal NTD (N=258); and group III, with a clinically diagnosed foetal NTD with known AFAFP concentrations (N=55).

**Results**
In 27 women of group I (0.4%), the MoM (multiple of the median) level was >2.5 times the median AFP concentration for the corresponding gestational age, and in two of these pregnancies this was related to NTD. In two pregnancies of group II (0.8%), an increased AFAFP was related to NTD. In group III, 44 of the 55 (80%) samples had an increased AFAFP.

**Conclusion**
In the near future, it is likely that imaging will replace AFAFP assays for the detection of foetal NTDs because high quality ultrasound imaging will detect NTDs accurately.

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**Introduction**
For more than 30 years, it has been a policy to always include amniotic fluid α-fetoprotein (AFAFP) measurement in invasive prenatal diagnostics for the detection of neural tube defects (NTDs), even when there is no increased risk of NTDs. There are two reasons for this: NTDs are considered to be severe disorders that often form the reason to request termination of pregnancy (WPDT, 2003); and the test is relatively simple, quick and cheap to perform. In the Netherlands, only 2% of the approximately 8,000 amniocenteses performed annually is because of an a priori increased risk of an isolated NTD. Besides measurement of the AFAFP concentration, these women receive advanced ultrasound examination in a foetal medicine unit to exclude NTDs.

There are three different types of isolated NTDs: spina bifida, encephalocele and anencephaly. A characteristic of spina bifida is the defective closure of the vertebrae. This is accompanied by the absence of skin in the case of spina bifida aperta (open spina bifida) or a defect covered by skin in the case of spina bifida occulta (closed spina bifida). In 90% of the foetuses with spina bifida, the defect is open and gives rise to increased AFAFP levels.

In general, NTDs are often accompanied by an increased AFAFP concentration. In the literature, a cut-off point of 2.5x the MoM (multiple of the median) is applied. Using a cut-off point >2.5x the MoM will enable the detection of 95% of the open NTDs (Milunsky, 1980). The Dutch Minister of Health has decided to make the prenatal second trimester foetal anomaly scan available to all pregnant women and to include it in basic health insurance from January 2006. The foetal anomaly scan is an advanced ultrasound scan and takes place between the 18th and 22nd week of pregnancy. The scan includes evaluation of the structures of the central nervous system (shape and sonographic density of the skull, presence of midline, cavum septum pellucidum, examination of cerebellum and lateral ventricular system, measurement of posterior ventricle) and examination of the spine and intactness of the skin to ensure that NTDs conform to protocol 3 of the Dutch Society for Obstetrics and Gynaecology (NVOG, 2005).

Now that the foetal anomaly scan is available to all pregnant women, the question arises as to whether a separate measurement of AFAFP to detect NTDs has any additional diagnostic value. To answer this question, we performed a retrospective study on women who had undergone amniocentesis for karyotyping and measurement of AFAFP. Reasons were sought for increased AFAFP concentrations, with special attention to the karyotype, foetal ultrasound findings and/or postnatal clinical findings.
Results

In group I, 6,188 samples of AF were karyotyped and the AFAFP concentrations determined. A total of 6,161 samples (99.6%) had normal AFAFP concentrations (<2.5×MoM). No cases of NTDs were reported during the follow-up of these pregnancies. In the remaining 27 samples (0.4%), the AFAFP concentrations increased (>2.5×MoM) for the corresponding gestational period. In this group, all increased AFAFP concentrations measured were in the AF of women with the referral reason of advanced maternal age. In the group of women whose foetus showed increased nuchal translucency at first trimester screening (N=111), no increased AFAFP was detected. Ultrasound examination revealed open spina bifida in two pregnancies, one of which was terminated and the other resulted in foetal demise. There was no evidence of NTDs in 17 of the remaining 25 cases, and the increased AFAFP concentration could possibly be explained by other AFP-related anomalies, such as foetal demise (N=9), chromosomal anomalies (trisomy 13, N=1; trisomy 21, N=1; trisomy 18, N=3), unilateral foetal hydronephrosis (N=1) and diamniotic twin pregnancies (N=2). In one twin pregnancy there was an increased AFAFP concentration in one foetus and both infants were born without complications, while in the other twin pregnancy there was a slightly increased AFAFP concentration in both samples, but ultrasound examination did not show any structural anomalies. In the eight pregnancies with unexplained increase in the AFAFP value, advanced ultrasound examination was normal in six cases. The follow-up data on the remaining two cases reported one foetus with a clubfoot (who died after premature delivery at a gestational age of 24 weeks). The other pregnancy showed an AFAFP value of 5.8×MoM. This pregnancy showed signs of utero placental insufficiency, but the pregnancy outcome was normal (normal birth weight).

In group II (women with an increased risk of NTDs), there was one case with an increased AFAFP value among the 63 users of anti-epileptic drugs. The subgroup with familial NTDs (N=141) also contained one case with an increased value. In these two cases with increased AFAFP, NTD was confirmed by advanced ultrasound examination, which made the parents opt for early termination of pregnancy. None of the women with diabetes mellitus (N=54) had increased AFAFP values.

In group III, among the women in whom foetal NTDs had been diagnosed using advanced ultrasound examination, 44 of the 55 cases (80%) had increased AFAFP concentrations. Table 1 represents an overview of the increased AFAFP values in groups I, II and III. Test performance tables are given in Table 2. The sensitivity in groups I, II and III is 100, 100 and 80%, respectively. The positive predictive value for group I is 7.4%.

Patients and methods

A retrospective study was performed between 1999 and 2005 on amniotic fluid (AF) samples obtained for prenatal cytogenetic evaluation and routine measurement of α-foetoprotein (AFP). The samples were grouped into three categories and included normal and abnormal karyotypes.

- Group I: women without any increased risk of foetal NTDs (referral reason was advanced maternal age (N=6,077) or increased nuchal translucency at first trimester screening (N=111)).
- Group II: women with an increased risk of foetal NTDs (women with diabetes mellitus (N=54), women using anti-epileptic drugs (N=63) and women with familial NTDs (N=141)).
- Group III: women in whom ultrasound examination showed a foetal NTD during pregnancy (N=55).

AF was collected at the outpatient clinics located in Nijmegen, Arnhem, Tilburg and Enschede. The samples (~20 ml) were split and amniocytes were cultured for karyotyping, and 2 ml cell-free supernatant was used for AFAFP measurements. All AFAFP measurements were performed in one laboratory (i.e., Department of Chemical Endocrinology, Radboud University Nijmegen Medical Centre). AFAFP was measured using a random access analyser type AxSYM from the Abbott Laboratories Diagnostics Division (Chicago, IL, USA). Under standard conditions the AF specimens were automatically diluted 101-fold by AxSYM. In case the initial measurement of AFAFP gave a result >25,000 g/l, the sample was manually diluted two-fold and four-fold and further processed with automatic predilution (101-fold) by the AxSYM. The AFAFP concentration was expressed in micrograms per litre. In a normal pregnancy, the AFAFP concentration can be expected to decrease by about 10% per week in the second trimester. Reference values were obtained by measuring the AFAFP concentrations per gestational week in a number of normal pregnancies and expressing the results in terms of the MoM. Using these median values, it was possible to establish an upper margin of the normal range per gestational week. From samples with an increased AFAFP concentration, the details of cytogenetic analysis, pregnancy follow-up and pregnancy outcome were obtained from their medical records when available, or by contacting the referring physician.

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Test performance values in group I, II and III

<table>
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<th>NTD +</th>
<th>NTD -</th>
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</tr>
<tr>
<td></td>
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Sensitivity = 100%, specificity = 99.6%
Positive predictive value = 7.4%, negative predictive value = 100%

Test performance values in group II

<table>
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<th>NTD +</th>
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<tr>
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Sensitivity = 100%, specificity = 100%
Positive predictive value = 100%, negative predictive value = 100%
Foetal anomaly scan potentially will replace routine AFAFP assays for the detection of neural tube defects

Chapter 2

There are other anomalies that can cause increased AFAFP values. These include abdominal wall defects, skin defects and foetal demise. NTDs and abdominal wall defects are often associated with the presence of the protein acetylcholinesterase (AChE) (Milunsky and Sapirstein, 1982). The AChE assay is performed incidentally and enables the distinction of NTDs from other AFAFP related defects. Renal anomalies can also give rise to increased AFAFP concentrations when renal AFP resorption is poor or absent. Chromosomal defects (trisomy 13, 18, 21 or 45,X) may be associated with elevated AFAFP in the absence of NTD by leakage of AFP through skin (Milunsky, 1992). Furthermore, in twin pregnancies and multiple pregnancies, AFAFP concentrations can be increased (Speroff et al., 1994) and may be difficult to interpret owing to foetal proteins that may diffuse across the membranes and cause false-positive or false-negative results (Johnson et al., 1989).

Isolated NTDs have a multifactorial aetiology. Genetic factors, environmental factors (ethnicity, diet, medication use) and periconceptional folic acid supplementation play important roles. It is well known that women with a previous NTD pregnancy have an increased risk, as well as women who take anti-epileptic drugs (e.g. valproic acid) (Frey and Hauser, 2003; Ornoy, 2006) or women with insulin-dependent diabetes (Loeken, 2005). There have been several clinical studies suggesting that the incidence and severity of diabetic pregnancy-induced malformations are correlated with poor glycemic control (Langer and Conway, 2003; Hauser, 2003; Ornoy, 2006) or women with insulin-dependent diabetes (Loeken, 2005). In addition, a number of chromosomal abnormalities are associated with the risk of congenital anomalies, including NTDs, but these make up only a small percentage of the NTDs in live births. Another prenatal screening method for NTDs is maternal serum AFP analysis (MSAFP as part of the triple test) (Canick et al., 2003). Little or no use is made of this prenatal procedure in the Netherlands owing to its limited sensitivity (80% in all NTDs, 60% for spina bifida) (Health Council of the Netherlands, 2001). The ultrasound detection of NTDs is high, partly because of the NVOG quality norm 6 for ultrasound technicians, prenatal screening and also because closed NTDs can be detected (Health Council of the Netherlands, 2004).

Over the past 10 years, the second trimester foetal scan (and consequently, screening for NTDs) has become an integral part of prenatal care in most West-European countries. Until recently, this did not include the Netherlands, but projects have now been started to train a sufficient number of ultrasound operators. The availability of high-resolution ultrasound equipment and expertise in scanning with the use of accurate examination protocols will improve the detection rate of NTDs. At present, there are regional differences in the progress to implement foetal anomaly scan screening. According to the literature, the sensitivity of the foetal anomaly scan to diagnose anencephaly is close to 100%, while for spina bifida the percentage is showing a steady increase, depending on technician training and strict quality control. In the most recent data, the sensitivity for spina bifida was 93% and for encephalocele it was 94% (Norem et al., 2005). Although the foetal anomaly scan seems very promising in terms of screening for structural anomalies, it also has limitations. For example, with the foetal anomaly scan, it is difficult to detect congenital nephrosis or skin defects such as aplasia cutis congenita (1:3,000) and epidermolysis bullosa (1:50,000) (Mandruzzato et al., 2002). The prevalence of the latter two anomalies is very low, so the only indication is to measure AFAFP concentrations on familial grounds. Furthermore, particularly in cases with persistent spine posterior position, or maternal morbid obesity, it is not always able to study NTDs.

Foetal anomaly scan screening provides direct indication about the presence of any NTDs (open and closed NTDs) and the diagnosis NTD can be confirmed by advanced ultrasound examination. This prevents patients with an increased risk of NTDs from having unnecessary invasive procedures with their inherent risk of miscarriage. In the near future, it is likely that imaging could replace AFAFP assays for the detection of NTDs, especially for patients without an increased risk, because high quality ultrasound imaging will detect open as well as closed NTDs accurately; however, the condition of implementing the 20-week foetal anomaly scan needs to be fulfilled in routine prenatal screening programmes. In the Netherlands, such programmes have been started and this work is currently in progress. We therefore intend to initiate a prospective population-based study in order to evaluate the efficacy of ultrasound screening data of the foetal anomaly scan before it is safe to abolish routine AFAFP assays. We hope that these findings will prompt other investigators in this field to perform prospective studies.

Acknowledgements

The authors wish to thank their colleagues of the Network Prenatal Diagnosis Nijmegen in Arnhem (Rijnstate Hospital), Tilburg (St. Elizabeth Hospital, TweeSteden Hospital), Enschede (Medical Spectrum Hospital Twente) and Nijmegen (UMC St. Radboud) for granting access to the research material and relevant follow-up data and Prof. Dr. Ad Geurts van Kessel for critically reading the article.
Lysosomal storage diseases in non-immune hydrops foetalis pregnancies

Kooper AJA, Janssens PMW, de Groot ANJA, Liebrand-van Sambeek MLF, van den Berg CJMG, Tan-Sindhunata GB, van den Berg PP, Bijlsma EK, Smits APT, Wevers RA

Abstract

Background
At least 20 inborn errors of metabolism may cause hydrops foetalis. Most of these are lysosomal storage diseases. The study proposes a diagnostic flowchart for prenatal diagnosis of non-immune hydrops foetalis.

Methods
This study contains a series of 75 non-immune hydrops foetalis pregnancies. Mucopolysaccharides, oligosaccharides, neuraminic acid and 21 lysosomal enzymes were measured in amniotic fluid and cultured amniotic cells.

Results
The study gives reference values for mucopolysaccharides and neuraminic acid at various stages of gestation. Four definite and two probable lysosomal diagnoses were found among the 75 investigated cases (=5.3–8%). Foetal death was found to cause false-positive values for mucopolysaccharides in amniotic fluid. In the galactosialidosis case, two novel mutations were found in the cathepsin A gene.

Conclusions
Reference values for mucopolysaccharides and neuraminic acid depend on gestational age. In a relatively high percentage of the hydrops foetalis pregnancies, a lysosomal aetiology is found. This study provides a strategy to diagnose lysosomal diseases in hydrops foetalis pregnancies. Awareness of lysosomal storage diseases causing hydrops foetalis is useful as it gives an opportunity for risk evaluation, genetic counselling to parents and targeted prenatal diagnostics for ensuing pregnancies.

Introduction

The diagnosis of hydrops foetalis (HF), the presence of excessive fluid in more than one body cavity in the foetus, is being made increasingly and at an earlier stage during pregnancy owing to routine prenatal ultrasound screening. Estimates of the incidence of HF vary between one in 600 and 4000 pregnancies (Norton, 1994; Stone and Sidransky, 1999). Estimates of mortality vary between 60% and 90% (Cassady, 2004). HF can have diverse and widely ranging causes due to disease processes in the cardiovascular or thoracic regions, foetal arrhythmia, monochorial twin pregnancies, foetal anaemia, chromosomal aberrations and genetic syndromes (Walkinshaw, 2000). Traditionally, HF is subdivided in immunological and non-immunological HF (NIHF). Inborn errors of metabolism are among the causes of NIHF and the group of lysosomal diseases is the most important subgroup. Prenatal diagnosis of a lysosomal disease in families at risk is well-established in chorionic villi and in amniocytes. For the prenatal diagnosis of a lysosomal disease in NIHF cases, the accumulating substrate of the defective enzyme and/or the enzymatic activity can be determined (Beck et al., 1984; Guibaud et al., 1985; Piraud et al., 1996; Bouvier and Maire, 1997). We have investigated a series of 75 pregnancies with NIHF at the metabolite level and at the enzyme level by measuring 21 lysosomal enzymes. Reference values for mucopolysaccharides (MPS) and neuraminic acid are not available in literature. This paper gives gestational age-related reference ranges for MPS and neuraminic acid and gives examples of abnormal oligosaccharide profiles of amniotic fluid. Four definite and two probable cases of NIHF pregnancies due to lysosomal disease are described.

Patients and methods

Patients
We have investigated a series of 75 pregnancies with NIHF. In all pregnancies, routine maternal antibody screening had excluded irregular antibodies. The classification of NIHF is based on the maternal blood group and the absence of irregular antibodies to red cell antigens Rhesus, C, E and Kell. Chromosomal abnormalities had been excluded in all cases. Investigations were carried out at the metabolite level and at the enzyme level to diagnose lysosomal diseases. In amniotic fluid these included the measurement of MPS, oligosaccharides and neuraminic acid. A panel of lysosomal enzyme determinations was performed in the cultured amniocytes. Forty control pregnancies were included in this study. Measurements on 75 NIHF pregnancies were performed as follows: in 42 pregnancies amniotic fluid was obtained and amniocytes were cultured; in 29 cases only amniotic fluid could be investigated and in four cases only cultured amniocytes were available. In our series four definite cases of lysosomal disease were found.
Basel, Switzerland; absorbance 550 nm). The assay requires correction for the presence of pyruvate in the sample. The protein content of the samples was measured with a modified Folin–Lowry method.

Oligosaccharides were analysed using thin layer chromatography (TLC) with modifications on the method described by Blom et al. (Blom et al., 1983). Samples were deproteinised first on a 10-kDa filter (Sartorius, Goettingen Germany; reagent no. 13239E) and subsequently desalted with an Amberlite mixed bed resin ion exchanger (Sigma A-5710; 200 mg/ml). After concentrating the sample by a factor 20, 5 μl sample/cm was applied on silicagel 60 TLC plates (Merck, Darmstadt, Germany; no. 5553). TLC plates were developed in n-butanol–acetic acid–water (2:1:1, by volume). Oligosaccharides were visualised by orcinol staining (100 mg orcinol (BDH Laboratory Supplies, Poole, UK; no. 29418) in 100 ml acetone and 5 ml sulfuric acid). Plates were heated for 10 min at 90 °C.

For biochemical studies a panel of 21 lysosomal enzymes was measured in 6×10⁶ amniocytes, washed three times with saline. These included arylsulfatase A, β-galactosidase, α-galactosidase A, N-acetyl β-glucosaminidase (total and A-isoform), β-glucosidase, sphingomyelinase, galactocerebrosidase, α-iduronidase, iduronate 2-sulfatase, heparine sulfaminidase, N-acetyl α-glucosaminidase, α-glucosaminide N-acetyltransferase, N-acetylglucosamine 6-sulfatase, arylsulfatase B, β-glucuronidase, neuraminidase, α-fucosidase, α-mannosidase, β-mannosidase and α-glucosidase. Methods used for these assays are essentially similar to the methods used for routine measurement of lysosomal enzymes in leucocytes.

Results

Reference ranges

No reference ranges are available for MPS and neuraminic acid in amniotic fluid. To establish these we have expressed both compounds per protein (MPS, Figure 1A; neuraminic acid, Figure 1B). Figure 1 gives the individual values for the control samples and the NIHF cases. The figure shows a gradual increase in concentration of both parameters with gestational age. The increase is more pronounced for neuraminic acid.

Amniotic fluid

Out of 75 NIHF pregnancies and 40 control pregnancies, 71 were investigated at the metabolite level (amniotic fluid obtained between 14 and 36 weeks of gestation). Standard investigations including quantitative analysis of MPS and neuraminic acid (bound + free), and oligosaccharide TLC revealed abnormal results in five samples (Figure
1 and Figure 2). Among these, two cases with highly increased MPS could not be followed up by additional investigations and remained without primary diagnosis. The protein concentration in these samples was normal. We consider these two cases as probable mucopolysaccharidoses (Figure 1A: MPS unspecified). The other three cases could be further investigated on amniocytes.

An increased MPS concentration was also found in two HF pregnancies with intrauterine foetal death (Figure 1A). The protein content was high amounting to 15.5 and 14.3 g/l, respectively. The neuraminic acid concentration in both samples was normal.

Amniotic cells

Data obtained at the metabolite level on amniotic fluid were confirmed by enzyme determinations on cultured amniotic cells in 42 out of 71 NIHF pregnancies. The enzyme determinations confirmed galactosialidosis (OMIM 256540) in case 1, GM₁-gangliosidosis (OMIM 230500) in case 2 and β-glucuronidase deficiency (OMIM 253220) in case 3. Additionally amniocytes from four other NIHF pregnancies (in which amniotic fluid was not available) were investigated revealing one additional case of β-glucuronidase deficiency. The enzyme assays did not reveal any deficiencies of lysosomal diseases that would not have shown up in the metabolite assays (for instance: arylsulfatase A, α-galactosidase A, β-glucosidase, sphingomyelinase, galactocerebrosidase). In the two foetal death cases, all mucopolysaccharidoses enzymes in amniotic cells showed normal activity. This excludes a primary defect in the catabolism of MPS in these cases and indicates intrauterine foetal death as an independent cause for increased MPS in amniotic fluid.

Pregnancies with lysosomal diagnoses

Case 1: Galactosialidosis

In amniotic fluid obtained at 27 weeks of gestation neuraminic acid was clearly increased (Figure 1B: 142 μmol/g protein, reference value for this gestational age <90 μmol/g protein). TLC of oligosaccharides in the amniotic fluid showed the abnormal presence of several neuraminic acid containing oligosaccharides (Figure 2, lane 4). β-d-Galactosidase and neuraminidase deficiency in cultured amniocytes suggested the diagnosis of galactosialidosis. It was confirmed by demonstrating the primary cathepsin A defect (Table 1). The final diagnosis of galactosialidosis (OMIM 256540) was performed on foetal fibroblasts and leukocytes from umbilical cord blood. Molecular analysis on the PPGB gene (GenBank ID no. 5476) identified two novel mutations: 1. c.292C > T (in exon 3 leading to replacement of His98 by Tyr); 2. c.707T > G in exon 8 leading to Arg substitution of Leu236 (nomenclature including the signal peptide). In a later pregnancy specific enzymes were measured in chorionic villi and found normal. This pregnancy resulted in the delivery of a healthy child.
Case 2: GM1-gangliosidosis

TLC showed abnormal oligosaccharide bands indicative for GM1-gangliosidosis (Figure 2, lane 2). Cultured amniocytes revealed decreased β-d-galactosidase activity but normal neuraminidase activity (Table 1). After birth, the diagnosis GM1-gangliosidosis (OMIM 230500) was confirmed in fibroblasts and leukocytes. In the GLB1 gene on chromosome 3p (GenBank ID no. 2720) encoding for β-d-galactosidase a novel homozygous mutation c.442C > T was found in exon 4 by direct sequencing of all exons of the genomic DNA. The mutation was confirmed with restriction enzyme analysis. It did not occur in 100 control alleles. At the protein level the mutation leads to replacement of Arg by Cys (R148C). A mutation in the same codon leading to R148S has been described in infantile GM1-gangliosidosis (Hilson et al., 1994). Due to the position of the mutation both gene products β-d-galactosidase as well as elastin binding protein are likely to be affected. Both parents were heterozygous for the mutation. In the subsequent pregnancy, specific enzyme analysis was performed on amniocytes at 16 weeks of gestation. A deficiency of β-d-galactosidase was found indicating an affected foetus. This led to the parents’ decision to terminate the pregnancy.
Cases 3 and 4: Mucopolysaccharidoses

Amniotic fluid from case 3 showed an MPS increase with a factor 1.5 compared to controls (Figure 1A). Further analysis with one-dimensional electrophoresis showed an increase in chondroitin sulfate and dermatan sulfate. A deficiency of β-glucuronidase was found in serum obtained by pericardial puncture of the foetus (6 nmol/h/mg protein; reference values 600–1200) and in foetal fibroblasts (6 nmol/h/mg protein; reference values 200–700). Based on these findings, mucopolysaccharidosis type VII (MPS VII, OMIM 253220) was diagnosed.

The same enzyme defect was found in case 4. The β-glucuronidase activity amounted to 7 in amniotic cells (reference 140–660). The deficiency was confirmed by enzyme analysis in leucocytes from cord blood (β-glucuronidase = 10 nmol/h/mg protein; reference 600–1200). At the molecular genetic level, a 27-nucleotide deletion c.1084_1107del was found in the cDNA of the GUSB gene (GenBank ID no. 2990). This mutation in exon 7 was present in heterozygous form. It leads to the loss of nine amino acids (p.362_370del) at the protein level. A second pathogenic mutation was found by sequencing the genomic DNA (c.1069C > T). This heterozygous mutation also is in exon 7. It results in reduced amounts of stable mRNA and to premature truncation at the protein level (p.R357X) (Vervoort et al., 1997).

Discussion

Lysosomal storage diseases (LSD) are extremely rare. However, the estimated combined birth incidence for all lysosomal diseases is 14 per 100,000 live births (Poorthuis et al., 1999). Deficiency of a lysosomal enzyme (nearly always) results in accumulation of the substrate of the specific enzyme in the lysosomes and leads to cell and tissue damage, swelling and organomegaly. The liver, spleen and bone marrow are among the targets. Damage to these organs and bone marrow may result in decreased haematopoiesis, hypoalbuminaemia, visceromegaly, damage of the myocardium, inhibited venous drainage of the heart and ascites due to portal hypertension. It is believed that these changes lead to the development of HF that represents the severe end of the wide spectrum of LSD phenotypes (Machin, 1989; Norton, 1994). Table 2 lists LSD and non-lysosomal diseases found in association with HF. Machin (Machin, 1989) and Jauniaux et al. (Jauniaux et al., 1997) reported that 1.0–1.4% of NIHF is due to LSD.

In the present study we have found four definite and two probable LSD diagnoses among 75 NIHF pregnancies (5.3–8%). Our figures on the prevalence of LSD are in line with three more recent studies presenting estimates ranging from 5.9% to 15% (Burin et al., 2004; Groener et al., 1999; Piraud et al., 1996). The highest estimate is from Burin et al. (Burin et al., 2004). When chromosomal abnormalities were first excluded in this series of 33 NIHF cases the estimate becomes even higher (21%). Burin et al indicate that their numbers probably are an overestimation as they are a national reference centre for lysosomal diseases. This is likely to cause a selection bias in their series which is illustrated by the fact that 3 of the 5 positive cases had a suggestive family history for a lysosomal disease. Comparing the various studies, the gestational age must be taken into account. We have analyzed NIHF pregnancies between 14 and 36 weeks of gestation, while the study of Piraud mainly included third trimester samples (Piraud et al., 1996). The low urinary volume production of the foetus in the first two trimesters may imply that LSD cannot be diagnosed at the metabolite level before a certain gestational age. These...
diagnoses will be missed if investigations are performed at the metabolite level only. Of course, these would also be found in the second trimester by enzyme testing in cultured amniocytes. As we have included 29 cases where we only tested at the metabolite level, this may explain partly why we found a somewhat lower percentage of lysosomal diagnoses than the French study by Piraud et al. (Piraud et al., 1996). The six diagnoses in our study all were found in the period between weeks 21 and 36 of gestation. This study describes a case of NIHF due to β-glucuronidase deficiency in the 21st week (case 3). This could be shown also at the metabolite level, suggesting that the urine production at this stage of the pregnancy is sufficient to find lysosomal diagnoses. However, it is uncertain whether all cases will be diagnosed at the metabolite level in these early stages.

In conclusion, our data suggest that the prevalence of LSD is significantly higher than the estimate of 1.0–1.4% in previous studies. Therefore, we recommend the inclusion of metabolic analyses of amniotic fluid and amniocytes in the routine diagnostic work-up of NIHF. Techniques described in this paper will facilitate establishing a diagnosis in cases that would have previously been considered idiopathic. Finding the primary cause of HF will not only provide better understanding of the mechanism, but will also enable more accurate risk estimates and genetic counselling in future pregnancies. The present study applied a new diagnostic strategy using a protocol of prenatal diagnostic procedures. Figure 3 presents a flowchart illustrating the strategy to detect LSD in HF pregnancies. At the metabolite level, the protocol relies on the measurement of MPS and neuraminic acid and on TLC of oligosaccharides. The cases with LSD gave unequivocal abnormal results that were well above the established reference ranges. Foetal death may cause increased MPS concentration in amniotic fluid and therefore complicates the interpretation of the laboratory result. The protein concentration of the amniotic fluid was clearly increased in the foetal death cases in our study while a normal value for protein was found in the β-glucuronidase deficient cases. At the enzyme level this study worked with a panel of 21 lysosomal enzymes. Obviously for routine diagnostic purposes this would not be feasible in most centres. The laboratory workload can be diminished by measuring only those lysosomal enzymes that are frequently involved in the aetiology of HF (such as β-glucuronidase, β-glucosidase). Before week 18 of gestation, urine production of the foetus is limited, bringing the risk that a lysosomal diagnosis would be missed at the metabolite level. Therefore, it may be considered to add β-galactosidase to the enzyme panel to pick up cases with GM₃-gangliosidosis and galactosialidosis is such cases. This combination of measurements at the metabolite and the enzyme levels will allow a diagnostic laboratory to pick up the most frequent LSD known to be associated with NIHF. However, defects that do not result in an increase of MPS, neuraminic acid or oligosaccharides in amniotic fluid will be missed with this strategy. Niemann-Pick types A and C, Wolman, Farber, mucolipidosis II and multiple sulfatase deficiency are among the diseases that will not be picked up. Diagnostic centres may want to include a selection of the enzymes involved in these diseases in their NIHF protocol.

In NIHF pregnancies that have not been investigated prenatally, postnatal tests for LSD should always be performed. This became even more important through the availability of enzyme replacement therapy for an increasing number of LSD (Desnick, 2004). Vacuolization in the placenta or in foetal cells may be further clues in the direction of a lysosomal etiology (Norton, 1994; Stone and Sidransky, 1999). A systematic approach of HF as proposed in this paper will contribute to our understanding of HF in individual cases, improve genetic counselling and provide chances for family planning in families at risk.
Acknowledgements

We thank Dr. M. Verjaal, clinical geneticist and Dr. C.M. Bilardo, gynaecologist of Academic Medical Centre Amsterdam for their comments in 3 cases and Dr. W. Kleijer, clinical biochemical geneticist Erasmus MC Rotterdam for measuring cathepsin A. The authors thank Dr. W. Lissens (Brussels, Belgium), Dr. E. Paschke (Graz, Austria) and Prof. A. D’Azzo (Memphis, USA) for molecular genetic analysis of the cases with β-glucuronidase, β-d-galactosidase and cathepsin A deficiency respectively. We are grateful to Dr. I. Maire (Lyon, France) for her gift of amniotic fluid of a case of sialidosis. The authors thank Jenne den Hartog (Nijmegen) for her studies in the initial phase of this project.
Multiplex ligation-dependent probe amplification (MLPA) as a stand-alone test for rapid aneuploidy detection in amniotic fluid cells

Kooper AJA, Faas BHW, Kater-Baats E, Feuth T, Janssen JCJA, van der Burgt I, Lotgering FK, Geurts van Kessel A, Smits APT

Abstract

Objective
This study aimed to determine the diagnostic application of multiplex ligation-dependent probe amplification (MLPA) as a stand-alone test for targeted detection of common chromosomal aneuploidies (i.e., 13, 18, 21, X and Y) in amniotic fluid cells in routine prenatal clinical practice.

Methods
In this evaluation study, the MLPA test using kit P095 was performed on 1,000 consecutive amniotic fluid samples and the results obtained were compared with traditional karyotyping (TK), the gold standard.

Results
The absolute specificity and sensitivity of the MLPA test were 100%. The test yielded a rapid reporting time: 94% within three working days and 5% within seven working days. The test failure rate was 0.8%. The percentage of abnormalities undetectable using this specific test was 2.4%: abnormal foetal ultrasound (N=9), increased risk first trimester screening (N=2), advanced maternal age (N=3) or other reason for referral (N=10). These abnormalities can be categorised in clinically significant (N=8), clinically uncertain (N=4) and clinically non-significant (N=12).

Conclusions
MLPA P095 is suitable as a stand-alone test for the rapid and efficient detection of the most common chromosomal aneuploidies in routine prenatal clinical practice. A flowchart for integrating the MLPA test into the cytogenetic laboratory workflow is presented.

Introduction
In recent years, several studies have reported that molecular techniques such as quantitative fluorescence-polymerase chain reaction (QF-PCR) and multiplex ligation-dependent probe amplification (MLPA) may represent accurate, rapid and cost-effective options for high-throughput testing of common chromosomal aneuploidies (i.e., 13, 18, 21, X and Y) in amniotic fluid cells (Grimshaw et al., 2003; Slater et al., 2003; Nicolini et al., 2004; Gerdes et al., 2005b; Hochstenbach et al., 2005; Cirigliano et al., 2006). An obvious next step would be the implementation of such molecular tests in routine prenatal clinical practice.

However, there is an ongoing debate on whether these targeted tests may serve as such (Leung et al., 2004a; Caine et al., 2005; Ogilvie et al., 2005b; Bui, 2007). Here we aimed to assess the suitability of targeted testing by MLPA as a stand-alone test for the detection of common chromosomal aneuploidies in amniotic fluid cells in prenatal clinical practice. Therefore, we first performed an MLPA evaluation study in a research setting, followed by a prospective evaluation study in a clinical diagnostic setting. In both studies, the results obtained were compared with traditional karyotyping (TK), the gold standard. Specifically, we evaluated the accuracy, reporting time and failure rates of the MLPA test in order to assess its technical suitability for routine diagnostic application. Additionally, the clinical significance of the chromosomal abnormalities that would have remained undetected when MLPA was used as a stand-alone test in a group of patients referred for an increased risk of Down syndrome was determined. Finally, relative merits of the MLPA test are discussed, including its putative implementation as a stand-alone test in prenatal clinical practice. In addition, a flowchart for integrating the MLPA test into the cytogenetic laboratory workflow is presented.

Material and methods
A total of 1,000 amniotic fluid samples from various gestational ages and with different referral reasons were collected at the outpatient clinics in Nijmegen, Arnhem, Tilburg, ’s-Hertogenbosch and Enschede (the Netherlands). First trimester risk assessment for trisomy 21 was based on maternal age, biochemical serum markers, i.e., maternal serum free beta-human chorionic gonadotrophin (hCG), fβ-hCG and pregnancy-associated plasma protein-A (PAPP-A), and a nuchal translucency (NT) measurement and TK was performed on all samples following standard procedures. DNA from amniotic fluid cells was isolated through lysis of cell pellets and proteinase K treatment using standard procedures. Subsequently, this DNA was purified using a QIAamp kit (Qiagen, Hilden,
The MLPA test, using kit P095, was performed according to the instructions of the manufacturer (www.mrc-holland.com). The polymerase chain reaction (PCR) products obtained were quantified by capillary electrophoresis on an ABI 3100 analyser, using Genescan analysis software (version 3.7) and Genemapper (version 4.0) software, all from Applied Biosystems. Samples were examined by visual inspection of the peak profiles in the electropherogram of the MLPA products. Substantial differences in peak heights in at least two consecutive loci were considered to be suspect for a structural chromosomal abnormality. Next, the MLPA peak areas were exported to a Microsoft Excel datasheet.

First an intra-sample normalisation of each probe peak area was performed, followed by an inter-sample normalisation with respect to the two normal reference samples (one male and one female) included in the same run. This normalisation was essential because variations in experimental conditions may lead to quantitative differences. Subsequently, MLPA results were expressed as the mean ratio per chromosome with a 95% confidence interval (CI). By doing so, the expected mean value of 1.0 represents two copies of the target sequence in the sample, whereas one or three copies of the target sequence result in expected mean values of 0.5 or 1.5, respectively.

On the 400 amniotic fluid samples collected in 2006, a single MLPA reaction was performed in a research setting, i.e. the results were compared with TK and not reported to the patient or the obstetrician. On the 600 consecutive samples collected in 2007, MLPA was performed in a clinical setting, i.e. the MLPA results were reported to both the patient and the obstetrician. Independent duplicate measurements were carried out to exclude sample mishandling. Repeated measurements were performed in case the duplicate measurements were discordant or when one of the MLPA tests failed. A test result was considered abnormal when the theoretical value (=expected value in a normal case) was not included in the CI 95% in both duplicate measurements and when at least one of the mean probe ratios differed 10% of the expected value. This notion was subsequently tested by extended karyotyping (all available clones were examined for the abnormality). Two different failure criteria were used in order to measure the effect of the test failure on the reporting time: technical failure and discordant results of the duplicate measurements.

Results

Patient characteristics

In Table 1, an overview of all referral reasons is given. The median age of the 595 patients with referral ‘advanced maternal age’ was 38 (range, 36-45) years. In addition, 145 of the patients were ‘screen positive’ after first trimester screening, resulting in a group of, in total, 740 pregnancies at risk for Down syndrome. In Figure 1, the karyotype results of all 1,000 amniotic fluid samples are shown compared with the MLPA results.

<table>
<thead>
<tr>
<th>Referral reason</th>
<th>N</th>
</tr>
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<tbody>
<tr>
<td>Advanced maternal age</td>
<td>595</td>
</tr>
<tr>
<td>Increased risk first trimester screening</td>
<td>83</td>
</tr>
<tr>
<td>&lt;36 years</td>
<td>62</td>
</tr>
<tr>
<td>≥36 years</td>
<td>179</td>
</tr>
<tr>
<td>Abnormal foetal ultrasound</td>
<td>81</td>
</tr>
<tr>
<td>Others</td>
<td>1,000</td>
</tr>
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</table>

### MLPA and traditional karyotyping in a research setting

The mean volume of amniotic fluid used for DNA isolation was 3.0 ml (range 1.0-8.0 ml). From all normal samples (N=370), the MLPA test was non-informative in 17 samples (4.3%), mostly due to low DNA concentrations. The MLPA was concordant with TK in 353 normal samples, and in 21 samples with trisomies 21, 18, 13 and/or copy number changes of the sex chromosomes, there were no false-positive or false-negative diagnoses. TK revealed nine chromosomal abnormalities that were not detected by MLPA: one 69,XXX and two unbalanced structural rearrangements in patients with foetal ultrasound abnormalities, four known familial balanced rearrangements and in two pregnancies at risk for Down syndrome de novo balanced translocations. In one of these latter samples, the MLPA test failed.

### MLPA and traditional karyotyping in a routine clinical setting

The mean volume of amniotic fluid used for DNA isolation was 4.0 ml (range 2.0-4.0 ml). Concordant MLPA measurements were obtained within three working days in 94% of the samples, 5% of the samples had a reporting time of seven days and in 0.8% of the cases, the MLPA test failed. Test failure occurred in three samples in which one of the duplicate measurements failed after repeated testing, and in two cases with an intrauterine foetal death, which resulted in (partial) DNA degradation. All normal samples (N=548) and cases with trisomies 21, 18, 13 and/or copy number changes of the sex chromosomes and male triploidies (N=30) were correctly identified by MLPA and there were no false-positive or false-negative results. TK revealed three unbalanced structural rearrangements in patients with foetal ultrasound abnormalities. These abnormalities represented one trisomy 21 due to a Robertsonian translocation,
46,XY,der(14;21),+21 and a 47,XY,t(11;18),+18 karyotype. The third sample was indicative for a partial deletion of chromosome 13, since three of the distal consecutive MLPA probes for chromosome 13 showed decreased peak heights. TK of this sample revealed a 46,XY,del(13)(q31.2) karyotype. Four samples resulted in a failure of TK, whereas in three of these cases a positive MLPA result (one with trisomy 21) was obtained. In the remaining cases, TK revealed chromosomal rearrangements that were not detected by MLPA: one 69,XXX and four unbalanced structural rearrangements in patients with foetal ultrasound abnormalities and eight balanced rearrangements (two of which were determined familial after karyotyping of the parents). In two pregnancies at risk for Down syndrome, a de novo balanced translocation was detected.

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Table 2 shows an overview of the referral reasons of the 54 samples with an abnormality detected with MLPA. The aneuploidies included trisomy 21 (N=24), trisomy 18 (N=15), trisomy 13 (N=5), 69,XXX and four unbalanced structural rearrangements in patients with foetal ultrasound abnormalities and eight balanced rearrangements (two of which were determined familial after karyotyping of the parents). In two pregnancies at risk for Down syndrome, a de novo balanced translocation was detected.

**Figure 1.**
Results of traditional karyotyping and MLPA in 1,000 amniotic fluid samples
*trisomy 21 (N=25) 48,XXY,+21 included in group with trisomy 21.

**Table 2.** Overview of the referral reasons of the 54 samples with an abnormality detected with MLPA

<table>
<thead>
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<tbody>
<tr>
<td>Advanced maternal age</td>
<td>8</td>
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<tr>
<td>Increased risk</td>
<td>5</td>
</tr>
<tr>
<td>Structural chrom aberrations</td>
<td>1</td>
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<td>Male trisomy</td>
<td>2</td>
</tr>
<tr>
<td>Structural chrom rearrangement</td>
<td>15</td>
</tr>
<tr>
<td>Male trisomy</td>
<td>5</td>
</tr>
<tr>
<td>Other</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
</tr>
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</table>

**High absolute specificity and sensitivity**
The capability to detect all abnormalities that the MLPA test is able to identify, compared with TK, was calculated based on all detectable euploidies (N=901) and aneuploidies

(N=51, the three samples with 46,XY,del(13)(q31.2), 46,XY,der(14;21),+21 and 47,XY,t(11;18),+18 were not included). All MLPA test results were concordant with TK resulting in a 100% absolute specificity (95% CI: 99.6-100) and sensitivity (95% CI: 93.0-100).

Table 2 shows an overview of the referral reasons of the 54 samples with an abnormality detected with MLPA. The aneuploidies included trisomy 21 (N=24), trisomy 18 (N=15), trisomy 13 (N=5), 69,XXX and four unbalanced structural rearrangements in patients with foetal ultrasound abnormalities and eight balanced rearrangements (two of which were determined familial after karyotyping of the parents). In two pregnancies at risk for Down syndrome, a de novo balanced translocation was detected.

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<tr>
<td>Male trisomy</td>
<td>14</td>
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<tr>
<td>Structural chrom rearrangement</td>
<td>1</td>
</tr>
<tr>
<td>Male trisomy</td>
<td>2</td>
</tr>
<tr>
<td>Clinical significant</td>
<td>2</td>
</tr>
<tr>
<td>No clinical significance</td>
<td>12</td>
</tr>
<tr>
<td>Uncertain clinical significance</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
</tr>
</tbody>
</table>

**High absolute specificity and sensitivity**
The capability to detect all abnormalities that the MLPA test is able to identify, compared with TK, was calculated based on all detectable euploidies (N=901) and aneuploidies...
Reduced relative sensitivity

The ability to detect any abnormality with MLPA within the range of all possible abnormalities that can be identified by TK was determined. This was based on 78 abnormal karyotypes from which 24 were detected by TK, but not by MLPA, and resulted in a relative sensitivity of 69.2% (95% CI: 57.7-79.2). Table 3 shows an overview of the referral reasons of the 24 samples with a MLPA result discordant with TK. Excluded were 26 samples: 22 with MLPA failures and 4 with TK failures. The number of abnormalities detected by TK in samples encompassing all referral reasons that were technically undetectable by MLPA was 24 (2.4%). Within this group, a categorisation can be made between clinically significant (N=8), uncertain clinical significance (N=4) and clinically non-significant (N=12). All clinically significant abnormalities were detected in amniotic fluid cells from pregnancies with a foetal ultrasound abnormality. For the group of pregnancies at risk for Down syndrome (N=740), the relative sensitivity was 77.3% (95% CI: 54.6-92.2), based on 22 abnormal karyotypes from which 17 were detected by MLPA. The five undetectable abnormalities included four balanced de novo translocations with uncertain clinical relevance and one sample with a familial translocation.
Discussion

It has been reported that QF-PCR and/or MLPA may serve as suitable methods for a rapid detection of the most common (non-mosaic) chromosome abnormalities in amniotic fluid cells (Grimshaw et al., 2003; Slater et al., 2003; Nicolin et al., 2004; Gerdes et al., 2005b; Hochstenbach et al., 2005; Cirigliano et al., 2006). It has also been reported that both the technologies have their benefits and limitations (Shaffer and Bui, 2007). The accuracy of QF-PCR in prenatal diagnosis has already been demonstrated in a number of large studies (Nicolini et al., 2004; Cirigliano et al., 2006). One of the rationales for introducing the MLPA P095 test in our laboratory setting was based on the availability of a commercial kit, produced by MRC Holland. MLPA tests are suited for further tailor-made applications, including the detection of common aneuploidies, microdeletions and single-gene mutations such as those encountered in cystic fibrosis. Also, a mental retardation-tailored assay can be devised including known microdeletion-associated and sub-telomeric targets which could be used for the genetic analysis of pregnancies with an ultrasound abnormality, but a normal karyotype (Northrop et al., 2005; Rooms et al., 2006; Faas et al., 2008). In reverse, as compared with QF-PCR, MLPA is relatively sensitive to DNA quality and does not allow the detection of maternal cell contamination in samples from females and/or the detection of female triploids. Since there is only one commercial supplier of MLPA kits, a sufficient amount of kits should be stored in order to guarantee a prenatal diagnostic service.

To assess the cost-effectiveness of introducing molecular tests for prenatal diagnosis of chromosome abnormalities, the costs of QF-PCR were calculated in an extensive report (Grimshaw et al., 2003). A comparable study will be performed for MLPA within a nationwide study M.A.K.E. (MLPA and karyotyping, an evaluation) in the Netherlands (Boormans et al., 2008). The use of interphase-fluorescence in situ hybridization (I-FISH) as stand-alone test for pregnant women at risk for Down syndrome compares unfavourably to MLPA and/or QF-PCR, i.e. with our standard routine quality assessment to perform a stand-alone test via independent duplicate measurements, I-FISH is not suitable for high-throughput analysis.

From our combined results, we conclude that the MLPA P095 test allows a fast, reliable and accurate detection of (an)euploidies of the chromosomes 13, 18, 21, X and Y in amniotic fluid cells. Although this MLPA kit was designed to detect copy number changes of the selected target chromosomes, partial deletions or duplications can also be detected through reduced or increased levels of individual peaks. On the basis of this notion, one of the MLPA results was indicative for a partial deletion of chromosome 13. This finding was subsequently confirmed with TK. Decreased levels of a single probe may be due to a point mutation. Theoretically, polymorphisms close to the probe ligation site may also result in a reduced relative peak level. However, MRC Holland has designed its probes such that, it avoids polymorphisms known from the Genbank databases.

Overall, we used independent duplicate measurements in order to reduce any risk of sample mishandling. It is relevant to note that results obtained with the uncultured amniotic fluid cells, as is the case with molecular tests such as MLPA, have stronger associations with pregnancy outcome than results obtained with the cultured amniotic fluid cells, as is the case with TK (Robinson et al., 2002). This notion is based on the fact that uncultured amniotic fluid cell populations contain different cell types and thus provide a better indication of the overall foetal status than cultured cell populations, which are clonal outgrowths of cellular subpopulations and thus affect the genetic make-up of a (mosaic) cell population studied (Donaghue et al., 2005). Although no mosaic samples were available in our prospective study, we performed a series of artificial dilution experiments. The results showed that the lowest detectable mosaicism for trisomy 21 was 20%, with a mean probe ratio of 1.17 with a CI 95%: 1.01–1.32 (Kooper et al., 2008). The expected mean value of 1.0 or 1.5 representing two or three copies for chromosome 21, respectively, was outside the CI 95% and was therefore indicative for a mosaic trisomy 21. Whenever a mosaicism for one of the target chromosomes is suspected, follow-up analysis is warranted.

In recent years, there has been ample debate on whether targeted testing can replace TK. The main argument against replacing TK is that targeted prenatal testing holds an a-priori risk of detection limitation. Several studies have addressed the issue of residual risk of undetected chromosome abnormalities in relation to clinical significance. As far as the clinical relevance of these anomalies is concerned, it is anticipated that at least a number of these pregnancies will result in miscarriages and/or will be associated with abnormalities detectable by ultrasound (Bui, 2007). For pregnancies with an increased risk of Down syndrome, a change of policy from full karyotype analysis to rapid molecular aneuploidy testing would result in a failure to detect chromosome abnormalities likely to have serious clinical consequences. This residual risk was estimated to be 0.06% (1 in 1,659) by Ogilvie (Ogilvie et al., 2005b), 0.1% (1 in 1,000) in a retrospective audit of about 14,000 invasive prenatal diagnoses by Bui (Bui, 2007) and 0.07% (1 in 1,500) in our own retrospective study of 7,140 women with an advanced maternal age (Kooper et al., unpublished data). Within this context, it is important to note that unavoidable detailed information obtained with TK with unpredictable outcomes requires intensive genetic counselling and may even result in unwarranted pregnancy terminations (Leung et al., 2004b; Ogilvie, 2003). It is also important to realise that a first trimester screening is designed to screen for Down syndrome and, therefore, that follow-up diagnosis with targeted testing alone should realistically fulfil the expectations of the couples and
obstetricians. In this study, four apparently balanced de novo structural rearrangements remained undetected by MLPA in the group of pregnancies with an increased risk of Down syndrome. The risk of a serious congenital anomaly is estimated to be 6.1% for de novo reciprocal translocations (Warburton, 1991). It should be noted, however, that such unexpected findings are mostly not foreseen and result inevitably in uncertainties about the pregnancy.

Since a few years, women in Stockholm, Sweden, with an increased risk of Down syndrome undergoing amniocentesis can choose between rapid aneuploidy detection (by QF-PCR) and full karyotyping. Experience based on over 6,000 clinical cases has shown that about 70% of these women choose rapid aneuploidy detection by QF-PCR (Bui, 2007). In the Netherlands, as yet, little is known about the patient’s preference (Leung et al., 2008). Therefore, we plan to implement the Swedish model in which women have the autonomy to choose between rapid aneuploidy testing (by MLPA) and TK. In Figure 3, we provide a laboratory flowchart for samples obtained from women at risk for Down syndrome. Inclusion of back-up protocols is needed in a targeted stand-alone policy to avoid pregnant women from having repeated amniocentesis.

The back-up protocols we use are i) storage of DNA in all samples with a normal MLPA test, this allows follow-up examination, in case a fetal anomaly is detected with the scan taken at 20 weeks and ii) TK when a MLPA test failure occurs. We recommend the suggested routine of duplicate analysis as a standard routine quality assessment for all types of stand-alone tests in a prenatal diagnostic setting, predominantly to prevent sample mishandling.

We anticipate that in the near future, next to QF-PCR, implementation of the MLPA P095 test as a stand-alone test will be one of the major steps forward in assessing the genetic constitution of the foetus.

**Acknowledgements**

The authors wish to thank the colleagues of the Network Prenatal Diagnostics Nijmegen in Arnhem (Rijnstate Hospital), Tilburg (St. Elizabeth Hospital, TweeSteden Hospital), Enschede (Medisch Spectrum Hospital Twente), ’s-Hertogenbosch (Jeroen Bosch Hospital) and Nijmegen (UMC St. Radboud) for granting access to the diagnostic samples. We are grateful to Dr Schouten from MRC Holland, supplier of the MLPA kits. The authors wish to thank the contributors of the nationwide study ‘MLPA And Karyotyping, an Evaluation, (M.A.K.E.)’ in the Netherlands for their participation in this discussion.
Individual probe performance of multiplex ligation-dependent probe amplification (MLPA) for aneuploidy detection in amniotic fluid cells

Kooper AJ, Faas BHW, Kater-Baats E, Feuth T, Geurts van Kessel A, Smits APT
Abstract

Objectives
To assess individual probe performances of the multiplex ligation-dependent probe amplification (MLPA) test kit P095 for the detection of aneuploidies and mosaics of chromosomes 21, 18, 13, X and Y in uncultured amniotic fluid samples.

Methods
A set of 199 euploid and 50 aneuploid uncultured amniotic fluid samples was used. Since exclusion of poor performing probes may improve overall sensitivity, individual MLPA performance of the probes in kit P095 were assessed. In addition, artificial dilution experiments were carried out to establish the (mosaic) detection limits of the test.

Results
In the euploid and aneuploid amniotic fluid samples tested, the MLPA test yielded an absolute sensitivity and specificity of 100% for the detection of trisomies of chromosomes 21, 18 or 13. The pooled standard deviation (SD) for the autosomal chromosomes was 0.044. Theoretical exclusion of the autosomal probes with the highest individual SD resulted in a slight decrease of this pooled SD to 0.037. In addition, we found that mosaics of approximately 20% are detectable by the MLPA test.

Conclusion
The MLPA test (kit P095) is suitable for the detection of non-mosaic (an)euploidies in amniotic fluid samples and yields abnormal results in manufactured mosaics when cell populations contribute at least 20% to the sample. Individual probes performed well; exclusion of the poorest performing probes only slightly affected the test sensitivity.

Introduction

Recently, rapid methods such as quantitative fluorescence (QF)-PCR and multiplex ligation-dependent probe amplification (MLPA) for the detection of common chromosome aneuploidies have been reported (Slater et al., 2003; Grimshaw et al., 2003; Nicolini et al., 2004; Mann et al., 2004; Hochstenbach et al., 2005; Gerdes et al., 2005a; Cirigliano et al., 2006). As a consequence, a validated QF-PCR test is currently offered to pregnant woman in a number of prenatal centres in Europe (Cirigliano et al., 2001; Ogilvie et al., 2005a).

Previously, Slater et al. reported that MLPA with kit P001 may serve as a rapid, flexible, sensitive and robust test for prenatal aneuploidy detection (Slater et al., 2003). Subsequently, Hochstenbach et al. reported on the performance of the P001 probe set in uncultured amniocytes (Hochstenbach et al., 2005). In 2005 MLPA kit P095, an improved version of the P001 kit, became available. Gerdes et al. developed software for fully automated analysis of the P095 kit and examined the probe reliabilities and corresponding SDs (Gerdes et al., 2005a). Very recently, van Opstal et al. (Van Opstal et al., 2009) reported that MLPA serves as a reliable method that could be used as a stand-alone test for rapid aneuploidy detection (RAD). Here we aimed to assess the individual probe performances of the P095 MLPA kit. Previously, mosaics for aneuploidies of chromosomes 21, 18, 13, X and Y have been reported to occur in 0.016% (Worton and Stern, 1984) and in 0.007% (Bui et al., 1984) of amniotic fluid samples. The phenotypic consequences of these mosaic aneuploidies span a broad range from full clinical manifestation to clinically unaffected. The detection of mosaics, however, represents a major problem in prenatal diagnostics and they may remain undiagnosed due to low-grade levels. Therefore, we also assessed the lowest limit of mosaic detection by MLPA kit P095.

Material and methods

Clinical samples
Amniotic fluid samples were collected at the outpatient clinics located in Nijmegen, Arnhem, Tilburg, ’s-Hertogenbosch and Enschede in the Netherlands. The referral reasons of these samples covered the whole range from low-risk to high-risk, with known clinical abnormalities detected by ultrasound and/or maternal serum screening.

Sample preparation and MLPA application
The MLPA test was performed through independent duplicate measurements using purified DNA from amniotic fluid cells. DNA from 2-4 ml amniotic fluid was isolated using a QIAamp kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.
The principle of the MLPA has been described in detail previously (Schouten et al., 2002). The MLPA test with the SALSA MLPA kit P095 Aneuploidy was performed as described by Schouten (MRC Holland, Amsterdam, the Netherlands). The P095 probe mix contains 36 different probes generating amplification products ranging in size between 136 and 454 nucleotides. Four of these probes (Y-chromosome) only generate signals from male samples, whereas for each of the chromosomes 21, 18, 13 and X eight probes are included in the mix. Each amplified probe product was identified and quantified by capillary electrophoresis on an ABI 3100 analyzer, using Genescan analysis software (version 3.7) and Genemapper (version 4.0) software, all from Applied Biosystems. Independent duplicate measurements were performed, i.e., all steps from DNA extraction, PCR to and Genemapper (version 4.0) software, all from Applied Biosystems. Independent electrophoresis on an ABI 3100 analyzer, using Genescan analysis software (version 3.7)

Peak examination and normalization

Routine visual examination of peak heights of individual MLPA probes in the electropherogram were compared with the peaks in the electropherogram of normal gender-matched reference samples. Subsequently, data analysis was carried out upon the transfer of GeneMapper results to a modified spreadsheet for normalization and ratio computation of the peak areas. First an intra-sample normalization of each probe peak area was performed, followed by an inter-sample normalization with respect to the two normal reference samples (one male and one female) included in the same run. Inter-sample normalization is essential because variations in experimental conditions may lead to quantitative differences. As a consequence of this normalization procedure, mean probe ratios per chromosome (called chromosome ratios) skew in case of a trisomy, since the probe ratios of the targets with two copies scale down to 80% when a trisomy for one of the targets is present. This decrease in chromosome ratios is not corrected for in the data analysis. The mean and standard deviation (SD) of each target chromosome was determined, followed by the 95% confidence interval (CI) defined as mean ± t_{0.05} * (SD/√n), where t_{0.05} is the percentage point of the t-distribution with (n-1) degrees of freedom, which gives a two-tailed probability of 0.05 and n is the number of targets on the chromosome (8 for chromosomes 13, 18, 21 and X and 4 for chromosome Y). In case of disomy (two copies of each target chromosome), these calculations will result in an expected or theoretical value of 1.0, representing two copies of the target sequence in the sample. One or three copies of the target sequence in a sample will result in theoretical values of 0.5 or 1.5, respectively. A test result is considered abnormal when the theoretical value (= expected value in a normal case) is not included in the 95% CI in both duplicate measurements and when at least one of the mean probe ratios differs 10% of the expected value. Follow-up analysis of these abnormal samples was performed by karyotyping. A full description of the data analysis procedure is shown in data supplement I an example given in data supplement II (see Supplemental Data I and II).

Inclusion criteria

A set of 249 amniotic fluid samples with an informative MLPA result, 199 normal results and 50 results indicative for an aneuploidy for one of the target chromosomes, was selected. This selection was based on the following eligibility criteria: input of 2-4 ml amniotic fluid (exclusion of heavily blood-stained samples) and sufficient input of DNA by visual fragment examination. Because tests results were obtained as two duplicate measurements per sample, one of these results was randomly included in this study. MLPA was performed prospectively in 239 of the 249 (96%) of the samples in a clinical setting, i.e., the MLPA results were reported to both the patient and the obstetrician. To extend the number of aneuploid samples, ten cases (seven with a trisomy 21 and three with a trisomy 18) were included from previous sampling in a preclinical setting.

Probe performance

Average, standard deviation (SD) and chromosome ratio were determined on a set of 199 euploid samples and 50 aneuploid samples with trisomy 21 (N=31), trisomy 18 (N=16), trisomy 13 (N=3), respectively. The standard deviations per probe were used to provide information on the performance of each probe. Based in this information, probes with the highest SD were theoretically excluded in order to assess its effect on the overall performance of the MLPA test.

Lower limit of detection mosaic

To determine the lowest limit of mosaicism, three step-wise dilution experiments (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100%) were performed using a normal sample and a sample with trisomy 21, trisomy 18 or trisomy 13, respectively. The standard deviations per probe were used to provide information on the performance of each probe. Based in this information, probes with the highest SD were theoretically excluded in order to assess its effect on the overall performance of the MLPA test.

Results

Statistics of the chromosome ratios

An overview of the mean chromosome ratios in the cohort of 249 amniotic fluid samples (199 euploid and 50 aneuploid) is presented in Table 1. The observed mean chromosome ratios for the presence of two copies of a target chromosome were in the range of 0.98 to 1.04 and, thus, close to the expected value of 1.0. The mean ratios for a single copy or three copies were in the range of 0.47 to 0.51 and 1.46 to 1.51, respectively. The SD for the
revealed that these outlier effects cannot be ascribed to the performance of specific individual probes. Results interpreted as inconclusive represented 95% CIs too wide to be useful for the chromosomes 13, 18, 21 and X in females, and were all confined to the same samples. After elimination of these inconclusive results, which are likely due to DNA impurities, we conclude that the MLPA test results yield a sensitivity for the detection of trisomies 13, 18 and 21 of 100% (95% CI: 29.2-100), (95% CI: 79.4-100) and (95% CI: 88.8-100), respectively, and a specificity of 100% (95% CI: 98.2-100) for the detection of disomies 13, 18 and 21.

Assessment of individual probe performance  
To evaluate the performance of the individual probes of all target chromosomes, the standard deviation (SD) of each probe was calculated in the euploid (Table 2) and for the autosomes in the aneuploid (Table 3) samples. In Table 2, the probes with the highest mean probe SD are highlighted: 13m7, 18m7, 21m8, Xm1 (in female), Xm8 (in male) and Ym3 with a SD of 0.281, 0.126, 0.135, 0.119, 0.084 and 0.085, respectively.

Individual probe performance of multiplex ligation-dependent probe amplification (MLPA) for aneuploidy detection in amniotic fluid cells

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Sample set</th>
<th>N</th>
<th>Expected mean</th>
<th>Observed mean</th>
<th>95% CI</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>Trisomy 13</td>
<td>3</td>
<td>1.5</td>
<td>1.51</td>
<td>-</td>
<td>0.07</td>
</tr>
<tr>
<td>13</td>
<td>Trisomy 13 (80%)</td>
<td>1</td>
<td>1.4</td>
<td>1.35</td>
<td>1.31-1.38</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>Trisomy 13 (20%)</td>
<td>1</td>
<td>1.1</td>
<td>1.11</td>
<td>1.10-1.12</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>Normal</td>
<td>199</td>
<td>1.0</td>
<td>0.99</td>
<td>-</td>
<td>0.05</td>
</tr>
<tr>
<td>18</td>
<td>Trisomy 18</td>
<td>16</td>
<td>1.5</td>
<td>1.46</td>
<td>-</td>
<td>0.04</td>
</tr>
<tr>
<td>18</td>
<td>Trisomy 18 (80%)</td>
<td>1</td>
<td>1.4</td>
<td>1.39</td>
<td>1.33-1.46</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>Trisomy 18 (20%)</td>
<td>1</td>
<td>1.1</td>
<td>1.13</td>
<td>1.06-1.19</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>Normal</td>
<td>199</td>
<td>1.0</td>
<td>0.98</td>
<td>-</td>
<td>0.04</td>
</tr>
<tr>
<td>21</td>
<td>Trisomy 21</td>
<td>31</td>
<td>1.5</td>
<td>1.49</td>
<td>-</td>
<td>0.04</td>
</tr>
<tr>
<td>21</td>
<td>Trisomy 21 (80%)</td>
<td>1</td>
<td>1.4</td>
<td>1.43</td>
<td>1.40-1.46</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>Trisomy 21 (20%)</td>
<td>1</td>
<td>1.1</td>
<td>1.17</td>
<td>1.01-1.32</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>Normal</td>
<td>199</td>
<td>1.0</td>
<td>1.04</td>
<td>-</td>
<td>0.04</td>
</tr>
<tr>
<td>X (female)</td>
<td>Normal</td>
<td>99</td>
<td>0.98</td>
<td>0.97</td>
<td>0.97-0.99</td>
<td>0.04</td>
</tr>
<tr>
<td>X (male)</td>
<td>Normal</td>
<td>100</td>
<td>0.51</td>
<td>0.51</td>
<td>0.51-0.52</td>
<td>0.03</td>
</tr>
<tr>
<td>Y (male)</td>
<td>Normal</td>
<td>100</td>
<td>0.47</td>
<td>0.46</td>
<td>0.46-0.48</td>
<td>0.06</td>
</tr>
</tbody>
</table>

To identify outliers in each sample, all individual chromosome ratios at 95% CIs were assessed. Subsequent evaluation of the outliers with a chromosome ratio >0.25 SD was calculated from the totals of euploid and aneuploid samples. The SDs were, for chromosomes 13, 18, 21 and X in the euploid samples, in the range of 0.03 to 0.05, and for the Y chromosome 0.06. The SDs of the chromosomes in the aneuploid samples were in the range of 0.04 to 0.07, which may be due to the relatively limited number of samples included in this set (50 versus 199).
discrimination between individual mean probe ratios for euploidy and aneuploidy results. There are two outliers, probes Xm7, in both female and male samples with an increased mean probe ratio of 1.092 (95% CI: 1.074-1.110) and 0.577 (95% CI: 0.562-0.591), respectively (Figure 4). Based on the highest mean SDs measured in 199 samples, we conclude that probes 21m8, 18m7, 13m7 and outlier Xm7 represent the least reliable probes in kit P095.

In the aneuploid samples (Table 3) probes for 13m5, 18m7 and 21m4 showed the highest mean SD, i.e., 0.187, 0.167 and 0.153, respectively. Probe 18m7 also showed a high mean SD in the euploid samples and, therefore, represents the least reliable probe. For chromosomes 13 and 21, the probes with the highest SD differ from the results of the euploid samples.

This inconsistency is probably due to the small number of the aneuploid samples included (see above). The mean ratios of all individual probes of all target chromosomes of the euploid and aneuploid samples are depicted in Figure 4, resulting in a complete

<table>
<thead>
<tr>
<th>Table 2. Continued.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chr</td>
</tr>
<tr>
<td>13m1</td>
</tr>
<tr>
<td>13m2</td>
</tr>
<tr>
<td>13m3</td>
</tr>
<tr>
<td>13m4</td>
</tr>
<tr>
<td>13m5</td>
</tr>
<tr>
<td>13m6</td>
</tr>
<tr>
<td>Xm1</td>
</tr>
<tr>
<td>Xm2</td>
</tr>
<tr>
<td>Xm3</td>
</tr>
<tr>
<td>Xm4</td>
</tr>
<tr>
<td>Xm5</td>
</tr>
<tr>
<td>Xm6</td>
</tr>
<tr>
<td>Xm7</td>
</tr>
<tr>
<td>Xm8</td>
</tr>
<tr>
<td>Xm1</td>
</tr>
<tr>
<td>Xm2</td>
</tr>
<tr>
<td>Xm3</td>
</tr>
<tr>
<td>Xm4</td>
</tr>
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<td>Xm5</td>
</tr>
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<td>Xm6</td>
</tr>
<tr>
<td>Xm7</td>
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<tr>
<td>Xm8</td>
</tr>
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<td>Ym1</td>
</tr>
<tr>
<td>Ym2</td>
</tr>
<tr>
<td>Ym3</td>
</tr>
<tr>
<td>Ym4</td>
</tr>
</tbody>
</table>

*Probes with the highest mean probe SD are highlighted.

Table 3. Statistics of the individual probes for chromosome 21, 18 and 13 in samples with an aneuploidy (trisomy 21, N=31, trisomy 18, N=16, trisomy 13, N=3).

<table>
<thead>
<tr>
<th>Chr*</th>
<th>Sample set</th>
<th>Mean</th>
<th>95% CI</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>21m1</td>
<td>Trisomy 21</td>
<td>1.506</td>
<td>1.465-1.547</td>
<td>0.111</td>
</tr>
<tr>
<td>21m2</td>
<td>Trisomy 21</td>
<td>1.528</td>
<td>1.491-1.564</td>
<td>0.099</td>
</tr>
<tr>
<td>21m3</td>
<td>Trisomy 21</td>
<td>1.520</td>
<td>1.479-1.561</td>
<td>0.112</td>
</tr>
<tr>
<td>21m4</td>
<td>Trisomy 21</td>
<td>1.417</td>
<td>1.361-1.473</td>
<td>0.153</td>
</tr>
<tr>
<td>21m5</td>
<td>Trisomy 21</td>
<td>1.427</td>
<td>1.386-1.467</td>
<td>0.110</td>
</tr>
<tr>
<td>21m6</td>
<td>Trisomy 21</td>
<td>1.506</td>
<td>1.453-1.600</td>
<td>0.146</td>
</tr>
<tr>
<td>21m7</td>
<td>Trisomy 21</td>
<td>1.464</td>
<td>1.411-1.516</td>
<td>0.140</td>
</tr>
<tr>
<td>21m8</td>
<td>Trisomy 21</td>
<td>1.570</td>
<td>1.521-1.620</td>
<td>0.131</td>
</tr>
<tr>
<td>18m1</td>
<td>Trisomy 18</td>
<td>1.423</td>
<td>1.389-1.457</td>
<td>0.064</td>
</tr>
<tr>
<td>18m2</td>
<td>Trisomy 18</td>
<td>1.358</td>
<td>1.296-1.421</td>
<td>0.118</td>
</tr>
<tr>
<td>18m3</td>
<td>Trisomy 18</td>
<td>1.400</td>
<td>1.355-1.445</td>
<td>0.085</td>
</tr>
<tr>
<td>18m4</td>
<td>Trisomy 18</td>
<td>1.522</td>
<td>1.465-1.576</td>
<td>0.102</td>
</tr>
<tr>
<td>18m5</td>
<td>Trisomy 18</td>
<td>1.448</td>
<td>1.397-1.498</td>
<td>0.094</td>
</tr>
<tr>
<td>18m6</td>
<td>Trisomy 18</td>
<td>1.538</td>
<td>1.478-1.600</td>
<td>0.115</td>
</tr>
<tr>
<td>18m7</td>
<td>Trisomy 18</td>
<td>1.532</td>
<td>1.443-1.621</td>
<td>0.167</td>
</tr>
<tr>
<td>18m8</td>
<td>Trisomy 18</td>
<td>1.440</td>
<td>1.372-1.509</td>
<td>0.128</td>
</tr>
<tr>
<td>13m1</td>
<td>Trisomy 13</td>
<td>1.398</td>
<td>1.271-1.524</td>
<td>0.051</td>
</tr>
<tr>
<td>13m2</td>
<td>Trisomy 13</td>
<td>1.577</td>
<td>1.402-1.753</td>
<td>0.070</td>
</tr>
<tr>
<td>13m3</td>
<td>Trisomy 13</td>
<td>1.546</td>
<td>1.340-1.753</td>
<td>0.083</td>
</tr>
<tr>
<td>13m4</td>
<td>Trisomy 13</td>
<td>1.553</td>
<td>1.305-1.801</td>
<td>0.100</td>
</tr>
<tr>
<td>13m5</td>
<td>Trisomy 13</td>
<td>1.564</td>
<td>1.095-2.032</td>
<td>0.187</td>
</tr>
<tr>
<td>13m6</td>
<td>Trisomy 13</td>
<td>1.459</td>
<td>1.355-1.562</td>
<td>0.042</td>
</tr>
<tr>
<td>13m7</td>
<td>Trisomy 13</td>
<td>1.364</td>
<td>1.164-1.564</td>
<td>0.022</td>
</tr>
<tr>
<td>13m8</td>
<td>Trisomy 13</td>
<td>1.482</td>
<td>0.569-2.395</td>
<td>0.102</td>
</tr>
</tbody>
</table>

*Probes with the highest mean probe SD are highlighted.
this pooled SD to 0.037. The exclusion of the Xm1 and Ym3 probes with the highest SD had no effect on the pooled SDs. Exclusion of probe Xm7 resulted in an increase of SD in normal female samples from 0.04 to 0.05 and had no measurable effect in normal male samples.

We conclude that the impact of this optimization step through probe selection has a minimal impact on the overall MLPA test performance.

The detection level of trisomy 21, 18 or 13 mosaics

In Figure 5 step-wise dilution experiments are depicted using samples with trisomy 21, 18, 13 and a normal disomic sample. In this graphical plot, chromosome 21, 18 and 13 show a shift from a normal ratio of 1.0 towards a complete trisomy with a ratio of 1.5. The lowest detectable manufactured mosaics (80% normal DNA - 20% trisomic DNA) in the dilution experiments yielded mean probe ratios of 1.17 (95% CI: 1.01-1.32), 1.13 (95% CI: 1.08-1.19) and 1.11 (95% CI: 1.10-1.12) for the chromosomes 21, 18 and 13, respectively. These chromosome ratios are significantly increased since the 95% CI in a 80-20% mixture does not include 1.0 and are ≥1.1. In Figure 5 the mean probe ratios of 1.43 (95% CI: 1.40-1.46) for chromosome 21, 1.39 (95% CI: 1.33-1.46) for chromosome 18, and 1.35 (95% CI: 1.31-1.38) for chromosome 13 in the three dilution experiments (80% trisomic DNA - 20% normal DNA) are marked. These 20-80% mixtures do not include probe ratios of 1.5, whereas the mean probe ratios of the target chromosomes are nearly 1.4. An overview of all ratios of the chromosomes 21, 18 and 13 and the theoretical profiles in the dilution experiments are shown in Table 4. Chromosome ratios and 95% CI of the 80-20% and the 20-80% mixtures are included in Table 1 next to the results of normal samples or samples with a non-mosaic aneuploidy.

<table>
<thead>
<tr>
<th>Trisomy (%)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
<th>90</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical profile</td>
<td>1.00</td>
<td>1.05</td>
<td>1.10</td>
<td>1.15</td>
<td>1.20</td>
<td>1.25</td>
<td>1.30</td>
<td>1.35</td>
<td>1.40</td>
<td>1.45</td>
<td>1.50</td>
</tr>
<tr>
<td>trisomy 21</td>
<td>1.01</td>
<td>1.06</td>
<td>1.17</td>
<td>1.21</td>
<td>1.26</td>
<td>1.31</td>
<td>n.a.</td>
<td>1.34</td>
<td>1.43</td>
<td>1.42</td>
<td>1.46</td>
</tr>
<tr>
<td>trisomy 18</td>
<td>0.98</td>
<td>1.02</td>
<td>1.13</td>
<td>1.12</td>
<td>1.19</td>
<td>1.23</td>
<td>1.22</td>
<td>1.34</td>
<td>1.39</td>
<td>1.42</td>
<td>1.45</td>
</tr>
<tr>
<td>trisomy 13</td>
<td>1.00</td>
<td>1.04</td>
<td>1.11</td>
<td>1.13</td>
<td>1.23</td>
<td>1.24</td>
<td>1.31</td>
<td>1.30</td>
<td>1.35</td>
<td>1.43</td>
<td>1.41</td>
</tr>
</tbody>
</table>

n.a. not available

Assessment of the optimal probe selection

In order to optimize the probe set, average and standard deviations for the chromosome ratios of the autosomes were re-calculated after a theoretical elimination of the probes with the highest SD, i.e., 21m8, 18m7 and 13m7. This optimization step resulted in a minimal decrease of the SD of the ratios for chromosomes 21, 18 and 13 from 0.04, 0.04 and 0.05 to 0.04, 0.03 and 0.04, respectively (Table 1). The pooled SD, calculated as the square root of the mean variance for these autosomal chromosomes, was 0.044. Theoretical exclusion of the probes with the highest SD resulted in a slight decrease of...
with Gerdes et al. (Gerdes et al., 2005a) a more detailed probe analysis revealed that probe 13m7 (Table 2) is the probe with the highest mean SD in this kit. Exclusion of the least reliable autosomal probes from the mix, however, resulted in only a slight decrease in the pooled probe SD of 0.007 and, therefore, does not contribute significantly to the reliability of the test.

Since our data analysis approach is based on statistics of mean probe ratios in conjunction with a 95% CI, it may provide a better diagnostic power for the detection of mosaic anomalies than the employment of fixed thresholds for normal and abnormal samples (Li et al., 2005). The criteria we used for the presence of a potential mosaicism were i) the expected mean probe ratios of both duplicate measurements were outside the 95% CI and ii) at least one of these mean probe ratios differed 10% from the expected value. In our experiments, the detection level of mosaicsms for trisomies 21, 18 or 13 with the MLPA test appeared to be approximately 20% and were therefore in the same range as traditional karyotyping of cultured cells. Within this context, it is relevant to mention that uncultured amniotic fluid cell populations contain different cell types and, thus may provide a better indication of the overall foetal status than cultured cell populations (Robinson et al., 2002), which are clonal outgrowths of sub-populations and thus have an effect upon the genetic make-up of a cell population (Donaghue et al., 2005). Therefore molecular tests such as MLPA, that make use of DNA from uncultured cells, may provide stronger associations with pregnancy outcome than traditional karyotyping. Although the main objective of the MLPA test kit P095 is to detect prevalent aneuploidies, it may also uncover unexpected partial cryptic chromosomal imbalances of the target chromosomes because the methodology involves relative quantification of single probes by examination of the electropherogram. This notion raises the question what criteria should be used for determining the significance of single losses or gains in probe signals. Van Opstal et al. (Van Opstal et al., 2009) encountered 0.08% (3 of 4,000) cases with a structural chromosome defect initially found by an amplification or a deletion of one or two of the MLPA probes. An example of the detection of a structural chromosomal imbalance by MLPA is depicted in Figure 6. The electropherogram shows three decreased peaks for chromosome 18. The ideogram of chromosome 18 illustrates the location of the MLPA probes on the distal q arm of chromosome 18 and is, therefore, indicative for a distal deletion of chromosome 18q. Follow-up cytogenetic analysis yielded a 46,XX,del(18)(q21.2) karyotype. From a clinical point of view, it may be advantageous to detect deletions or duplications of one or two probes for the identification of structural chromosomal defects. On the other hand, however, these deletions or duplications may equally well represent harmless sub-microscopic copy number variations. Since the MLPA performance of kit P095 has not yet been validated at this level, we agree with van Opstal et al. (Van Opstal et al., 2009) that if MLPA is used as a stand-alone test (for

Overall, we conclude that these three dilution experiments yield abnormal results when either the abnormal or the normal cell population contributes at least 20% to the sample.

**Discussion**

We tested a cohort of 199 euploid and 50 aneuploid amniotic fluid samples for the performance of the MLPA kit P095 and observed an absolute specificity and sensitivity of 100% with a 95% CI of 98.2-100 and 92.9-100, respectively. This means that this MLPA kit is designed in such a way that a good probe performance is obtained. In agreement
pregnant women with an increased risk for Down syndrome), the interpretation of single
probe quantification should be neglected. Furthermore, we propose that only in cases
where at least two flanking probes are amplified or deleted in both duplicate
measurements, further tests are warranted to confirm or disprove the suspected
structural chromosomal anomaly.

In addition, it should be noted that the MLPA test kit P09S does not allow the detection
of maternal cell contamination (MCC) in female prenatal samples and/or the detection of
female triploidies and is not able to distinguish a complete male triploidy from a male
sample with MCC. Therefore, heavily blood-stained samples are considered unsuitable
for MLPA testing and amniotic fluid from pregnancies suspected for a triploidy should
be subjected to other detection methods. Prenatal microarrays provide the potential to
scan for genomic abnormalities at a high resolution (Rickman et al., 2006) and may be
used in pregnancies with a foetal ultrasound abnormality and a normal karyotype.
Molecular genetic tests like FISH and QF-PCR are already available to clinical cytogentic laboratories for the rapid detection of the most common aneuploidies in amniotic fluid
cells. In several laboratories within Europe (Stockholm, Sweden (Bui, 2007) and London,
UK (Mann et al., 2008)). QF-PCR is already used as a targeted stand-alone test for
pregnancies with an increased risk for Down syndrome. Similarly, the MLPA test appeared
to be suitable for the rapid and efficient detection of the most common chromosomal
aneuploidies in routine prenatal clinical practice (Kooper et al., 2008). Within a nationwide
study M.A.K.E. (MLPA And Karyotyping, an Evaluation) the patient’s preference for either
rapid aneuploidy testing or traditional karyotyping are evaluated (Boormans et al., 2008).

Taken together, we conclude that the MLPA probe kit P09S shows a good probe
performance and that exclusion of the least reliable probes only slightly affects the
sensitivity of the assay.

Acknowledgements

The authors wish to thank the colleagues of the Network Prenatal Diagnostics Nijmegen
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Enschede (Medisch Spectrum Hospital Twente), ’s-Hertogenbosch (Jeroen Bosch
Hospital) and Nijmegen (UMC St. Radboud) for granting access to the patient material
and relevant follow-up data. We are grateful to Dr. Schouten from MRC-Holland, supplier
of the MLPA kits.

![Figure 6. Electropherograms illustrating a normal female reference sample (A) and a patient sample (B). The eight series of four peaks represent MLPA probes for chromosomes 21, 18, 13 and X, respectively. Three peaks in sample B, representing 18m2, 18m4 and 18m6, are decreased as compared to the peaks of the normal reference sample and, therefore, indicative for a partial imbalance of chromosome 18. The corresponding probe ratios showed a mean of 1.09 (95% CI: 0.82-1.35) for 21, 0.84 (95% CI: 0.47-1.21) for 18, 1.14 (95% CI: 0.83-1.45) for 13 and 1.09 (95% CI: 0.93-1.25) for the X chromosome, respectively. The ideogram of chromosome 18 (C) shows the locations of the three decreased probes (18m2, 18m6 and 18m4) on the distal region of the long arm of chromosome 18. Follow-up karyotyping revealed a 46,XX,del(18)(q21.2) karyotype (D).]
**Data supplement II**  Example of peak normalization procedure.

Intra-sample normalization with flanking probes (on either side or on one side of the probe) was performed as follows: normalized value of probe 21m1=observed peak area 21m1 divided by sum of observed peak areas of 18m1 and 13m1; normalized value of probe 18m1=observed peak area 18m1 divided by sum of observed peak areas of 21m1 and 13m1; normalized value of probe 13m1=observed peak area 13m1 divided by sum of observed peak areas of 18m1 and 21m1; normalized value of probe X 1=observed peak area X 1 divided by sum of observed peak areas of 21m1, 18m1 and 13m1; normalized value of probe Y 1=observed peak area Y 1 divided by sum of observed peak area of 21m2, 18m2 and 13m2 (for males). For females the normalized value of the Y probes was set to 0. The same procedure was followed for the normalized value of probe 21m2=observed peak area 21m2 divided by sum of observed peak areas of 18m2 and 13m2, and so on. The next step was to further normalize these probe values with respect to reference values derived from the tray-specific references (one male and one female). The inter-sample normalization with tray-specific references was performed as follows: the reference value for a probe on chromosome 21, 18 or 13 was the mean of the corresponding intra-sample normalized probe values in both tray-specific references; the reference value for a probe on the X chromosome was 2/3 times the sum of the corresponding intra-sample normalized probe values in both tray-specific references; the reference value for a probe on the Y chromosome was 2 times the corresponding intra-sample normalized probe value of the male reference. Finally, an inter-sample normalized probe value was defined to be the ratio of the intra-sample normalized value and the reference value derived from the tray-specific references as described above. The mean and standard deviation (SD) of each target chromosome was determined, followed by the 95% confidence interval (CI) defined as mean ± t_{0.05} * (SD/√n), where t_{0.05} is the percentage point of the t-distribution with (n-1) degrees of freedom, which gives a two-tailed probability of 0.05 and n is the number of targets on the chromosome (8 for chromosomes 13, 18, 21 and X and 4 for chromosome Y).

<table>
<thead>
<tr>
<th>marker ad</th>
<th>marker chr</th>
<th>locus Y</th>
<th>Yn</th>
<th>ref</th>
<th>refm</th>
<th>refm</th>
<th>Ynn</th>
</tr>
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<td>21m1</td>
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<td>18m1</td>
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<td>Ym2</td>
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<td>21m5</td>
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<td>16</td>
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<td>18250</td>
<td>0.42072</td>
<td>0.39031</td>
<td>0.40086</td>
<td>0.39559</td>
</tr>
</tbody>
</table>

This example gives for the first two series of markers (m1 and m2) and for all eight chromosome 21 markers of one particular sample, the observed peak areas (Y), the intra-sample normalized values (Yn), the female and male controls (reff respectively refm), the reference values (ref) and finally the inter-sample normalized values (Ynn).
Yn for the first marker of chromosome Y was calculated as the observed peak area divided by the sum of the observed values of Y on the second markers of chromosome 21, 18 and 13: 
\[ \frac{19620}{32938+30488+30285} = 0.20937. \]

The observed peak areas for the female and male controls are not displayed, only the intra-normalized values obtained in the same manner as described above: ref and refm.

The reference value (ref) for a probe on chromosome 21, 18 or 13 is the mean of the corresponding intra-sample normalized probe values in both references: 
\[ \text{ref} = \frac{\text{reff} + \text{refm}}{2}. \]

The reference value for a probe on the X chromosome is \( \frac{2}{3} \) times the sum of the corresponding intra-sample normalized values within the controls: 
\[ \text{ref} = \frac{2}{3} (\text{reff} + \text{refm}). \]

The reference value for a probe on the Y chromosome is 2 times the corresponding intra-sample normalized values of the male control: 
\[ \text{ref} = 2 \times \text{refm}. \]

Finally, the inter-sample normalized values Ynn are defined to be the quotients Yn / ref.

For example, the intra-sample normalized peak area for the first chromosome 21 repeat: 
\[ 0.98523 = \frac{0.47657}{0.48373}. \]

In order to construct a 95% confidence interval, for example for the 'true' value of chromosome 21 in this example, the mean and the standard deviation of the 8 Ynn values are needed:

Mean (0.98523, 1.11778, 1.14324, 0.97437, 1.00403, 0.98914, 0.94777, 1.06354) = 1.02814.

The corresponding standard deviation is SD = 0.07155. Applying the formula for the 95% CI yielding a 95% CI: 0.97 – 1.09.
Detection of chromosome aneuploidies in chorionic villus samples by MLPA

Kooper AJA, Faas BHW, Feuth T, Creemers JWT, Zondervan HH, Boekkooi PF, Quartero RWP, Rijnders RJP, van der Burgt I, Geurts van Kessel A, Smits APT

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Abstract

The objective of this study was to examine the suitability of multiplex ligation-dependent probe amplification (MLPA) in chorionic villus samples as a replacement for traditional karyotyping for the detection of (an)euploidies of chromosomes 21, 18, 13, X, and Y. Chorionic villus samples were diagnosed by traditional karyotyping using short-term cultures (STC) and long-term cultures (LTC), and by MLPA using kit P095. DNA was extracted after digestion of whole villi with proteinase K and/or trypsin and collagenase. Different cell-dissociation procedures were tested to obtain MLPA results representative of the cytotrophoblast layer and the mesenchymal core. Over 95% of the MLPA results were in concordance with the traditional karyotyping of STC and LTC. Traditional karyotyping revealed seven mosaics. After digestion of whole villi with proteinase K, only abnormal cell lines confined to the STC gave rise to abnormal MLPA results. In one sample, the complete discrepancy between STC and LTC was resolved after enzymatic dissociation of cells from the cytotrophoblast layer and the mesenchymal core. MLPA in chorionic villus samples was found to be a reliable test for the detection of (an)euploidies of chromosomes 21, 18, 13, X, and Y. Whole villi digestion with proteinase K resulted in the over-representation of cytotrophoblasts in the DNA pool. To obtain MLPA results representative for STC and LTC, enzymatic dissociation of cells from the cytotrophoblast layer and mesenchymal core is required.

Introduction

Chorionic villus sampling has been widely accepted as a technique for first trimester prenatal diagnosis and is performed from 11 weeks of gestation. Until recently, prenatal diagnosis of chorionic villus samples (CVS) was accomplished through tissue culture and subsequent cytogenetic analysis. This procedure is labour-intensive and time-consuming. Therefore, more rapid and comprehensive methods for the prenatal diagnosis of CVS are currently being developed and implemented. In a number of prenatal centres in Europe, quantitative fluorescent PCR (QF-PCR) analysis is already being offered to women undergoing invasive testing by chorionic villus sampling (Cirigliano et al., 2004). In parallel, we have implemented multiplex ligation-dependent probe amplification (MLPA) for the rapid detection of (an)euploidies of chromosomes 21, 18, 13, X, and Y in amniotic fluid cells (Slater et al., 2003; Hochstenbach et al., 2005; Kooper et al., 2008). A general disadvantage of the use of CVS in comparison with amnion fluid is the extra-embryonic nature of this tissue. Although foetus and placenta originate from the same zygote, a discrepancy between the chromosomal constitution of cells in the placenta and cells in the foetus, known as chromosomal mosaicism, can occur. Such mosaics are well documented in the literature and are detected in 1% to 2% of the CVS (Kalousek and Vekemans, 1996; Stetten et al., 2004). Abnormal mosaic cells can be found in both foetal and placental tissues, or may be confined to either the placenta (confined placental mosaicism, CPM) or the foetus (Simoni and Fraccaro, 1992). Karyotypes of CVS represent cells from chorionic ectoderm (cytotrophoblasts) in short-term cultures (STC) and chorionic mesoderm (mesenchymal core) in long-term cultures (LTC). In molecular testing of CVS it is, therefore, of obvious importance to establish that both cell lineages are adequately represented by the pool of cells from which the DNA is extracted (Mann et al., 2007). In this study we investigated the suitability of the MLPA test for the detection of (an)euploidies in CVS and assayed to what extent this test compares to traditional karyotyping (TK) of STC, LTC, or both.

Materials and methods

Clinical samples

CVS with a weight of >30 mg (N=152), were collected at the outpatient clinics located in Nijmegen, Arnhem, Tilburg, ‘s-Hertogenbosch and Enschede (Network Prenatal Diagnostics Nijmegen, the Netherlands) from pregnant women at 11 to 21 weeks of gestation. From these 152 CVS, 125 were consecutively collected between May 2006 and June 2007. Additionally, twenty CVS with known aneuploidies for one of the target...
chromosomes and seven CVS diagnosed by TK as mosaic were added. The referral reasons of the pregnant women ranged from low-risk to high-risk. The CVS were washed in PBS and the villi were separated from maternal decidua and blood clots under an inverted microscope.

Karyotyping
Approximately 20 to 30 mg of the villi was used for conventional karyotyping according to standard STC and LTC procedures. Briefly, 10 to 15 mg of the villi was used for STC and, subsequently, incubated for 30 minutes in colcemid, followed by a short hypotonic treatment after which the cells were fixed in methanol/acetic acid (3:1) and rehydrated. Finally, the trophoblast (interphase and metaphase) cells were released from the villus core using 60% acetic acid and spread on microscopic slides. The remaining 10 to 15 mg of the villi were used for LTC, after incubation for one hour in trypsin-EDTA and a 40-minute incubation in collagenase. Metaphases were harvested in situ using standard procedures on Labtek II chamber slides. Cytogenetic investigation of STC and LTC was routinely performed and 4 and 8 metaphases were analyzed, respectively, to exclude discrepancies between STC and LTC (Mellink et al., 2003). Cytogenetic examination of the LTC was expanded to 29 metaphases when an abnormal karyotype was detected in STC or LTC cells. CVS karyotypes with a tetrasomy or triploidy were excluded from this study.

Definition of mosaic levels
CVS encompass cells from both the trophoblasts and the mesenchymal core. Discordant findings between STC and LTC and/or foetal tissues have been either referred to as pseudomosaics or true mosaics (Gardner and Sutherland, 1996). Here, three levels to define mosaicism were used: level I, detection of a single abnormal cell (single cell pseudomosaicism), level II, the same abnormality was detected in two or more cells in the same culture vessel (multiple cell pseudomosaicism) or level III, the same abnormality was observed in two or more independent culture vessels (true mosaicism) (Gardner and Sutherland, 1996). Discrepancies between karyotypes of villus and foetal tissues may occur as a result of a cpm. Three types of CPM can be discerned and categorized by the placental cell lineage exhibiting the abnormal cell line, i.e., confined to the cytotrophoblast (type I), the mesenchymal core (type II), or both (type III)(Kalousek and Vekemans, 1996).

Fluorescence in situ hybridization analysis
Interphase-fluorescence in situ hybridization (I-FISH) analyses were performed on nuclei (N=100) of STC villi cells in samples exhibiting a mosaicism of any of the target chromosomes using an AneuVysion prenatal detection kit (Vysis Inc, Downers Grove, Illinois) for an accurate establishment of the distribution of normal and abnormal cells.

DNA extraction following proteinase K treatment of whole villi
This method was used for all CVS. DNA from at least two chorionic villi was extracted using a QIAamp DNA Mini Kit (Qiagen) following the protocol “Isolation of DNA from soft tissues using the TissueLyser and QIAamp DNA Mini Kit” (Qiagen, Westburg bv, the Netherlands). In this procedure, incubation at 56°C with proteinase K results in lysis of the villi before DNA extraction. Proteinase K has a specific activity and degrades tissue to facilitate the purification of the DNA. Finally, DNA was eluted in 50 µl AE buffer (10 mmol/l Tris-HCl, 0.5 mmol/l EDTA, pH 8.0). The DNA was quantified using a Nanodrop spectrophotometer (NanoDrop Technologies Wilmington, Delaware) and varied from 2.5 to 650 ng/µl elution buffer.

DNA extraction following enzymatic dissociation of villi
Two methods were designed to obtain cell populations from the cytotrophoblast layer and the mesenchymal core separately. In the first method, a modification of the method described by Mann (Mann et al., 2007), ie, digestion of cleaned villi with collagenase (800 units/ml; 37°C, 30 minutes), was followed by trypsin digestion (0.5% trypsin/EDTA, 37°C, 30 minutes). After collagenase digestion, the suspension was separated from the remaining villi and transferred to a tube containing 4 ml PBS and 10% foetal calf serum to stop the digestion. After centrifugation (1200 rpm, 5 minutes) the supernatant was removed and the cell pellet was re-suspended in 300 µl PBS (fraction C). After digestion of the remaining villi with trypsin, 4 ml PBS + 10% foetal calf serum were added to stop the reaction. After centrifugation (1200 rpm, 5 minutes) the supernatant was removed and the cell pellet was re-suspended in 300 µl PBS (fraction M). Finally, 100 µl of fraction C and 100 µl of fraction M were mixed (1:1) (fraction T) and used for DNA isolation as described below.

In the second method, digestion of cleaned villi was first performed with trypsin (0.5% trypsin/EDTA, 37°C, 1 hour) followed by collagenase digestion (800 units/ml; 37°C, 40 minutes). After both digestion steps, cell fractions C, M, and T were obtained as in the first method. DNA extractions were performed by incubation of the cell population with proteinase K at 56°C. DNA was purified using a QIAamp DNA Mini Kit (Qiagen) following the protocol “Blood and body fluid spin protocol.” Finally, DNA was eluted in 50 µl AE buffer (10 mmol/l Tris-HCl, 0.5 mmol/l EDTA, pH 8.0). Both methods were performed on CVS case 7 with discrepant results in STC and LTC, i.e., 46,XY and 47,XY,+18, respectively.

MLPA test
The MLPA test was performed with SALSA MLPA kit P095 for aneuploidy detection as described by Schouten (MRC Holland, Amsterdam, the Netherlands) (Schouten et al., 2002). The MLPA analyses were performed blinded to the cytogenetic analyses until

Detection of chromosome aneuploidies in chorionic villus samples by MLPA
In the first method, the MLPA test was performed with SALSA MLPA kit P095 for aneuploidy detection as described by Schouten (MRC Holland, Amsterdam, the Netherlands) (Schouten et al., 2002). The MLPA analyses were performed blinded to the cytogenetic analyses until
A summary of the test results on all 152 samples is displayed in Figure 1, showing a box plot with the mean ratios for the chromosome 21, 18, 13, X, and Y probes in euploid (N=124: normal male N=58 and normal female N=66), aneuploid (N=21), and (pseudo) mosaic samples (N=7). In all normal samples, the mean ratios for the autosomes were

Figure 1.
Quantitative MLPA analysis with mean probe ratios of the 21, 18, 13, X, and Y targets with 50% of the samples within the box. The outliers are cases with values between 1.5 and 3 box-lengths from the boundaries of the box. Case 3 shows an increased mean probe ratio for chromosome 21 of 1.24 (95% CI: 1.02-1.46). Inspection of individual probe ratios of this chromosome showed an increase for all individual probes, indicative for a mosaic of trisomy 21. Case 7 is illustrated as an extreme (*) in the box plot with an increased mean probe ratio for chromosome 18 of 1.18 (95% CI, 1.08–1.28), i.e., this ratio is increased for the expected value of 1.0 for a disomy 18 and decreased for the expected ratio of 1.5 for a trisomy and, therefore, indicative for a mosaic trisomy 18. Case 1 is illustrated as an extreme (*) in the box plot with a decreased mean probe ratio for the Y chromosome of 0.17 (95% CI: 0.14–0.21). Case 2 is illustrated as an outlier ‘o’. The mean probe ratio for X is 0.80 (95% CI: 0.70–0.90), i.e., this ratio is increased for the expected value of 0.5 for a disomy and decreased for the expected ratio of 1.5 for a trisomy and, therefore, indicative for a mosaic 45,X/46,XX. The other outliers in the box plots result in increased or decreased mean probe ratios, all related to broad confidence intervals. The expected values of 0.5, 1.0, or 1.5 are within the confidence intervals in all outliers.

The Levene’s test was used to determine equality of variances of the pooled standard deviations of the mean probe ratios for the target chromosomes 13, 18, 21, and X between CVS and amniotic fluid samples.

Results
Proteinase K treatment of whole villi
In all 152 CVS, the MLPA test was performed successfully. The results were compared with cytogenetic analyses of both STC villi and LTC villi. There was a correct assignment of sex in all 152 samples. No evidence for the presence of maternal contamination was noted in male samples, i.e., the expected normalization effect of a decreased mean probe ratio for the Y chromosome combined with an increased mean probe ratio for the X chromosome was observed in none of the male samples.

completion. Data analyses were performed on the transfer of electropherogram-based GeneMapper results to a modified spreadsheet for normalization and ratio computation of the peak areas (Kooper et al, submitted). First an intra-sample normalization of each probe peak area was performed, followed by an inter-sample normalization with tray-specific references. Normalization is essential because variations in experimental conditions may lead to quantitative differences.

As a consequence of our normalization procedure, mean probe ratios skew in case of a trisomy, since the probe ratios of the targets with two copies scale down to 80% when a trisomy for one of the targets is present. This decrease in mean probe ratios is not corrected in the data analysis. The mean and SD of each target chromosome were determined, followed by a 95% confidence interval (CI). In cases of disomy (two copies of each target chromosome), these calculations will result in a theoretical (expected) value of 1.0, representing two copies of the target sequence in the sample. One or three copies of the target sequence in a sample will result in theoretical values of 0.5 or 1.5, respectively. A result is considered abnormal when the theoretical value (= expected value in a normal case) is not included in the confidence interval and the mean probe ratio is more or less than 10% of the expected value. Follow-up of these samples is performed by karyotyping and/or amniocentesis.

MLPA does not detect female triploidies. As a consequence of the normalization procedure, relative peak areas do not differ in intra-sample normalizations as the copy numbers of all of the targets is the same. A male triploidy will yield normal mean probe ratios for 21, 18, and 13, an increased mean probe ratio for X and a decreased mean probe ratio for Y. In addition, there is limited potential to discriminate maternal contamination in a normal male sample from 69,XXY and, therefore, careful CVS cleaning before sample preparation is of critical importance.

The Levene’s test was used to determine equality of variances of the pooled standard deviations of the mean probe ratios for the target chromosomes 13, 18, 21, and X between CVS and amniotic fluid samples.
The mean probe ratios for samples with a trisomy 21, 18, or 13 were all significantly increased (mean ratios of >1.0 with 95% CI not including 1.0) and close to the expected value of 1.5. In all normal male and female samples the mean probe ratios for X and Y were close to the expected values: 1.0 for X in female samples and 0.5 for X and Y in male samples, and for X in samples with a monosomy X. The variation in mean probe ratios was slightly higher for Y, which may be due to the limited number of targets included in the P095 kit for this chromosome.

Normal karyotypes

In 124 of the CVS the normal results of MLPA and TK were in concordance for all five target chromosomes (18 and 66 male and female samples, respectively).

Aneuploidies in both STC and LTC

In total, 21 aneuploidies for one of the target chromosomes were detected; one with trisomy 13, five with trisomy 18, ten with trisomy 21 and five with monosomy X. There was complete concordancy between the MLPA and TK results. In all 145 non-mosaic samples, the unexplained SD of the mean probe ratio for the target chromosomes 13, 18, 21 and X turned out to be 0.041. The Y chromosome was excluded in this calculation because of the presence of only four probes (see above); this residual variation was significantly lower (P<0.001, using F test to compare two variances) than the corresponding value of 0.068 determined in a set of amniotic fluid samples (Kooper et al, submitted).

(Pseudo)mosaicisms

Seven samples displayed discordancies between TK (STC and LTC) and MLPA. The results of TK (STC and LTC) and MLPA for these samples are summarized in Table 1. The MLPA results of these cases were compared with the TK results (golden standard) and are summarized in Table 2.

Case 1: STC villi showed a mosaic karyotype 45,X[2]/46,XY[1] and a normal karyotype in LTC. I-FISH of STC nuclei showed a normal probe ratio for X. A normal mean probe ratio for X indicates that the decrease of Y is not related to the presence of maternal contamination in this male sample. Case 1 is illustrated as an extreme (*) in the box plot in Figure 1 (with a mean probe ratio of more than three box-lengths from the boundaries of the box and indicative for a mosaic 45,X/46,XY).

Case 2: STC villi showed a mosaic karyotype 45,X[3]/47,XXX[1] and LTC villi showed a non-mosaic X chromosome. I-FISH of STC nuclei resulted in a mosaic monosomy X.

Table 1. Results of traditional karyotyping of STC, LTC, and I-FISH for one of the target chromosomes in seven CVS, including mosaic and pseudomosaic cases

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Referral reason</th>
<th>Gestation weeks</th>
<th>STC karyotype</th>
<th>STC I-FISH (% in 100 nuclei)</th>
<th>LTC karyotype</th>
<th>Diagnosis amnio-centesis</th>
<th>Mosaicism level (STC / LTC)</th>
<th>CPM type</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>Maternal age &gt;35 years</td>
<td>46,XX</td>
<td>III / I</td>
<td>III</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MA, maternal age >35 years; CPM II, confined placental mosaicism type II; CPM III, confined placental mosaicism type III; US-abn, ultrasound abnormality; RS, at risk for Down after first trimester screening; n.a., not available (not performed); STC, short-term culture; LTC, long-term culture; DNA, primary reason for invasive testing was the presence of a familial DNA mutation.
Table 2. Overview of the chromosome mean probe ratios in seven mosaic cases after preparation of whole villi with proteinase K treatment.

<table>
<thead>
<tr>
<th>Case</th>
<th>STC Karyotype</th>
<th>LTC Karyotype</th>
<th>Mean probe ratios target chromosomes (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>#18: 0.97 (0.94-1.01)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#21: 1.04 (0.95-1.13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#X: 0.52 (0.48-0.56)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#Y: 0.17 (0.14-0.21)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#18: 0.93 (0.82-1.04)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#21: 1.05 (0.91-1.19)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#X: 0.80 (0.70-0.90)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#Y: 0.17 (0.14-0.21)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#18: 0.93 (0.75-1.10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#18: 1.24 (1.02-1.46)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#X: 0.55 (0.45-0.65)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#Y: 0.43 (0.34-0.52)</td>
</tr>
<tr>
<td>4.</td>
<td>46,XX[4]</td>
<td>45,X[3]/46,XX[27]</td>
<td>#13: 1.00 (0.84-1.15)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#18: 0.97 (0.83-1.10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#21: 1.07 (0.93-1.21)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#X: 0.89 (0.80-0.99)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#Y: 0.17 (0.14-0.21)</td>
</tr>
<tr>
<td>5.</td>
<td>46,XY[4]</td>
<td>47,XY,+21[2]/46,XY[27]</td>
<td>#13: 1.02 (0.91-1.13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#18: 0.97 (0.89-1.05)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#21: 1.02 (0.94-1.11)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#X: 0.49 (0.45-0.52)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#Y: 0.48 (0.46-0.50)</td>
</tr>
<tr>
<td>6.</td>
<td>45,X[3]/46,XX[1]</td>
<td>45,X[1]/46,XX[28]</td>
<td>#13: 1.05 (0.96-1.13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#18: 0.96 (0.90-1.03)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#21: 1.01 (0.92-1.10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#X: 0.90 (0.83-0.96)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#Y: 0.17 (0.14-0.21)</td>
</tr>
<tr>
<td>7.</td>
<td>46,XY[12]</td>
<td>47,XY,+18[29]</td>
<td>#13: 0.91 (0.83-0.99)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#18: 1.18 (1.08-1.28)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#21: 0.92 (0.85-0.99)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#X: 0.51 (0.47-0.56)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#Y: 0.45 (0.35-0.54)</td>
</tr>
</tbody>
</table>

disomy X/trisomy X in 68%, 3%, and 9% of the cells, respectively. The MLPA results showed a decreased mean probe ratio for X of 0.80 (95% CI: 0.70-0.90). The ratio is increased for the expected value of 0.5 for a monosomy X and decreased for the expected ratio of 1.0 for a disomy and, therefore, indicative for a mosaic 45,X/46,XX. Case 2 is illustrated as an outlier ‘o’ in the box plot in Figure 1 with a mean probe ratio between 1.5 and 3 box-lengths from the boundaries of the box.

Case 3: STC villi showed a mosaic karyotype 47,XY,+21[2]/46,XY[5] and LTC villi showed a non-mosaic trisomy 21 karyotype. I-FISH of STC nuclei resulted in a mosaic trisomy 21 in 76% and 24% of the cells, respectively. The mean probe ratio for chromosome 21 was increased to 1.24 (95% CI: 1.02-1.46). Inspection of individual probe ratios of this chromosome showed an increase for all individual probes indicative for a mosaic trisomy 21. There was discordance between the MLPA results and the results of TK of the LTC villi: the expected value of 1.5 was not within the 95% CI.

Case 4: LTC villi showed a mosaic karyotype 45,X[3]/46,XY[27] and STC villi showed a normal female karyotype. I-FISH of STC nuclei showed disomy X in 95% of the nuclei. The MLPA resulted in normal mean probe ratios of all target chromosomes (mean probe ratio × 0.89, 95% CI: 0.80-0.99, this value is in the gray zone and interpreted as normal). Follow-up cytogenetic analysis of amniotic fluid cells was normal, indicating that the abnormal cell line was probably confined to the placenta and the LTC (CPM type II).

Case 5: LTC villi showed a mosaic karyotype of a low-grade mosaicism 47,XY,+13[2]/46,XY[27] and STC villi showed a normal male karyotype. I-FISH of STC nuclei resulted in a test failure. The MLPA resulted in normal mean probe ratios of all target chromosomes. Follow-up cytogenetic analysis of amniotic fluid cells was normal, indicating that the abnormal cell line was confined to the placenta and the LTC (CPM type II).

Case 6: both STC and LTC villi showed a mosaic karyotype 45,X/46,XX. I-FISH of STC nuclei confirmed a mosaic monosomy X/disomy X in 26% and 74% of the cells, respectively. The MLPA resulted in a slight decrease of all probe ratios for the X chromosome of 0.90 (95% CI: 0.83-0.96). Follow-up cytogenetic analysis of amniotic fluid cells was normal, indicating that the abnormal cell line was confined to the placenta, both the STC and LTC (CPM type III).

Case 7 showed a complete discrepancy between STC and LTC: the STC showed a 46,XY and the LTC a 47,XY,+18 karyotype. The MLPA resulted in a slight increase of the mean probe ratio for chromosome 18 of 1.18 (95% CI: 1.08-1.28). The MLPA results of DNA from...
whole villi were compared to the results of the enzymatically dissociated cell pools (Table 3). The mean probe ratios for chromosome 18 in fraction C of both methods showed a 95% CI including the value of 1.0, indicating that the cell pool isolated in fraction C represented a pool of cytotrophoblastic cells. The mean probe ratios for chromosome 18 in fraction M of both methods showed a 95% CI including the value of 1.5, indicating that the cell pool isolated in fraction M represented cells from the mesenchymal core. Fraction T, a mixture of fraction C and M (1:1), yielded results indicative for a mosaic trisomy 18, using both methods.

We conclude that these modified protocols for CVS preparation have resulted in an accurate representation of the cytotrophoblast layer and the mesenchymal core, which is in full concordance with the results obtained by karyotyping of STC and LTC.

Abnormalities undetectable by MLPA

In six samples abnormalities were revealed by TK that remained undetected by MLPA. These included three samples with a familial chromosomal rearrangement, two samples with a mosaicism with an extra marker chromosome with no clinical relevance (reason for referral nuchal translucency and advanced maternal age) and one sample with a mosaic trisomy 10 (reason for referral advanced maternal age). Follow-up by ultrasound in this latter pregnancy revealed an oligohydramnion.

Discussion

The suitability of the MLPA test on CVS as a replacement for TK in the detection of prevalent aneuploidies of chromosomes 21, 18, X, and Y was assayed in 152 samples. By doing so, we found a complete concordance between the MLPA and TK results for the diagnosis of all euploidies and non-mosaic aneuploidies, respectively, yielding an absolute specificity and sensitivity of the MLPA test of 100%. However, a reliable prenatal diagnosis based on CVS may be complicated by several factors related to the cellular composition of CVS tissue, which at the cytogenetic level may be expressed as (pseudo) mosaicsisms and/or maternal contaminations. Between 1 and 2 percentage of the CVS karyotypes is mosaic, and in more than 80% of the cases the mosaicism is confined to the placenta (Hahnemann and Vejerslev, 1997; Grati et al., 2006). Mosaicism for trisomies 21, 18 and 13 have been reported to occur in 0.26% of CVS (Smith et al., 1999). It is important to bear in mind that mosaicsisms for one of these target chromosomes may also extend into the foetal cell lineages and, as such, they must be considered as risk factors for foetal abnormalities. Therefore, it is relevant to establish the detection level of
The potential presence of maternal cell contamination (MCC) poses a serious risk for prenatal misdiagnosis (Schrijver et al., 2007). We have taken into account that MCC in an over-representation of DNA from cytotrophoblastic cells and, as such, closely resembles STC cell DNA. This notion is in line with previous studies demonstrating the cellular complexity of CVS tissues and the relevance of CVS preparation methods for molecular testing to minimize the risk of false-positive or -negative results (Allen et al., 2006; Waters et al., 2007). As such, MLPA analysis of CVS on DNA isolation of whole villi digested with proteinase K as presented here, is only appropriate as a replacement for TK of STC villi. In case 7, in which the abnormal cells were confined to the LTC, the MLPA result for chromosome 18 was indicative for a mosaic trisomy 18, with a 95% CI of 1.08-1.28. Neither 1.0 or 1.5 was included in the 95% CI. This indicated that only a small proportion of the cells digested with proteinase K was of mesenchymal origin. In the other two cases, with an abnormality confined to the LTC (case 4 and 5), this phenomenon was not observed probably because the abnormalities in the LTC were present as low-grade mosaicism and, therefore, were not observed in the MLPA results.

In case 7, we tested two different methods for enzymatic dissociation of the villi to obtain cells from both the cytotrophoblast and the mesenchyme cell lineages. From our data, we conclude that these two methods resulted in proper representations of the cytotrophoblast layer and the mesenchymal core, and that the MLPA results obtained are in concordance with the results obtained by karyotyping of STC and LTC, respectively. Enzymatic dissociation with trypsin/EDTA followed by collagenase showed that the total cell population (fraction T) consisted predominantly of cells from the mesenchymal core. This notion was confirmed by the MLPA electropherogram from fraction C. Digestion with collagenase followed by trypsin/EDTA gave the best results, representing a total cell population (fraction T) with a distribution of 60% cytotrophoblastic layer and 40% mesenchymal core cells. QF-PCR results obtained with this CVS preparation method were consistent with the presence of mesenchyme and cytotrophoblastic cells in almost equal proportions (Mann et al., 2007), it was proposed by Mori et al. that the cytotrophoblast layer of chorion villi becomes thinner during gestation, resulting in a gradual over-representation of the mesenchymal lineage (Mori et al., 2007). Since in case 1 we found that the CVS in the 21st week of gestation displayed MLPA results representative of the cytotrophoblast lineage, we could not confirm this latter notion.

The potential presence of maternal cell contamination (MCC) poses a serious risk for prenatal misdiagnosis (Schrijver et al., 2007). We have taken into account that MCC in female samples cannot be detected by MLPA (see material and methods). In the male samples tested by MLPA, however, no evidence was found for the presence of maternal contamination. Additionally, we tested two of the male DNA samples for the presence of MCC using another PCR-based system (AmpFLSTR Identifiler Kit, Applied Biosystems). Again, we failed to obtain any evidence for MCC. It appears that the MCC problem can be minimized if maternal deciduas are carefully removed from the villi and/or if sample (DNA) preparation procedures are thoroughly optimized. Nevertheless, we suggest that validation studies are required to determine a further assessment of MCC as potential cause of misdiagnosis in prenatal testing of CVS. Enzymatic dissociation of CVS yields cell pools representative for the cytotrophoblast layer and mesenchymal core separately. Therefore, rapid aneuploidy testing with MLPA on these cell pools is comparable to karyotyping of STC and LTC.

Although QF-PCR has the same inherent limitations as MLPA in that it will not detect structural chromosome aberrations (Cirigliano et al., 2004; Shaffer and Bui, 2007) it is sensitive for the detection of MCC and triploidy (69,XXX). In reverse, MLPA has the advantage of being able to assess the copy number of up to 50 loci in a single assay. As such, MLPA can easily be extended to other genomic regions of known clinical relevance (Faas et al., 2008) and can also be used as a highly efficient technique for the detection of sub-telomeric imbalances (Ahn et al., 2007). The MLPA technology involves ligation of probes corresponding to a chromosome-specific sequence that is unique within the genome. In contrast to polymorphic loci used for QF-PCR, these chromosome-specific sequences show little or no variation, which avoids non-informativeness of the targeted sequences. Therefore, the MLPA technology may be well-suited for combining speed and targeted testing of specific chromosomal inter- and/or intragenic regions. Until recently, rapid aneuploidy detection was mostly performed by I-FISH. MLPA and QF-PCR have some advantages over I-FISH, i.e., the tests are less labour-intensive, more cost-effective and/or better suited for high throughput analyses. QF-PCR and MLPA are considered to be valid alternatives to karyotyping for specific referral reasons, although some clinically significant abnormalities will remain undetected. In a retrospective study of 3,700 CVS of women with referral reason elevated maternal age, we determined that for every 1,000 CVS performed up to 25 chromosomal aberrations, of which six with potential clinical significance, would remain undetected if MLPA were to replace TK (unpublished data). As far as the clinical relevance of these anomalies is concerned, it should be borne in mind that 70% of the (structurally) unbalanced chromosomal anomalies lead to intrauterine death or miscarriage before birth, and/or are associated with abnormalities detectable by ultrasound (Leung et al., 2004b).

The development and implementation of additional novel molecular-cytogenetic techniques, such as array CGH, is continuously increasing the resolution of the detection of chromosome abnormalities (Shaffer and Bejani, 2004). In prenatal diagnostics array...
CGH may be applied to pregnancies with ultrasound abnormalities and a normal karyotype. However, retrospective validation studies have indicated that more insight into normal versus abnormal copy number variation within the human genome (Redon et al., 2006; Rickman et al., 2006) and a comprehensive detection of mosaicisms is required before such a technology can be applied into routine prenatal diagnostic care. We conclude that MLPA is a powerful technique for the detection of aneuploidies of chromosomes 21, 18, 13, X, and Y in CVS. In addition, we conclude that DNA extraction methods for CVS have a major impact on the genetic make-up of the DNA pool and affect the reliability of the molecular diagnosis of aneuploidies. In anticipation of molecular targeted testing for prevalent aneuploidies in CVS, we have designed a laboratory flowchart (Figure 2). In this flowchart, the implementation of rapid aneuploidy detection is based on DNA extracted from the cytotrophoblasts and the mesenchymal core, respectively. In contrast to Gerdes et al. (Gerdes et al., 2005b) concordant abnormal results in these duplicate measurements are considered as final test results, irrespective of whether an abnormality is detected by ultrasound or serum screening. Taken together, we conclude that MLPA as targeted stand-alone CVS test in pregnancies with an increased risk for Down syndrome is a fast and reliable alternative for traditional karyotyping on STC and LTC.

**Acknowledgements**

We thank Judith Derks-Willemsen for technical support and database development. We are grateful to Dr. Schouten from MRC Holland, who supplied the MLPA kits.

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**Figure 2.**

Laboratory flowchart for integrating rapid aneuploidy detection (RAD) in CVS into the cytogenetic diagnostic service. Approximately 30 mg of cleaned villi is split into three fractions (10 mg each). Two independent cell preparation procedures based on enzymatic digestion with collagenase and/or trypsin/EDTA are performed on fractions I and II to obtain suspensions from cytotrophoblasts (fraction C) and the mesenchymal core (fraction M), separately. A small amount of fraction M is used for LTC. Fraction III is stored for back-up. DNA is extracted from fractions C and M and RAD by MLPA or QF-PCR are assayed independently. The blue and red arrows indicate the routing for RAD as stand-alone test and the routing for TK, respectively. In this flow chart, TK of the STC is replaced by RAD on DNA from the cytotrophoblast fraction. Discordant results with RAD between fractions C and M or test failures is indicative for TK of the LTC, or a repeat experiment using back-up fraction III. When the results of RAD are abnormal, follow-up karyotype analysis is performed to confirm the nature of the aneuploidy.
Chapter 7

Clinical application of microarray-based genomic profiling in prenatal diagnosis

Kooper AJA, Faas BHW, de Leeuw N, Smits APT, Geurts van Kessel A
Introduction

In recent years, several prenatal diagnostic centres have, next to traditional karyotyping (TK), implemented molecular cytogenetic techniques such as fluorescence in situ hybridization (FISH), quantitative fluorescence polymerase chain reaction (QF-PCR) and multiplex ligation-dependent probe amplification (MLPA) for targeted rapid aneuploidy detection (RAD) in either amniotic fluid cells or chorionic villus samples (Bryndorf et al., 1996; Ogilvie et al., 2005a; Kooper et al., 2008; Cirigliano et al., 2009; Kooper et al., 2009; Van Opstal et al., 2009). In case foetal ultrasound examination reveals developmental anomalies that are highly suspected for a chromosomal abnormality and its karyotype is normal, additional molecular tests have become available to detect sub-microscopic aberrations (deletions and/or duplications smaller than 5 Mb). At present, many recurrent developmental disorders are known to be associated with such sub-microscopic deletions and/or duplications. With the recent development of microarray-based comparative genomic profiling techniques, the relative DNA copy numbers of thousands of genomic regions can be measured simultaneously (Guillaud-Bataille et al., 2004; Veltman, 2006). Through the application of this technology, sub-microscopic abnormalities were e.g. detected in ~9% of patients with mental retardation and/or congenital anomalies (de Vries et al., 2005; Menten et al., 2006; Koolen et al., 2009). Similarly, the application of microarray-based comparative genomic profiling technologies (using either BAC, oligo or Single Nucleotide Polymorphism (SNP) arrays) has the potential to increase the diagnostic yield in pregnancies with foetal malformations. Cryptic or sub-microscopic imbalances of sub-telomeric regions in foetuses with ultrasound abnormalities are known to substantially contribute to anomalous phenotypes (Bendavid et al., 2006; Faas et al., 2008). Additionally, in case a specific foetal malformation is present, its interpretation may be facilitated by the application of advanced molecular cytogenetic methods (Batista et al., 2007; Friedman, 2009). For targeted microarrays probes are selected that are known to be involved in disease-causing (pathogenic) copy number variants (CNVs), whereas genome-wide microarrays with an overall high genome coverage may detect CNVs with unknown clinical relevance. Recently, a few studies have been published dealing with the application of targeted and/or genome-wide microarrays in prenatal diagnosis (Batista et al., 2007; Darilek et al., 2008; Tyreman et al., 2009; Van den Veyver et al., 2009). The clinical interpretation of the analyses, however, appeared to be hampered by the increasing awareness that a plethora of polymorphic CNVs may be present within the genomes of apparently healthy individuals (Redon et al., 2006). Before its clinical implementation, this notion therefore requires carefully designed clinical studies and the inclusion of parental (microarray) results in order allow a distinction between benign and pathogenic CNVs.

Abstract

Microarray-based genomic profiling allows the detection of chromosomal gains and losses at a resolution that is several magnitudes higher than that of traditional karyotyping (TK). As such, this technology allows the identification of relatively small genomic imbalances that may cause severe congenital birth defects and/or mental retardation. The diagnostic application of prenatal microarray analysis offers the promise of efficiently detecting clinically relevant sub-microscopic genomic gains and/or losses in a foetus. The chance of encountering genomic anomalies of uncertain clinical significance, however, is considered high. In this chapter, we report the application of microarray analysis using the Affymetrix NspI 250k SNP array platform to four foetuses. Prenatally, these four foetuses presented with ultrasound abnormalities that were highly suspect for a chromosomal aberration. In three of the four foetuses, however, TK did not reveal any chromosomal aberration. In the fourth foetus an apparently balanced translocation was encountered. Subsequent microarray analysis revealed genomic losses of 2.9 Mb (17p13) and 5 Mb (3q26q27) in two cases, respectively. In two other cases an atypical loss of ~1.5 Mb (22q11) and a loss of the ARSA region (22q13) were detected postpartum by other methods, respectively. Subsequent microarray analysis allowed the exact demarcation of the respective deletion breakpoints in these latter two cases. Our results underscore the relevance of the detection of sub-microscopic chromosome aberrations through microarray analysis in foetuses with an ultrasound abnormality. The potential clinical implications for the application of this technology as a prenatal test are discussed.
Here, we report four prenatal cases with ultrasound abnormalities in whom, through high-resolution copy number profiling, CNVs were detected. The clinical interpretation of these CNVs is discussed in the context of what may be considered as ‘good clinical practice’ in future prenatal diagnostics.

Material and methods

Case 1
Amniocentesis was performed at 22+6 weeks of gestation since ultrasound examination had revealed an oesophageal atresia with fistula, polyhydramnion and hydrocephalus. Rapid aneuploidy detection (RAD) in amniotic fluid (AF) cells by I-FISH revealed a male foetus with disomy 13, 18 and 21. Additional cytogenetic analysis of these cells using standard techniques revealed a normal 46,XY karyotype. In addition, a normal AFP concentration of 8.9 mg/l was measured in the AF sample. Foetal demise occurred in the 24th week of gestation.

Case 2
A 37-year-old pregnant female was referred at 16+2 weeks of gestation for amniocentesis because of advanced maternal age. RAD on DNA isolated from AF cells by MLPA revealed a female foetus with disomy 13, 18 and 21. Additional cytogenetic analysis of these cells using standard techniques revealed an apparently balanced de novo 46,XX,t(3;18)(q26.2;q21.3) karyotype (Figure 2A). In addition, a normal AFP concentration of 16.7 mg/l was measured in the AF sample. Follow-up ultrasound examination at 20 weeks of gestation revealed an hydrocephalus and aqueduct stenosis, upon which the pregnancy was terminated. Subsequent foetal autopsy did not reveal any additional abnormalities.

Case 3
Amniocentesis was performed at 22+5 weeks of gestation since ultrasound examination had revealed a heart defect, suspect for tetralogy of Fallot and 22q11 microdeletion syndrome. RAD in AF cells by MLPA revealed a female foetus with disomy 13, 18 and 21. I-FISH testing was performed with probe LSI TUPLE1 (HIRA), a 117 kb (spectrum orange) probe that maps to 22q11, and the LSI ARSA (spectrum green) control probe (both from Vysis, Downers Groove, USA) that maps to the telomeric end of 22q (22q13). Results showed no aberration in the 22q11 and 22q13 regions. Additional cytogenetic analysis using standard techniques revealed a normal 46,XX karyotype. A normal AFP concentration of 7.8 mg/l was measured in the AF sample. Because postpartum examination revealed clinical features suspect for a 22q11 microdeletion, MLPA analysis was performed using the P250 kit (MRC Holland, Amsterdam, the Netherlands) on DNA from blood according to the manufacturer’s specifications. An atypical 22q11.2 deletion was detected which was flanked by the low-copy repeats LCR22-C and LCR22-D.

Case 4
Amniocentesis was performed at 21+3 weeks of gestation since ultrasound examination had revealed a hypoplastic right heart and soft markers for Down syndrome. RAD in AF cells by MLPA revealed a female foetus with disomy 13, 18 and 21. Additional cytogenetic analysis using standard techniques revealed a normal 46,XX karyotype. A normal AFP concentration of 4.0 mg/l was measured in the AF sample. Postpartum examination revealed a hypertrophic right ventricle of the heart, and a perimembranous ventricular septal defect (VSD) combined with a slight dilatation of the right kidney. Dual-color FISH testing was performed on metaphases from cultured blood cells with the LSI TUPLE1 (HIRA) and LSI ARSA probes (see under case 3) to exclude the 22q11 microdeletion syndrome. Remarkably, no aberrations were detected within the 22q11 region (HIRA) but, instead, the ARSA probe revealed a deletion of the 22q13 region.

Cytogenetic and fluorescence in situ hybridization analyses
Cytogenetic analyses were performed on metaphase spreads harvested from cultured amniotic fluid cells or peripheral blood-derived (parental) lymphocytes using standard techniques. For fluorescence in situ hybridization (FISH) analyses, BAC probe (BACPAC Resources) labelling, slide preparation, and hybridization were performed essentially as described elsewhere (de Bruijn et al., 2001). Visual examination of the slides was performed using a Zeiss Axiophote-2 microscope equipped with appropriate filters. Digital images were captured using a high-performance Leica DC 350FX camera coupled to a Leica CW 4000 software package.

Multiplex ligation-dependent probe amplification (MLPA)
MLPA was performed using kit P250 (www.mrc-holland.com) according to the manufacturer’s specifications. Briefly, sample DNAs were isolated using a QIAamp kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The MLPA probes were hybridized to the sample DNAs, ligated and amplified by PCR. Each resulting amplified probe product had a unique length and was identified and quantified by capillary electrophoresis on an ABI 3100 analyser, using Genescan analysis software (version 3.7) and Genemapper (version 4.0) software, all from Applied Biosystems.

Genome-wide microarray-based genomic profiling
Microarray analyses were carried out on DNA from the four foetuses after foetal demise...
Results

Identification of a chromosome 17p13 microdeletion in case 1

250k SNP array analysis was performed on DNA extracted from cultured amniotic fluid (AF) cells, which initially exhibited a normal 46,XY karyotype. This analysis revealed a DNA copy number loss on the p-arm of chromosome 17 (Figure 1), resulting in a revised karyotype denoted as 46,XY.arr snp 17p13.2p13.1(SNP_A-1870741→SNP_A-2216514)x1. Further analysis of our data using the UCSC Genome Browser indicated that the deletion observed encompasses a region of ~2.9 Mb on 17p13.2p13.1, including 443 SNPs and more than 50 genes. The Ensembl v37 database indicated that the deleted region contained the following known disease causing genes: CHRNE (slow-channel congenital myasthenic syndrome: MIM #254200, #601462, #608930, #608931, *100725), GP1BA (Bernard-Soulier syndrome: MIM *606672, #153670, #231200, #177802), ACADVL (very long chain fatty acid dehydrogenase deficiency: MIM *609575, #201475, *609575), SLC2A4 (diabetes mellitus, noninsulin-dependent: MIM #125853, *138190) and KCTD11 (hyperproreninemia: MIM not present). Subsequent region-specific FISH analysis of the cultured AF cells, using BAC probes RP11-374L01, RP11-220M19, RP11-636N17 (17p13.2) and RP11-558E15 (17p13.1), revealed deletions of these probes, thereby confirming the genomic loss indentified by SNP array analysis. FISH analysis with the same probes on parental blood cells did not reveal any anomalies, thus indicating that the deletion detected in the AF cells was de novo.

Identification of a chromosome 3q26q27 microdeletion in case 2

SNP array analysis was performed on DNA extracted from cultured AF cells, which exhibited a 46,XX,t(3;18)(q26.2;q21.3) karyotype (Figure 2A). Since both parental karyotypes were found to be normal, we conclude that the translocation had arisen de novo. SNP array analysis revealed a genomic loss on the q-arm of chromosome 3 encompassing the 3q26.33q27.2 breakpoint region, resulting in a 46,XX(t(3;18)(q26.2;q21.3)dn.arr snp 3q26.33q27.2(SNP_A-2205925→SNP_A-4237540)x1 karyotype (Figure 2B). Data from the UCSC Genome Browser indicated that the genomic loss is ~5 Mb in size, containing 307 SNPs and encompassing more than 50 genes, including the known disease causing gene THPO associated with thrombocythemia and thyroid iodine peroxidase deficiency (MIM *600041, *600044, #187950). Subsequent region-specific FISH analysis of the cultured AF cells, using BAC probes RP11-379M20 and RP11-624A13, revealed deletions of these probes, thereby confirming the loss in the 3q26.33 region indentified by SNP array analysis.

Data analysis

Genomic copy numbers were calculated using the public domain software package CNAG (Copy Number Analyzer for Affymetrix GeneChip Mapping arrays), version 2.0. The underlying algorithm of CNAG strongly improves the signal-to-noise ratios of the final copy number output by i) correcting for length and GC content of the individual polymerase chain reaction (PCR) products using quadratic regressions and by ii) providing fully automated optimal sample selection. The normalized ratios were analyzed for loss and gain of regions by a standard Hidden Markov Model (HMM), which was optimized in order to maximize the detection of the known validated copy number aberrations, while minimizing the false-positive rate, as described before (de Vries et al., 2005; Kuiper et al., 2007). The set HMM parameters were 0 for N=2, -0.38 for N=1, 0.3 for N=3, and 0.55 for N=4. An average of five or more consecutive SNPs showing a single copy number loss (N=1) and an average of seven or more consecutive SNPs showing a single copy number gain (N=3) provided a 95% confidence for representing a true copy number variation (Hehir-Kwa et al., 2007).

Web resources

Database of Genomic Variants (March 2006): http://projects.tcag.ca/variation/
UCSC Genome Browser (March 2006): http://genome.ucsc.edu/
Decipher (March 2009): https://decipher.sanger.ac.uk/
Ecaruca (July 2009): http://www.ecaruca.net/
Ensembl (Feb 2009): http://www.ensembl.org/
Further analysis of the SNP array data using the UCSC Genome Browser and Ensembl indicated that the deletion observed encompasses 784 SNPs covering >30 genes, including the ARSA gene (MIM *607574, #250100, #272200) and the ARC gene (MIM *102480) associated with metachromatic leukodystrophy and male infertility due to acrosin deficiency, respectively. FISH analysis with the ARSA probe on parental blood samples did not reveal any anomalies, thus indicating that the deletion detected in the AF cells was de novo.

Identification of a chromosome 22q11.2 microdeletion in case 3

Postpartum examination of DNA extracted from blood with MLPA kit P250 revealed deletion of the low-copy repeats LCR-C and LCR-D on chromosome 22q11.2 (see also Figure 5), indicating an atypical deletion within this region (Figure 3A). Subsequently, SNP array analysis was performed on DNA extracted from uncultured AF cells for further characterisation of the breakpoints. By doing so, a loss of the 22q11.21q11.22 region was detected resulting in a 46,XX.arr snp 22q11.21q11.22(SNP_A-1859774  SNP_A-1788873)x1 karyotype (Figure 3B). Data from the UCSC Genome Browser indicated that the genomic loss was 1.3 Mb in size, containing 44 SNPs, and included the known disease causing genes SERPIND1 (serpin peptidase inhibitor clade D, associated with thrombophilia due to heparin cofactor II deficiency: MIM #188050, *142360) and GGT2 (gamma-glutamyl-transferase, familial high serum: MIM *137181).

Identification of a chromosome 22q13.3 microdeletion in case 4

In addition to previous prenatal karyotyping of AF cells and the postpartum detection of a deletion of a FISH probe encompassing the ARSA gene in the 22q13.3 region (see case 3), SNP array analysis was performed to more precisely map the breakpoints of this deletion. This analysis, performed on DNA from uncultured AF cells, revealed a DNA copy number loss of 6.1 Mb (Figure 4), resulting in a karyotype denoted as 46,XX.ish del(22)(qter)(HIRA+,ARSA-)arr snp 22q13.31q13.33(SNP_A-2284753  SNP_A-4279731)x1. Further analysis of the SNP array data using the UCSC Genome Browser and Ensembl indicated that the deletion observed encompasses 784 SNPs covering >30 genes, including the ARSA gene (MIM *607574, #250100, #272200) and the ARC gene (MIM *102480) associated with metachromatic leukodystrophy and male infertility due to acrosin deficiency, respectively. FISH analysis with the ARSA probe on parental blood samples did not reveal any anomalies, thus indicating that the deletion detected in the AF cells was de novo.
The four prenatal cases with ultrasound abnormalities reported here illustrate the clinical relevance of SNP array analysis for high resolution genomic profiling. In case 1, the relatively large size of the deletion (~2.9 Mb) and the fact that it encompasses several genes is highlighted. Clinical application of microarray-based genomic profiling in prenatal diagnosis

**Figure 3.**
MLPA and SNP array results of case 3. Panel A shows the electropherograms of the normalized MLPA ratios (Y-axis) of a negative control sample without a 22q11 deletion (blue), a positive control sample with a common 22q11.2 deletion (red) and case 3 (green) with an atypical 22q11.2 deletion. The markers in kit P250 are chosen between 15,959,700 to 23,283,730. Case 3 shows single copy losses of five probes, two in the LCR22-C region (SNAP29, LZTR1) and three in the LCR22-D region (HIC2, PPIL2 and TOP3B), represented by reduced peak ratios of ~0.5 in the electropherogram. In panel B the copy number profile revealed by SNP array analysis is shown, including a loss of 1.3 Mb at 22q11.21q11.22.

**Figure 4.**
FISH analysis in case 4 showing the HIRA (red) and ARSA (green) regions, respectively (A). The arrow indicates the chromosome with the ARSA deletion. GTG banded chromosomes 22 from blood cells (B). The deletion chromosomes are marked by arrows. SNP array analysis (C) revealing a loss of 6.1 Mb in 22q13.31q13.33 ranging from SNP_A-2284753 to SNP_A-4279731.

**Discussion**

The four prenatal cases with ultrasound abnormalities reported here illustrate the clinical relevance of SNP array analysis for high resolution genomic profiling. In case 1, the relatively large size of the deletion (~2.9 Mb) and the fact that it encompasses several genes is highlighted.

[Diagrams and graphs are shown, illustrating the results from the cases described.]

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Deletions of the 22q13.3 region (sizes varying from 100 kb to 8 Mb) on chromosome 22 are associated with a phenotype that includes minor facial abnormalities, frequent neonatal hypotonia, normal or accelerated growth, and developmental and speech delay. The prevalence of the condition is unknown. The 22q13.3 deletion has previously been found in patients referred for the diagnosis of DiGeorge syndrome / velocardiofacial syndrome (VCFS) and has remained under-diagnosed because of the non-specificity of the phenotype (Maitz et al., 2008). The results from case 3 indicate that in case of cardiovascular defects 22q11 deletion diagnosis is best performed with MLPA kit P250 for the detection of common and atypical 22q11 deletions. With the unexpected finding of the 22q13.3 deletion in case 4, suspect for a 22q11 deletion, we show the relevance of microarray analysis for the detection of this microdeletion.

Severe disease causing genes, highly suggests a causative role for this deletion in the intrauterine death in this pregnancy. As such, the application of SNP array analysis likely provides a diagnostic cause for the foetal pathology in this case. In case 2, SNP array analysis clearly showed that the cytogenetically detected apparently balanced de novo translocation is unbalanced in nature. Up till now, this 3q26.3q27.2 deletion has not been observed in healthy individuals and is, therefore, likely to be causally related to the congenital anomalies observed in the foetus. Apparently balanced translocations remain a challenge for geneticists, especially when they are detected prenatally. With an incidence of about 1 per 2,000 newborns it has been reported that the risk for a congenital anomaly in this population is 2-3 times higher than that in an unselected population of newborns, for which the risk of anomalies is 2-3% (Warburton, 1991). In cases with de novo translocations disease-causing copy number variants (CNVs) may be present not only at the breakpoints but also anywhere in the genome. This notion stresses the need for whole genome profiling approaches (Sismani et al., 2007). A major limiting factor for such approaches in prenatal diagnostics, however, is the chance of detecting CNVs with uncertain clinical relevance. Therefore, in terms of improvement of prenatal care, more information is required on the overall clinical relevance of CNVs.

The MLPA P250 probe mix, used in case 3, contains 30 different probes in the 22q11 region in addition to probes for a number of other regions such as 10p14, 4q35 and 17p13, all associated with features of the DiGeorge anomaly. In addition, smaller deletions outside the typically deleted 22q11 region can be identified with this probe mix, and the MLPA test is more suited for the detection of duplications in this region than FISH (Jalali et al., 2008). The proximal 22q11.2 region is rich in low copy repeats (LCRs), genomic structures that are known to mediate meiotic recombination (Koolen et al., 2006; Vissers et al., 2009). The most common recombination event occurs between LCR-A and B, which gives rise to a 3 Mb deletion and the concurrent 22q11 deletion syndrome (Shaikh et al., 2000). The deletion observed in case 3 is similar in size to the one reported by Ogilvie et al. encompassing approximately 28 genes (Figures 3 and 5) (Ogilvie et al., 2009a). The latter authors provided preliminary evidence for the involvement of one or more of the genes located within the deleted interval distal to LCR-D in cardiac defects and, more specifically, that haplo-insufficiency of the CRKL gene may cause abnormal cardiac development. The incidence of these atypical deletions has been considered low. It has been reported, however, that since only probes covering the TUPLE1/HIRA loci are generally used in routine diagnostics, about 6% of the 22q11.2 deletions may be missed (Rauch et al., 2005). A refined analysis of the exact deletion sizes in additional cases with variant breakpoints and/or atypical deletions is expected to facilitate the elucidation of the molecular basis of the 22q11 DiGeorge microdeletion syndrome (Stachon et al., 2007).
Taken together, the application of genome-wide high resolution microarray analysis will result in the detection of otherwise undetectable CNVs as shown in these four examples. On the other hand, the technique also visualizes CNVs in the human genome frequently encountered in healthy individuals. It has been estimated that such CNVs cover ~12% of the human genome (Redon et al., 2006). Discriminating between benign CNVs and pathogenic CNVs is a first step in the diagnostic process after having identified CNVs in an affected foetus or patient. The use of in-house control CNV data and publicly available databases such as the Database of Genomic Variants or the UCSC Genome Browser may provide a first clue regarding the possibility that a CNV may be associated with disease. At present, however, these data are still too limited to exclude uncommon (rare) CNVs from clinical significance. In addition, ethnicity-associated CNVs are known to exist, but as yet only limited control CNV data are available to discriminate between ethnic populations (Redon et al., 2006; White et al., 2007). Our understanding of benign and pathogenic CNVs is still in its infancy and, therefore, both clinicians and cytogeneticists are encouraged to deposit such information into databases such as DECIPHER and ECARUCA to increase common knowledge on phenotype/genotype correlations (Vermeesch et al., 2007). Currently, methods are being developed to evaluate and/or update CNVs generated by a classification scheme where the probability of a region being denoted as disease causing can be calculated (Marioni et al., 2008). Such information may also be of help in obtaining better insights into the extent and role of CNVs in health and disease.

Preliminary data on the detection of causative CNVs in pregnancies with ultrasound abnormalities have been provided by several authors. An additional 6% of CVS and AF cases with a normal karyotype would be diagnosed using whole genome microarrays (Batista et al., 2007) assuming that post- and prenatal detection rates are similar. With targeted microarrays in two (2.4%) of 84 prenatal samples with (major or minor) ultrasound abnormalities causative CNVs were detected (Van den Veyver et al., 2009). The latter authors suggested that reserving prenatal microarray analysis for pregnancies with an abnormal prenatal ultrasound result may not be optimal since many genomic disorders represented on the targeted microarray were not associated with clinical features that are detectable upon prenatal ultrasound examination. Congenital heart disease (CHD) combined with multiple congenital anomalies (MCA) showed a highest abnormality rate (28.6%) after microarray analysis of neonates (aged ≤ 28 days) in a study of the Baylor College of Medicine (Lu et al., 2007). In order to provide a better coverage of the targeted regions, and to reduce the number of genes identified for which termination would be ethically questionable (for example BRCA, AZF) and to reduce the number of regions of uncertain clinical relevance, targeted arrays in prenatal diagnosis may be preferred (Le Caignec and Redon, 2009). In a recent whole-genome microarray study using the Affymetrix GeneChip 6.0 array, however, in 10% of 106 consecutive pregnancies with abnormal ultrasound findings and a normal karyotype putative pathogenic CNVs were detected (Tyreman et al., 2009). Preliminary data from our own prenatal service using whole genome 250k NspI SNP arrays in 24 foetuses with ultrasound abnormalities highly suspected for a sub-microscopic aberration revealed 4 (16%) imbalances that otherwise would have remained undetected, all of which are likely to be clinically relevant (Faas et al., submitted).

Bejjani and Shaffer stated that ‘diagnostic laboratories should always remain at least one step behind the cutting edge of research’ (Bejjani and Shaffer, 2006) and argued that the use of genome-wide microarray analyses in clinical laboratories should await further understanding of the genome and its architecture. As yet, however, we agree with Tyreman and Friedman that prenatal diagnosis by genome-wide microarray analysis is valid for pregnancies known to be at risk for having a pathogenic CNV, and that careful implementation of genome-wide microarrays will yield relevant results in at least 10% of obstetric patients with abnormal ultrasound findings and a normal karyotype (Friedman, 2009; Tyreman et al., 2009). It should be kept in mind, however, that with the clinical introduction of this technique result interpretation and genetic counselling strategies must be adopted to allow its implementation with a maximum benefit and a minimum risk (Darilek et al., 2008). The possibility to detect CNVs of uncertain clinical relevance necessitates agreements between patients, clinicians and laboratory geneticists on what should be reported. These agreements could be offered in a consent form to explain the risks and benefits of having prenatal microarray analysis. In the near future, national guidelines will be formulated in the Netherlands for reporting microarray results to prospective parents. Based on our preliminary results and those from others, we propose that in pregnancies with an ultrasound abnormality and a normal aneuploidy test result, genome-wide microarray analysis may replace traditional karyotyping. In addition, we recommend to store DNA of foetuses with a de novo balanced translocation in order to enable whole genome profiling in case a foetal ultrasound abnormality is detected during the pregnancy. Karyotyping and/or FISH, however, remain obligatory for a further delineation of the detected microarray alterations.

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Chapter 8

General discussion and future prospects

8.1 The efficacy of protein tests
8.2 The efficacy of molecular tests
8.3 Appropriate prenatal care
8.4 Future prospects
In recent years, significant advances have been made in the resolving power and speed of prenatal diagnosis, including an increased resolution in ultrasound scan (US) examination, the introduction of first trimester screening (FTS), the possibility to biochemically detect metabolic disorders and, last but not least, the implementation of novel molecular tests. In particular, the continuous development of more advanced molecular tests has had a major impact on invasive prenatal diagnosis. In the early 1990s, fluorescence in situ hybridization (FISH) and, later on, quantitative fluorescent polymerase chain reaction (QF-PCR) and multiplex ligation-dependent probe amplification (MLPA) have entered the field of prenatal diagnostic testing, thereby obviating the need to culture foetal cells. These techniques allow a rapid aneuploidy detection (RAD) of pre-selected chromosomal regions (Mann et al., 2004; Slater et al., 2003) and, as such, are particularly suited for high throughput targeted testing. Next to RAD, recent advances in microarray analysis have created the possibility to efficiently detect chromosomal imbalances at a genome-wide scale with a significantly higher resolution than traditional karyotyping (TK). High quality prenatal and obstetric care require well-considered diagnostic choices. In light of the above mentioned technical developments and its putative clinical applications in prenatal diagnosis, the question rises which test should be offered to assure that pregnant women receive appropriate prenatal care. Both these technical developments and its putative clinical implications are discussed below.

8.1 The efficacy of protein tests

**AFP measurement in amniotic fluid**

Abnormal alpha foetoprotein (AFP) levels in amniotic fluid (AF) were historically employed to diagnose neural tube defects (NTDs) but, in recent years, AFP levels have been superseded by foetal US examination (Cameron and Moran, 2009). From an historical point of view, AF samples have been collected for TK and, simultaneously, for routine AFP level measurements in cases with and without an increased risk for a NTD. In Chapter 2, we show that routine AFP assays in AF samples, in pregnancies without an increased risk for a NTD, generate a significant number of false-positive results. Based on this observation, and taking the advances in US examinations into account, we expect that the 20-week scan will gradually replace AFP measurement as a screening tool for NTD detection. In 2004, the overall prenatal detection rate for NTDs through a 18 to 22 week routine US examination in 18 European countries was already 88% (range 25-94%), with the highest detection rates in countries using standards determined by a national screening policy (Cameron and Moran, 2009).

In case of an increased risk for a NTD, the preferred strategy for NTD detection is an
advanced (targeted) US examination. This strategy is expected to lead to a reduction of iatrogenic miscarriages in pregnancies at risk for a NTD. US examination prevents prospective parents from uncertainty and anxiety concerning the health of the foetus caused by the high rate of false-positive AFP test results. In contrast to AFP measurement, US examination also allows the detection of closed NTDs. Using 2D and 3D protocols, trained sonographers can even delineate the lesion level of a NTD. Such information will be imperative for informing prospective parents about its implications for their unborn child. It is anticipated that, in the long run, AFP measurement in AF will be limited to particular foetal AFP-related disorders and/or perinatal distress conditions.

Identification of novel biomarkers

Proteomics-based identification of novel biomarkers for foetal abnormalities in maternal plasma, AF and reproductive fluids has made significant progress over the past five years. This progress is attributed mainly to advances in various technology platforms associated with mass spectrometry-based techniques (Choolani et al., 2009). In particular, AF is a significant contributor to foetal health and, therefore, it constitutes a potential rich source of biomarkers for the diagnosis of maternal and foetal disorders. The biochemical detection of metabolic disorders combined with gene mutation analysis has indicated that stipulated hereditary disorders can be detected early in pregnancy. In Chapter 3, we show that the measurement of 21 lysosomal enzymes in AF of foetuses with non-immune hydrops foetalis (HF) and a normal karyotype, in conjunction with gene mutation analysis, identified at least 5% of foetuses with a lysosomal storage disease. Application of this strategy to the diagnosis of lysosomal diseases in pregnancies with a HF is, therefore, useful for risk assessment, genetic counselling and targeted prenatal diagnostics for ensuing pregnancies. As such, we recommend the inclusion of metabolic analyses in the routine diagnostic work-up of non-immune HF.

Most of the current prenatal proteomic research is focussing on premature birth, eclampsia, foetal cardiopathology and chromosomal aneuploidy cases. The major challenge to overcome, however, lies between protein discovery and target validation. Therefore, potential biomarkers should be subjected to further comparative analyses of both protein expression and structural modification using samples from chromosomally normal and abnormal pregnancies. Tsangaris et al. (2006) applied proteomics to the identification of proteins differentially expressed in AF samples derived from pregnancies with Down syndrome (DS) and from chromosomally normal cases. This comparison revealed significant quantitative and qualitative differences in protein expression levels in both groups. Proteins that were up-regulated in AF of DS pregnancies included alpha-1-microglobulin, collagen alpha I, III and V chains and basement-membrane-specific heparin sulphate proteoglycan core protein, whereas the insulin-like growth factor binding protein 1 precursor was decreased in these AFs. Possibly of more significance was the discovery that splicing factor arginine/serine-rich 4 was only found in the DS samples and not in the normal ones. The proteins identified are encoded by genes located outside chromosome 21, implying that increases in copy numbers of genes located on chromosome 21 may affect the expression of genes on other chromosomes, possibly mediated by increased activities of certain transcription factors. The study of Tsangaris et al. illustrates the potential of proteomics for biomarker discovery in the AF of aneuploid pregnancies (Tsangaris et al., 2006).

It must be emphasized, however, that the protein content of AF samples largely depends on its developmental stage. Therefore, certified reference materials must be developed, next to sample quality control and quality assurance protocols (Nagalla et al., 2007). Although the application of proteomic technologies in AF is still in its infancy, the identification and characterization of disease-related biomarkers in AF is expected to significantly improve in the near future. Furthermore, because differentially expressed proteins and peptides are likely to cross the placental barrier and, thus, are shed into the maternal serum, proteomic analysis has the potential to be employed for non-invasive prenatal testing of aneuploidies and pregnancy complications as well (Kollai et al., 2008), without subjecting the developing foetus to the potential harm impinged by current invasive prenatal procedures (see below, future prospects).

8.2 The efficacy of molecular tests

RAD in prenatal diagnosis

In recent years FISH, MLPA, and QF-PCR techniques have been developed and implemented for RAD in prenatal diagnosis. In Chapter 4, we show that the MLPA kit P095 exhibits a specificity and sensitivity of 100% for the detection of non-mosaic (an) euploidies of the chromosomes 13, 18, 21, X and Y. In addition, we provide in this chapter a laboratory flowchart for the implementation of MLPA as a stand-alone diagnostic RAD test in AF for pregnancies at risk for Down syndrome (DS). At the national and international level, the clinical application of RAD in prenatal diagnostic centres is variable: RAD is either used as an alternative for I-FISH for pregnancies with US abnormalities, as stand-alone test for pregnancies at risk for DS, or as an adjunct to TK. The chance of approximately 1:1200 of missing a clinical relevant chromosomal finding detectable with TK but not by RAD has been used as a major argument against RAD testing as replacement for TK.

Up till now, the option for pregnant women to choose for RAD as stand-alone test in pregnancies at risk for DS in our clinic has led to a reduction in TK of ~60%. This result is
concordant with an experience based Swedish study covering >6000 clinical samples, which indicated that ~70% of women choose RAD instead of TK (Bui, 2007). Remarkable is that choices for TK or RAD ranged from 40% to 90% between different prenatal clinics in our (Dutch) region. Whereas in all prenatal clinics the parents received identical brochures with information on genetic testing, differences were noted in transferring this information to the parents. In some of the prenatal clinics, in addition to the brochure, oral consults were held by genetic advisors and/or gynaecologists, whereas in other clinics the parents’ decisions were based on the information in the brochure only. Obviously, communication of information to parents may play a key role in its final outcome (Chapter 4).

In an attempt to further optimise the MLPA test, we assessed the individual probe performances of MLPA kit P095 (Chapter 5). The results obtained indicated that exclusion of the poorest performing probes only slightly affected the overall sensitivity of the test, thereby underscoring the robustness of the test. We additionally applied MLPA to chorionic villus samples (CVS) (Chapter 6). The cellular complexity of CVS tissue and the possibility of discrepant results between QF-PCR and TK was previously reported (Waters et al., 2007). This has led to a change in laboratory practice in the UK for RAD in CVS through the implementation of a specific enzymatic dissociation protocol (Mann et al., 2007). We adapted this dissociation protocol for the application of RAD on DNA derived from cytotrophoblasts and the mesenchymal core, respectively. By using this adapted protocol, we found that complete discrepancies between cytotrophoblasts and mesenchymal core can be confirmed by MLPA (Chapter 6). QF-PCR, which is based on microsatellite genotyping, has advantages over MLPA, i.e., for MLPA more high quality DNA is required and, in contrast to MLPA, QF-PCR has the potential to detect all triploidies (Hulten et al., 2003). Major advantage of QF-PCR, however, is the possibility to detect the presence of a second cell line. This was nicely demonstrated by the detection of both maternal cell contaminations (MCC) and mosaicsisms (Cirigliano et al., 2004). Cell lines contributing at least 20% to the total cell population were confidently identified. MLPA (and i-FISH) also have the capacity to detect mosaics at a 20% level, but MCC can only be detected in case of a male foetus. This makes QF-PCR particular suitable for the application of RAD on CVS. Next to the detection of MCC, STR markers also allow a distinction between meiotic and mitotic nondisjunction events in CVS. As such, a confined placental mosaicism (CPM) can be suspected in CVS when only di-allelic patterns are observed for all informative markers. Additionally, true mosaic foetuses can be identified by a tri-allelic STR pattern, which is indicative for a meiotic origin of the extra chromosome (Cirigliano et al., 2009). Nevertheless, the MLPA technology still serves as an efficient tool for RAD and for the targeted detection of sub-microscopic deletions and/or duplications in pregnancies suspected for a chromosomal aberration.

Microarray analysis in prenatal diagnosis

The clinical application of microarray-based genomic profiling in prenatal diagnosis (Chapter 7) has provided the capacity to detect more clinical relevant abnormalities than TK and/or RAD. Retrospective results obtained through 250k NspI SNP array analyses in 24 foetuses with ultrasound abnormalities and a normal karyotype showed 16.6% chromosomal imbalances, all of which are likely clinically relevant (Faas et al., submitted). These data support a recent retrospective study in which it was concluded that genome-wide high resolution microarray analysis may allow the detection of at least 10% of all pathogenic copy number variants (CNVs) in pregnancies with abnormal US findings and a normal karyotype (Tyreman et al., 2009). It should be noted, however, that its prenatal use may also result in uncertain clinical significance leading to parental anxiety and, in the worst case scenario, to the termination of a normal pregnancy (Van den Veyver et al., 2009).

In Chapter 7, several prenatal cases with ultrasound abnormalities in whom sub-microscopic CNVs were detected are discussed in the context of what may be considered as ‘good clinical practice’ in prenatal diagnostics. In recent reviews of the potential application of microarray platforms for clinical use in prenatal diagnosis, implementation of targeted arrays has been favoured (Le Caignec et al., 2006; Manning and Hudgins, 2007; Stankiewicz and Beaudet, 2007). Targeted microarray platforms are recommended for the detection of interstitial microdeletions/duplications and known congenital syndromes, including those affecting sub-telomeric and pericentromeric regions, but need to be reviewed regularly since new microdeletion/duplication syndromes continue to be identified (Tyreman et al., 2009). The higher coverage of genome-wide microarrays allows the detection of more and smaller aberrations than targeted microarrays. Before diagnostic application, however, result interpretation and genetic counselling strategies must be refined to allow maximum benefit and minimum risk (Darilek et al., 2008). The possibility to detect CNVs of uncertain clinical relevance necessitates agreements between patients, clinicians and laboratory geneticists on what should be reported. These agreements could be offered in a consent form to explain the risks and benefits of having prenatal microarray analysis. In the near future, national guidelines will be formulated in the Netherlands for reporting microarray results to prospective parents. Based on our preliminary results and those from others, we propose that in pregnancies with an US abnormality and a normal RAD result, genome-wide microarray analysis may replace TK. TK and/or FISH, however, remain obligatory for further delineation of a genomic gain or loss. In the near future, the application of advanced next-generation sequencing platforms will provide digital profiling information for the detection of copy numbers and positional alterations at the ultimate (basepair) resolution (Shen and Wu, 2009). There is no doubt that this capacity will add yet another level of complexity to its ultimate (prenatal) clinical implementation.
8.3 Appropriate prenatal care

Conditions of appropriate care

Until the end of the 19th century, medical care for pregnant women was almost exclusively limited to the delivery itself. Improvements therein began with the acquisition of new basic knowledge, which was subsequently implemented in routine medical practice. These developments created a multidisciplinary approach for prenatal care, involving obstetricians, neonatologists, clinical and laboratory geneticists, and US experts in centres for prenatal diagnosis. Nowadays, prenatal screening for Down syndrome and NTDs is an integral part of many routine screening programs. Additional invasive diagnostic tests are available to detect and/or confirm the presence of genetic defects before birth. In order to establish what can be considered as the most suited diagnostic laboratory test, understanding of its basic principles is a prerequisite. At least two conditions may be considered for suitable invasive diagnostic laboratory tests:

1. Desirable findings must be avoided to optimize decision making
2. Results must be rapidly available early in gestation

1. Undesirable findings must be avoided to optimize decision making

In general, diagnostic tests should provide relevant information appropriate for clinical use. Within the current spectrum of prenatal tests, AFP measurements for the detection of NTDs and the application of genome-wide microarray analyses for the detection of chromosomal anomalies may yield abnormal AFP levels and CNVs with unknown clinical relevance, respectively. Obviously, such uncertainties must be avoided as much as possible. With TK chromosome abnormalities >5 Mb can be detected, thus providing information on abnormalities related to the referral reason for invasive testing. But, TK may also detect unrelated chromosomal abnormalities, including unexpected findings. Unexpected findings can be considered as undesirable when the clinical impact of the finding is unclear and/or when uncertainty about the phenotype of the child is imposed, thus presenting the prospective parents with a serious dilemma, i.e., whether or not to continue the pregnancy (van Zwieten et al., 2005). In contrast to TK, prenatal tests using QF-PCR and MLPA are limited to specific regions on chromosomes 13, 18, 21, X and Y, thus focussing on the most common chromosomal aneuploidies. This largely relieves prospective parents from the burden of unexpected and incomprehensible results and, by doing so, contributes to well-informed decision making. The inclusion of targets for the X and Y chromosomes is still a matter of debate. In most prenatal centres, pregnant women undergoing invasive testing have the option to know the sex of their unborn child, even if there is no medical reason for it. Sex chromosome analysis through TK or molecular testing (MLPA/QF-PCR) may lead to the, unexpected, detection of sex chromosomal alterations. In a cohort of 19,517 AF samples from pregnancies with advanced maternal age, 18% of the detected 333 chromosomal abnormalities involved sex chromosomes (Leung et al., 2008). In a retrospective study we revealed a rate of 19%, thus confirming the above findings (Kooper et al., unpublished data). Although it has been reported that the prenatal detection of sex chromosome anomalies may facilitate the identification of foetuses with Turner syndrome and/or 47,XY syndrome (Vaknin et al., 2008), most individuals with sex chromosomal aneuploidies are only mildly affected. Nevertheless, the UK National Screening Committee (UKNSC) has recommended that in pregnancies at risk for Down syndrome, FISH or PCR tests should only include targets for the chromosomes 13, 18 and 21 (Caine et al., 2005). Additional research is needed to unravel the (non-medical) reason for prospective parents, and the (medical) reason for professionals, to know the foetal sex, taking into account that foetal sex determination by a 20 week scan has the potential to replace foetal sex determination by invasive genetic testing (Kooper et al., unpublished data).

2. Results must be rapidly available early in gestation

Based on time constraints in prenatal care, tests results should be rapidly available. The desire of prospective parents to obtain confirmation of the good health of the foetus during pregnancy plays a central role. In psychological terms, prenatal diagnosis is an anxiety-inducing procedure (Kowalcew et al., 2003), and a significant reduction in anxiety is obtained after a normal result. In this light, two types of stress can be discerned (Weimann and Johnston, 1988). The first is linked to the invasive nature of procedures, such as amniocentesis and CVS, and the attendant risk of a miscarriage. The second is related to the outcome of the test. RAD by QF-PCR takes 24-48 hours to complete, compared to 2-3 days for MLPA or up to 21 days for TK. It is, therefore, essential that prospective parents with a positive Down screening result qualify for RAD, which can effectively alleviate their anxiety (Leung et al., 2008) short after the invasive procedure. This targeted information is often preferred over more comprehensive information as long as the results are received rapidly (Ryan et al., 2005). In a randomized trial (ARA; Amniocentesis Results: Investigation of Anxiety) held in twelve hospitals in the UK the issuing of RAD versus TK results was performed (Hewison et al., 2007). By doing so, it was found that women having RAD exhibited significantly less anxiety during the waiting period than those having TK. Anxiety levels were comparatively low in all groups a month after having received normal results. Another advantage of RAD is that it allows parents for earlier decision making in cases in which the foetus exhibits a significant anomaly. It has previously been shown that an earlier gestational age at termination acts as an independent factor for a lower level of posttraumatic stress symptoms (Korenromp et al., 2007).
Parents’ autonomy to choose

There are national and international differences in offering prospective parents, with an increased risk of a foetus with DS, the autonomy to choose between different prenatal tests, i.e., TK or RAD. Questions that have been raised include: who is making the choice, i.e., parents or doctors or other health professionals, and are choices made according to receiving the best possible care and/or other criteria? Decisions made within the prenatal genetic testing domain entail potentially far-reaching consequences, including test-related risks to the foetus. Grimshaw (2003) assessed the attitude of pregnant women, medical professionals and the general public regarding this aspect, using a questionnaire-based approach (Grimshaw et al., 2003). Most obstetricians (57%), midwives (71.4%) and pregnant women (67%) preferred RAD to TK, whereas the majority of the non-pregnant general public (60%) expressed a preference for TK. Clearly there is disagreement, even among each category of respondents. Offering women the autonomy to choose may turn out to be clinically impractical, due to the overall complex nature of the counselling procedures (Leung et al., 2008), however, reported that parents should have the autonomy to choose after being fully informed about the pros and cons of the different prenatal tests. Greener (2007) has presented a thematic review of the assumptions underlying patient choice in medical care, based on who is meant to be making choices, what choices are meant to be made and how choices are meant to be made (Greener, 2007). It was concluded that doctor-patient relationships are usually asymmetric and that patients are usually not as well informed as doctors about the referral conditions. In times of increasing patient involvement and medically equivalent treatment options, shared decision-making has become an integral part of good medical practice (Smets et al., 2007) in which both patients and clinicians participate in discussions on treatment options, thereby reaching mutually agreed decisions. In comparison to the paternalistic model (where the clinician is seen as acting in the patient’s best interest) or the informed model (where the clinician increases the patient’s knowledge so that the control over the decision making process lies with the patient), this shared decision-making approach provides an opportunity to go beyond simply presenting relevant facts in a value-free way (Hunt et al., 2005). Further research, however, will be needed to assess whether this general model of shared decision-making will comply to actual prenatal practice.

8.4 Future prospects

Referral reasons for prenatal testing

Based on the fact that a higher detection rate for DS and less iatrogenic miscarriages can be obtained when first trimester screening (FTS) risk assessment will be implemented as integral part of routine prenatal care, regardless the age of the pregnant women, we suggest to reconsider the use of advanced maternal age as a referral reason for invasive testing. In addition, we suggest to reconsider the addition of routine TK in referrals for molecular or metabolic testing and recommend microarray analysis as a complement (or replacement) of TK to obtain a higher detection rate of sub-microscopic aberrations in pregnancies with foetal ultrasound abnormalities.

The decision to use an age cut-off point was historically based on an attempt to balance the risk of a foetal chromosomal abnormality with the risk of a procedure-related pregnancy loss. However, maternal age is a poor screening criterion, since the majority of children with DS are born to women younger than 35 years of age. The American College of Obstetricians and Gynaecologists (ACOG) no longer recommends the use of a maternal age of 35 years as a cut-off point for eligibility for CVS or amniocenteses (Driscoll and Gross, 2009). Instead, they recommend to first refine the risk assessment with a FTS test. Maternal age is, next to nuchal translucency and biochemical measurements in maternal blood, the third parameter in the FTS test. When women undergo invasive testing because of their advanced maternal age, the number of iatrogenic miscarriages is even higher than the number of diagnosis of DS (detection/miscarriage ratio 0.7). The use of the FTS test considerably improves the ratio (from 0.7 to 3.3) (Health Council of the Netherlands, 2004). Therefore, compared to invasive testing, FTS has the potential to substantially lower screening-related miscarriages, which raises the question of whether invasive testing should still be offered in a screening program for DS based on maternal age only (Bornstein et al., 2008). An increased risk for FTS is, therefore, likely to replace the referral reason ‘advanced maternal age’ as primary criterion for invasive testing. The impact of such a policy change, when calculated retrospectively for the year 2005 in the Netherlands, would result in a reduction of 93% (6,040 to 423) of invasive tests for pregnancies referred for ‘advanced maternal age’ (cut-off level of 1:250 at term and a screen positive rate of 7%), see Figure 1 (Wortelboer et al., 2009) and a reduction of iatrogenic miscarriages from 30 to 2 (based on an average miscarriage risk of 0.9% after invasive testing for amniocentesis or CVS). Wapner already reported that if FTS would replace advanced maternal age as the primary criterion whereby to recommend TK, 85% of women aged >36 years could avoid an invasive diagnostic procedure (Wapner et al., 2003). If FTS would replace advanced maternal age parents should be informed that screening tests do not detect all cases of aneuploidy and that diagnostic tests are also available to definitively determine whether the foetus has a major chromosomal abnormality. After a review of the potential risks and benefits of screening and diagnostic testing, parents may alternatively decide for screening and/or testing in this pregnancy.

While women over the age of 36 have routinely been offered additional screening or testing options during pregnancy, 66% of the children with DS are born to women below...
the age of 35 (Howe et al., 2000). Based on this information, we suggest that FTS tests should be available (free of charge) for all pregnant women. For the Dutch situation, this would result in 181,336 pregnancies that may opt for FTS (Statistics Netherlands, 2007).

We are aware of the fact that clinically significant, less common chromosome abnormalities may remain undetected when TK is no longer performed in referrals for molecular or biochemical testing and that the invasive procedure puts the pregnancy at risk. However, karyotypes with uncertain prognosis, such as mosaic findings and small marker chromosomes, have over twice this prevalence, thereby generating anxiety and potentially needless terminations (Ogilvie et al., 2009b).

According to their opinion some women/families would prefer to have maximum information and maximum autonomy. In a Dutch prospective study, Pieters et al. showed that only a minority of women with a low-risk pregnancy would opt for genome-wide prenatal testing (Pieters et al., 2009). Based on this result, the ethical-moral question was raised whether genome-wide testing should be offered to pregnant women at all. The application microarray analysis as a complement (or replacement) of current TK in pregnancies with foetal multiple congenital anomalies, however, can provide a higher detection rate of sub-microscopic aberrations.

**Down syndrome screening in the Netherlands**

In 2007, 23% of all pregnant women in the Netherlands requested a FTS test. This uptake rate is relatively low compared to for example the UK, where uptake percentages were over 95% (Spencer, 2003). The relatively low uptake rate in the Netherlands may be related to the fact that until recently prenatal care was not considered as something "medical" and was not part of customary care. Participation was based on informed choice and women <36 year had to pay for FTS. Once implemented in routine medical care, FTS will probably result in a lower participation in invasive testing of pregnant women >36 years when FTS risk assessment is lower than the risk based on age only. This, in turn, will result in a reduction of iatrogenic miscarriages (see above).

FTS detection rates in the Netherlands have been estimated to be 75.9% at a cut-off level of 1 in 250 at term, with a screen positive rate of 3.3%. The detection and false positive rates were comparable to results reported from screening programs in other countries, such as France, Scotland and Canada (Wortelboer et al., 2009). The performance, however, was lower compared to the UK with a detection rate of ~90% and a false-positive rate of 5% (Nicolaides, 2005). Higher detection rates in DS screening and reductions in iatrogenic miscarriages can be obtained through the incorporation of a sequential second trimester screen into routine obstetric practice. Both stepwise sequential screening and fully integrated screening yield high detection rates for DS, with low false-positive rates (Malone et al., 2005). The FTS test, however, is preferred by the majority of women over a test with a marginally higher detection rate that provides test results later in pregnancy (Spencer and Aitken, 2004).

An incidental consequence of FTS for DS is the possibility to obtain screening results for an increased risk of trisomy 18 or 13. Trisomy 18 and 13, which are the second and third most common trisomies after 21, are lethal and the rate of spontaneous abortions or foetal deaths between 12 and 40 weeks of gestation is ~80%. The remaining one-year
survival rate is approximately 5-10% (Rasmussen et al., 2003). Because of this lethality and high foetal death rate, it may be argued whether there is a diagnostic benefit of reporting screening results concerning these chromosomes to prospective parents when applying FTS test for DS risk assessment. The alternative view is that since many trisomy 18 and 13 foetuses can be identified during the second trimester by foetal US, women have the option of second trimester termination and thus of avoiding the risk of invasive testing if the FTS result proves to be false positive (Kagan et al., 2008). Within the Netherlands, there is a discussion ongoing whether screening for DS should be extended with screening of trisomy 13 and 18. A WBO license is required for such an application and has recently been submitted.

Genomic characterization of Down syndrome
In order to understand the phenotypic consequences of DS, it is crucial both to understand the genomic content of human chromosome 21 and to evaluate how the expression levels of its genes are altered by the presence of a third copy of this chromosome. The length of 21q is 33.5 Mb and approximately 3% of its sequence codes for proteins. Although the phenotype of DS may be complex and variable, it often includes congenital heart defects, craniofacial abnormalities, gastrointestinal anomalies, cognitive impairment, and the development of leukemia and/or Alzheimer’s disease (Roizen and Patterson, 2003). Over-expression of genes on chromosome 21 by 50% in many tissues is thought to elicit DS. However, there is currently no explanation for how this relatively small increase in transcript levels would result in any specific phenotypic feature of DS. Therefore, a central goal of DS research is to understand which of the genes on chromosome 21, when present in three copies, may lead to different DS-associated phenotypes, and how its increased expression levels lead to the molecular, cellular and physiological changes underlying DS. Two distinct approaches are being taken to address these issues; i) the application of genomic association (GWA) studies which may point at genes that play an important regulatory role in DS pathology and ii) the assessment of animal models with trisomy 21. Animal models have shown that trisomy 21 has a significant impact on the development of many tissues, most notably the heart and the brain (Wiseman et al., 2009). Additional GWA studies (Lyle et al., 2008) have pointed at genomic regions harbouring genes that may be relevant for DS, but most of the identified DS critical regions (DSCRs) are still large (several Mb), and more cases are needed to narrow down the phenotypic maps to a reasonable number of candidate genes per phenotype. Two of the candidate genes in the DSCRs are DYRK1A and DSCR1. The latter encodes a known inhibitor of calcineurin (Arron et al., 2006). DYRK1A is a priming kinase that facilitates the further phosphorylation of numerous proteins by other kinases and is up-regulated in a number of tissues from patients with DS (Dowjat et al., 2007; Liu et al., 2008). DYRK1A over-expression in brains of DS patients strongly suggests that in humans the associated kinase needs to be tightly regulated and that both an increase and a decrease in its dosage could result in a disease phenotype (Moller et al., 2008). Gwack et al. (2006) noted that DYRK1A and DSCR1 are located in DSCRs, and suggested that their findings might aid in understanding the immunological and neurological defects in DS. The increased activity of DYRK1A and DSCR1 may contribute, not only to mental retardation, but also to many other features of DS (Arron et al., 2006; Epstein, 2006; Gwack et al., 2006), including molecular and cellular mechanism leading to neurological phenotypes and mental retardation in DS (Rachidi and Lopes, 2007).

Although knowledge of brain abnormalities in DS models is evolving and various phenotypic expressions in mouse models have been studied, little information is available about other organs. Additional mouse DS model studies revealed a higher incidence of lymphomas (Levine et al., 2009). This observation is of interest in relation to the increased incidence of malignancies in human DS. The additional use of chromosome engineering to generate new trisomic mouse models are likely to significantly contribute to our future understanding of the aetiology of DS (Wiseman et al., 2009). Additionally, more accurate information on genotype-phenotype relationships in DS is needed to improve health care. Such information will allow more accurate predictions concerning the ultimate DS phenotype and, as such, may help parents and professionals to make better informed decisions about the pregnancy and its prospects.

Future progress in prenatal diagnostics
The emerging possibilities of non-invasive prenatal diagnostics (NIPD) may imply another significant change to the testing and screening of pregnancies. Research on non-invasive testing began by examining the placental barrier between mother and foetus. The results obtained indicated that the foetus could release its DNA and RNA into the mother’s circulation. So, initially, interest was focused on genes, gene products and/or mutations therein passed on to the foetus by the father and which were distinguishable from those of the mother. Although some work has been carried out using foetal cells obtained from the cervical mucus (Fejgin et al., 2001; Mantzaris et al., 2005) or foetal DNA in maternal urine (Shekhtman et al., 2009), most research has been focused on strategies to detect cell-free nucleic acids (cffNA, i.e. DNA and RNA) from the foetus in the maternal circulation. Foetal cell-free DNA in maternal plasma originates from apoptotic placental cells (trophoblasts) (Tjoa et al., 2006; Alberry et al., 2007) and comprises about 3-6% of the total cell-free DNA during early and late pregnancy, respectively (Lo et al., 1998b). A number of clinical applications of cffDNA analysis in prenatal screening and/or diagnosis have been developed, based on distinct and detectable differences between foetal and maternal genomes like foetal sex or foetal Rhesus D. Foetal sex determination is feasible
and reliable using cffDNA from 7 weeks of gestation through the detection of sequences on the Y chromosome and has been made available to all women at risk of X-linked disorders (Costa et al., 2002) and congenital adrenal hyperplasia (Rijnders et al., 2001). It has been estimated that this application may reduce the need for invasive testing by 50% (Finning and Chitty, 2008). In addition, foetal RhD status determination has widely been used in pregnancies involving RhD-negative women (Lo et al., 1998a).

The additional discovery of cell-free mRNA in the maternal circulation holds great promise for NIPD. Lo et al. discovered mRNA molecules that were specific to the foetus and that could be used for the diagnose foetal chromosomal aneuploidies (Lo et al., 2007). They showed that the SNP allele ratio of PLAC4 mRNA released in the maternal plasma reflects the allele ratio of chromosome 21 in the placenta itself and, therefore, in the foetus. Both the sensitivity and the specificity of the test were found to be high (90% and 96.5%, respectively), and it seems possible to use this test throughout all three gestational trimesters. However, the technique is only applicable to cases that are informative (i.e., heterozygous) for the SNP studied. But, obviously, the test can be extended with SNPs in other candidate genes. Since foetal-derived mRNA is rapidly cleared from the maternal circulation following delivery, antepartum and postpartum samples were compared with paired newborn umbilical cord blood samples to identify unique foetal markers in maternal whole blood. Through gene expression analyses of such whole blood samples a unique set of biologically diverse foetal transcripts could be identified. These transcripts may serve to identify foetuses affected by a variety of pathologic conditions (Maron et al., 2007). In a very recent report, the same group showed that amniotic fluid samples may provide unique molecular windows into developmental disorders, i.e., in addition to identifying genes relevant to the DS phenotype by transcriptional profiling, they were also able to identify several disrupted biological pathways (Slonim et al., 2009).

In recent years, groups led by Dennis Lo and Stephen Quake have applied next generation sequencing (NGS) to the detection of foetal chromosomal aneuploidies (Chiu et al., 2008; Fan et al., 2009). They independently showed the feasibility of converting cell-free DNA from maternal blood into genomic libraries, followed by sequencing and mapping the reads to the reference human genome. Subsequent assessment of the number of reads that map to each chromosome allowed a determination of the relative dosage of each chromosome to be ascertained. These studies have opened up new avenues for non-invasively assessing foetal aneuploidies and have provided a foundation for NGS-based analyses of cell-free DNA (Lo and Chiu, 2009). There is no doubt that NIPD will be the future and, as such, will form a basis for the establishment of large scale prenatal screening programs (Kooij et al., 2009). Over the next few years, several clinical trials involving at least some of the NIPD strategies mentioned above are anticipated.

The future in reproduction
As more pregnancies are undergoing testing, the benefits and limitations of prenatal testing are becoming more pertinent. At an increasing pace prenatal tests will allow the assessment of its potential outcomes including prognoses, recurrence risks, options for additional testing, and long-term management but, at a similar pace, they will create uncertainties when the test results remain ambiguous: progress in prenatal tests may raise both hopes and worries. Already, modern societies are entering an era of personalized genetics, in which anyone can opt for a read-out of known risk genes or, soon, a complete personal genome sequence. There is no doubt that these technologies will make their way into the fertility clinic. True, with thousands of genetic risk variants contributing to multiple different conditions, no embryo will have the ‘perfect’ genetic future. But these techniques may allow prospective parents to create a top-five wish-list of the characteristics they most want for their child - avoiding, for example, Parkinson's disease that plagues the family - and choose the embryo most likely to meet those criteria. Or the parents may focus on non-health-related aspects such as intelligence and/or ambition. Clearly, the ethical debate about genetic selection is likely to intensify over the next years, as it should (Editorial Nature, 2008). Realizing the potential of genetics in prenatal care as a potential source of continuing anxiety, we will have to keep the ultimate goal of genetic testing in mind, especially its prenatal diagnostic application. After all, advances in technologies need to serve as triggers for higher-quality prenatal care, beneficial to prospective parents and healthcare professionals.
Chapter 9

Summary / samenvatting
Summary

The aim of invasive prenatal diagnosis is to detect foetal anomalies by examination of chorionic villi and/or amniotic fluid (Chapter 1). Traditionally, cytogenetic analysis of foetal cells is performed by karyotyping, with reporting times ranging from 10 days (chorionic villi) to 3 weeks (amniotic fluid). However, the development and implementation of new molecular tests such as fluorescence in situ hybridization (FISH), multiplex ligation-dependent probe amplification (MLPA) and quantitative fluorescent polymerase chain reaction (QF-PCR) have changed prenatal diagnostics. Through these tests it has become possible to detect the most common aneuploidies of chromosomes 13, 18, 21, X and Y within 24 to 48 hours. The additional application of microarray technologies provides almost unlimited possibilities for the detection of sub-microscopic chromosomal aberrations. Potential pathogenic genetic defects, such as sub-microscopic deletions and/or duplications can efficiently be identified. These advances in prenatal diagnostics, in conjunction with advances in ultrasonography and the implementation of prenatal screening programs, lead to more detailed (genetic) knowledge of the unborn child. In this thesis new diagnostic tests and their concomitant clinical applications are described in relation to improved prenatal care.

In Chapter 2, the relevance of routine measurement of α-fetoprotein (AFP) in amniotic fluid (AF) for the detection of neural tube defects (NTD) is discussed. The improved quality of ultrasound examinations and the implementation of foetal anomaly scans in the 2nd trimester of pregnancy, as part of a national screening program, provide new possibilities for the detection of NTD.

In Chapter 3, the relevance of measuring lysosomal enzymes in AF supernatants and amniocytes for the detection of lysosomal storage diseases in pregnancies with a non-immune hydrops foetalis is described. In 5% of the pregnancies a lysosomal storage disease was detected as causal factor. In conjunction with gene mutation analysis, prenatal testing can be offered in a next pregnancy. A laboratory workflow for routine metabolic analyses of foetuses with a non-immunological hydrops foetalis and a normal karyotype is proposed.

In Chapter 4, the detection of the most common foetal aneuploidies in AF samples with a rapid DNA test, based on MLPA kit P095, is described. The application of this DNA test as a stand-alone test for rapid aneuploidy detection (RAD) in pregnant women at risk for a child with Down syndrome is debated, since in approximately 11,500 of the cases clinical relevant chromosomal abnormalities will remain undetected. With a rapid
DNA test as stand-alone test, however, fast and targeted answers can be provided to the referral question.

In an attempt to further optimise the MLPA test, individual probe performances of MLPA kit P095 were assessed (Chapter 5). The results obtained indicated that exclusion of the poorest performing probe only slightly affected the overall sensitivity of the test, thereby underscoring the robustness of the test.

In Chapter 6, the application of the MLPA test for chorionic villus samples (CVS) is described. Routinely, cytogenetic analyses are performed on extra-embryonic chorionic tissue, composed of cytotrophoblasts and mesenchymal cells. Metaphases from these cells can be analysed in short-term cultures (STC) and long-term cultures (LTC), respectively, generating reliable test results representing the foetal karyotype. In order to assess DNA from both cell types with the MLPA test, a chorionic villi dissociation protocol was tested. By doing so, cytotrophoblasts and mesenchymal cells could be obtained separately for DNA isolation, thus creating the possibility to offer RAD as a CVS option to pregnant women at risk for having a child with Down syndrome.

In Chapter 7, clinical prenatal examples with ultrasound abnormalities and a normal karyotype are presented in which sub-microscopic aberrations were detected by MLPA and/or microarray analysis. This retrospective study has shown that with the microarray technology the level of detection of clinically relevant sub-microscopic aberrations may increase substantially. However, prenatal application of the microarray technology is as yet debated, since this technology may generate test results of which the clinical relevance is uncertain.

Finally, in Chapter 8 a number of factors is described that contributes to improved prenatal diagnosis in relation to patient healthcare. A central dogma herein is that the reason of referral must determine the choice for a diagnostic test. Uncertain test results should be avoided as much as possible, the test should preferably be rapid and its results available early in pregnancy. The chapter ends with a preview on future developments in prenatal diagnostics. Via increases in knowledge on the aetiology of hereditary and congenital diseases acquired through the Human Genome Project, and its concomitant technological innovations, it is anticipated that prenatal diagnostics will continue to change in the coming years. In particular, major changes are expected with respect to foetal detection of Down syndrome in maternal plasma (non-invasive diagnostics). A number of aspects of this new form of diagnostics, and its relation to developments in mass spectrometry and/or next-generation-sequencing, are discussed. Additional genome association studies and animal models to assess genotype-phenotype associations will in the future generate possibilities to predict the phenotype of an unborn child with Down syndrome. This will enable prospective parents and professionals to make better informed choices about the pregnancy and the future of their child.

In summary, it can be concluded that it is of utmost importance to join technological advances and changing insights into prenatal diagnostics in order to (continue to) provide optimal patient healthcare.
Samenvatting

Invasieve prenatale diagnostiek, door middel van een vlokkentest of vruchtwateronderzoek, is gericht op het detecteren van afwijkingen bij de foetus (Hoofdstuk 1). Chromosomen onderzoek in foetale cellen gebeurt vanouds door middel van karyotypering, met rapportagetijden variërend van 10 dagen (vlokkentest) tot 3 weken (vruchtwateronderzoek). Echter, de ontwikkeling en implementatie van nieuwe moleculaire testen zoals fluorescentie in situ hybridisatie (FISH), multiplex ligatie-afhankelijke probe amplificatie (MLPA) en kwantitatieve fluorescente polymerase kettingreactie (QF-PCR) hebben de prenatale diagnostiek veranderd. Via deze testen is het mogelijk geworden om de meest voorkomende aneuploïdieën van chromosomen 13, 18, 21, X en Y binnen 24 tot 48 uur op te sporen. Daarnaast biedt de toepassing van microarray technieken ongekende mogelijkheden voor de detectie van submicroscopische chromosomale afwijkingen. Potentiële pathogene genetische afwijkingen, zoals submicroscopische deleties en/of duplicaties, kunnen op efficiënte wijze worden geïdentificeerd. Deze ontwikkelingen in de prenatale diagnostiek, in combinatie met verbeteringen in ultrageluidonderzoek en de implementatie van prenatale screeningsprogramma’s, leiden tot meer gedetailleerde (genetische) kennis over het ongeboren kind. In dit proefschrift worden nieuwe diagnostische testen en de daarmee samenhangende klinische toepassingen beschreven in relatie tot kwaliteitsverbetering van prenatale zorg.

In Hoofdstuk 2 wordt de relevantie van het routinematig meten van α-foetoproteïne (AFP) in vruchtwater voor de detectie van neurale buisdefecten (NBD) ter discussie gesteld. De verbeterde kwaliteit van ultrageluidonderzoek en de implementatie van structureel echoscopisch onderzoek (SEO) in het 2de trimester van de zwangerschap, als onderdeel van een nationaal screeningsprogramma, bieden nieuwe mogelijkheden voor de detectie van NBD.

In Hoofdstuk 3 wordt de relevantie van het meten van lysosomale enzymen in vruchtwater voor de detectie van stapelingsziekten in zwangerschappen met een niet-immunologische hydrops foetalis beschreven. In 5% van de zwangerschappen kon een lysosomale stapelingsziekte als oorzakelijke factor worden aangemerkt. In combinatie met genmutatie analyse kan bij een eventuele volgende zwangerschap prenatale diagnostiek worden aangeboden. Een laboratoriumprotocol voor het routinematig uitvoeren van metabole analyses bij foetussen met een niet-immunologische hydrops foetalis en een normaal karyotype wordt voorgesteld.
In **Hoofdstuk 4** wordt de detectie van de meest voorkomende foetale aneuploïdieën in vruchtwater met behulp van een DNA sneltest, gebaseerd op MLPA kit P095, beschreven. De toepassing van deze DNA test als ‘stand-alone’ test voor sneldetectie bij zwangere met een verhoogd risico op een kind met Down syndroom wordt ter discussie gesteld, omdat in ongeveer 1:1500 van de gevallen klinisch relevante chromosomale afwijkingen niet zullen worden gedetecteerd. Echter, met deze DNA sneltest als stand-alone test kan wel snel en gericht antwoord worden gegeven op de onderzoeks vraag.

In een poging om de MLPA test verder te optimaliseren werden prestaties van individuele probes uit de MLPA kit P095 onderzocht (**Hoofdstuk 5**). De verkregen resultaten lieten zien dat het uitsluiten van de probe met de grootste standaarddeviatie de diagnostische gevoeligheid van de test vrijwel onveranderd liet, hetgeen de robuustheid van de test bevestigt.

In **Hoofdstuk 6** wordt de toepassing van de MLPA test op vlokken (chorionvilli) beschreven. Chromosomenonderzoek in vlokken wordt standaard uitgevoerd op extraembryonaal weefsel, bestaande uit cytotrofoblasten en mesenchymale cellen. Metafasen van deze cellen kunnen respectievelijk uit de STC (short-term culture) en de LTC (long-term culture) worden bestudeerd, welke gezamenlijk op betrouwbare wijze het foetale karyotype representeren. Om met de MLPA test het DNA van beide celtypen te onderzoeken werd gebruik een celdissociatieprotocol getest. Gebleken is dat op deze manier cytotrofoblasten en mesenchymale cellen gescheiden kunnen worden verkregen voor DNA isolatie. Daarmee werd de mogelijkheid gecreëerd om zwangere met een verhoogd risico op een kind met Down syndroom, nu ook de DNA sneltest op vlokken als optie aan te bieden.

In **Hoofdstuk 7** worden prenatale voorbeelden met een echoscopische afwijking en een normaal karyotype gepresenteerd waarin met behulp van de MLPA en/of microarray technologie submicroscopische afwijkingen werden gevonden. Dit retrospectieve onderzoek heeft laten zien dat door middel van de microarray technologie het detectieniveau van klinisch relevante submicroscopische afwijkingen substantieel kan toenemen. Echter, prenatale toepassing van de microarray technologie wordt vooral nog ter discussie gesteld omdat deze technologie kan leiden tot onderzoeksresultaten waarvan de klinische betekenis onduidelijk is.

Tenslotte wordt in **Hoofdstuk 8** een aantal factoren beschreven die bijdragen aan verbeterde prenatale diagnostiek in relatie tot patiëntenzorg. Centraal hierbij staat het dogma dat de onderzoeksvraag bepalend is voor de keuze van de diagnostische test.
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Curriculum Vitae
List of publications
2006


2007


2008


2009


2010

Mundhofir FEP, Kooper AJA, Winarni TI, Smits APT, Faradz SMH, and Hamel BCJ. A small supernumerary marker (sSMC) chromosome 22 due to a maternal translocation between chromosomes 8 and 22: a case report. Journal of Genetic Counseling (submitted).
Chapter 1

Figure 1.

(A) GTG banded karyotype. One chromosome 9 homolog exhibiting an enlarged heterochromatic region is marked by an arrow. (B) Metaphase spread from the same sample again showing a chromosome 9 homolog (arrow) with an enlarged heterochromatic region. (C) C-banding confirming the heterochromatic nature of the enlarged region of one of the chromosome 9 homologs (arrow). Together, these analyses result in a 46,XY,9qh+ karyotype.
Figure 2.
Overview of the participation of pregnant women in first trimester screening and invasive testing in the period 2000-2007.

Figure 3.
Examples of FISH on interphase (A) and metaphases (B, C and D). FISH probes can be classified into centromere-specific (A), locus-specific (B), chromosome-specific (C), or telomere-specific (D).

Figure 4.
Genotyper profile of a male trisomy 21 sample obtained after QF-PCR and subsequent processing using a genetic analyzer. One marker (D21S1414) on chromosome 21 shows a trisomic tri-allelic pattern (1:1:1), two markers (D21S1411 and D21S1446) show 2:1 ratios and one marker (D21S1435) shows a homozygous (non-informative) pattern. Both the X- and Y-specific products (AMXY) are present in a normal 1:1 ratio, together with the SRY-specific product. The XY male chromosome constitution is also evident from the normal heterozygous pattern of both pseudo-autosomal markers (X22 and DXYS218) and the single product obtained from the X-linked HPRT locus.
Figure 5.
Detection of trisomy 18 by MLPA. Capillary electrophoresis patterns from a normal female sample (upper panel) and a female sample with a trisomy 18 (lower panel) analysed with kit P095 are shown. The P095 probe-mix contains 36 different markers with amplification products ranging in size from 136 to 454 bp. Four of the probes will only generate a signal on male DNA samples (i.e., Y chromosome-specific). Every set of four peaks represents markers on chromosome 21, 18, 13 and X, respectively. The arrows mark the alterations: an increase of the fluorescent signals for 18m1-18m8 in the trisomy 18 sample relative to the same markers in the normal sample.

Figure 6.
Microarray profile showing a deletion of a 2.7 Mb region on chromosome 12. Each probe present on the array is arranged along the X-axis according to its location on the chromosome, with the distal p-arm clones towards the left and the distal q-arm clones towards the right. The log2 test-over-reference (T/R) ratio values are plotted on the Y-axis. The red dots in the upper panel represent individual probes. In the lower panel (blue) each dot represents the averaged T/R value of 10 neighbouring probes.
Chapter 3

Figure 2.
Thin layer chromatography of oligosaccharides in amniotic fluid from two pregnancies affected by HF (orcinol dye). Lane 1: dextran hydrolysate, Lane 2: GM1-gangliosidosis (case 2), Lane 3: normal amniotic fluid, Lane 4: galactosialidosis (case 1), Lane 5: reference containing (from top to bottom) fucose/xylose, glucose, galactose, maltose/glucuronic acid, lactose, raffinose, tetragalacoside, sialylactose. Abnormal lanes of both HF amniotic fluid samples are indicated by arrows. In both HF cases, amniotic fluid was aspirated in the 27th week of pregnancy, shortly after HF had been established.

Figure 3.
Proposed flowchart for prenatal diagnosis of non-immune hydrops foetalis
Figure 2.
Mean probe ratios of the chromosome 21, 18, 13, X and Y targets, with 50% of the mean probe ratios within the box.

Chapter 5

Figure 5.
Three dilution experiments in which samples with a trisomy 21, 18 or 13 were mixed with a normal sample. Eleven dilutions are plotted on the horizontal axis representing mixtures of (from left to right) 100-0, 90-10, 80-20, 70-30, 60-40, 50-50, 40-60, 30-70, 20-80, 10-90, 0-100% normal DNA and DNA with trisomy 21, 18 or 13, respectively. In this plot, the mean probe ratios for chromosome 21, 18 and 13 show a shift from a normal value of 1.0 towards a complete trisomy with a value close to 1.5. The black circle marks the lowest detectable mosaics (80-20% mixtures), with a mean probe ratio of 1.17 (95% CI: 1.01-1.32) for chromosome 21, a mean probe ratio of 1.13 (95% CI: 1.08-1.19) for chromosome 18 and a mean probe ratio of 1.11 (95% CI: 1.10-1.12) for chromosome 13. The red circle marks the dilutions of 20% normal DNA with 80% of trisomy 21, 18 or 13 DNA. These mixtures show a mean probe ratio of 1.43 (95% CI: 1.40-1.46) for chromosome 21, 1.39 (95% CI: 1.33-1.46) for chromosome 18 and 1.35 (95% CI: 1.31-1.38) for chromosome 13.
Figure 6. Electropherograms illustrating a normal female reference sample (A) and a patient sample (B). The eight series of four peaks represent MLPA probes for chromosomes 21, 18, 13 and X, respectively. Three peaks in sample B, representing 18m2, 18m4 and 18m6, are decreased as compared to the peaks of the normal reference sample and, therefore, indicative for a partial imbalance of chromosome 18. The corresponding probe ratios showed a mean of 1.09 (95% CI: 0.82-1.35) for 21, 0.84 (95% CI: 0.47-1.21) for 18, 1.14 (95% CI: 0.83-1.45) for 13 and 1.09 (95% CI: 0.93-1.25) for the X chromosome, respectively. The ideogram of chromosome 18 (C) shows the locations of the three decreased probes (18m2, 18m6 and 18m4) on the distal region of the long arm of chromosome 18. Follow-up karyotyping revealed a 46,XX,del(18)(q21.2) karyotype (D).

Figure 2. Laboratory flowchart for integrating rapid aneuploidy detection (RAD) in CVS into the cytogenetic diagnostic service. Approximately 30 mg of cleaned villi is split into three fractions (10 mg each). Two independent cell preparation procedures based on enzymatic digestion with collagenase and/or trypsin/EDTA are performed on fractions I and II to obtain suspensions from cytotrophoblasts (fraction C) and the mesenchymal core (fraction M), separately. A small amount of fraction M is used for LTC. Fraction III is stored for back-up. DNA is extracted from fractions C and M and RAD by MLPA or QF-PCR are assayed independently. The blue and red arrows indicate the routing for RAD as stand-alone test and the routing for TK, respectively. In this flow chart, TK of the STC is replaced by RAD on DNA from the cytotrophoblast fraction. Discordant results with RAD between fractions C and M or test failures is indicative for TK of the LTC or a repeat experiment using back-up fraction III. When the results of RAD are abnormal, follow-up karyotype analysis is performed to confirm the nature of the aneuploidy.
Chapter 7

Figure 1.
Chromosome copy number variations identified in case 1 by 250k SNP array analysis. The log₂ T/R (test-over-reference) ratio values are plotted on the Y-axis versus the genomic position on the respective chromosome represented by the idiogram on the X-axis in the lower part of the figure. The red dots in the upper panel represent individual SNP values. The thin blue line in this panel represents the effective HMM outcome with a normal [N=2] T/R ratio of 0. A significant imbalance is indicated by a rise or fall of this line by 0.3 identifying a single copy number gain and -0.38 identifying a single copy number loss. In the lower panel (blue) each dot represents the averaged value of 10 neighbouring SNPs. The array result revealed a loss of 2.9 Mb at 17p13.2p13.1. The four encircled gains are common CNVs (i.e., polymorphisms).

Figure 2.
GTG banded karyotype (A) of a cultured AF cell in case 2, showing a de novo apparently balanced translocation t(3;18)(q26.2;q21.3) (B). Chromosome copy number changes, identified by SNP array analysis, resulting in a loss of ~5 Mb at 3q26.3q27.2 (C).
Figure 3.
MLPA and SNP array results of case 3. Panel A shows the electropherograms of the normalized MLPA ratios (Y-axis) of a negative control sample without a 22q11 deletion (blue), a positive control sample with a common 22q11.2 deletion (red) and case 3 (green) with an atypical 22q11.2 deletion. The markers in kit P250 are chosen between 15,959,700 to 23,283,730. Case 3 shows single copy losses of five probes, two in the LCR22-C region (SNAP29, LZTR1) and three in the LCR22-D region (HIC2, PPIL2 and TOP3B), represented by reduced peak ratios of ~0.5 in the electropherogram. In panel B the copy number profile revealed by SNP array analysis is shown, including a loss of 1.3 Mb at 22q11.21q11.22.

Figure 4.
FISH analysis in case 4 showing the HIRA (red) and ARSA (green) regions, respectively (A). The arrow indicates the chromosome with the ARSA deletion. GTG banded chromosomes 22 from blood cells (B). The deletion chromosomes are marked by arrows. SNP array analysis (C) revealing a loss of 6.1 Mb in 22q13.31q13.33 ranging from SNP_A-2284753 to SNP_A-4279731.
Figure 5.
Schematic representation of the 22q11.2 region. The positions of FISH probe TUPLE/HIRA and the eight low copy repeat (LCR) clusters are marked (A to H from centromere to telomere). The positions the disease causing genes reported in the OMIM database are indicated. Several atypical 22q11.2 deletions that were previously reported (Mikhail et al., 2007; Shaikh et al., 2007; Ben-Shachar et al., 2008) are indicated. Case 3 and a recently reported case (Ogilvie et al., 2009a) exhibited deletions encompassing LCR-C and LCR-D (~1.3 Mb). These atypical deletions are not detected by FISH probe TUPLE/HIRA. Adapted from Descartes et al., 2008.