FETAL mRNA IN MATERNAL PLASMA

The studies described in this thesis were performed at the Department of Clinical Chemistry and at the division of Prenatal Diagnosis and Therapy, Department of Obstetrics and Gynaecology, VU University Medical Center, Amsterdam, the Netherlands.

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Fetal mRNA in maternal plasma

Development of a non-invasive prenatal test for trisomy 21
A quest of a Holy Grail

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Wheresoever you go, go with all your heart.

Conficius, Chinese philosopher

Aan mijn ouders †††
Aan mijn tante Atty

CONTENTS

CHAPTER 1

General Introduction

Prenatal diagnosis has become an integral part of obstetric care in the Western world. In the Netherlands since about 1975 women of 38 years and older were offered invasive testing like amniocentesis or chorionic villus sampling, because of the increased change on fetal aneuploidy due to advanced maternal age. In 1985 this indication was linked to a maternal age of 36 years. The first prenatal diagnosis of Down syndrome was reported in 1968.[1] Nowadays, about 40 years later, the main reason for invasive procedures in the Netherlands is still maternal age.[2] Since the end of the 1980's non-invasive screenings tests on Down syndrome have been developed. The last 10 years research mainly focused on the first trimester combination test as screening test on Down syndrome. [3-6] This test is a combination of a serum test and the nuchal translucency measurement. For the serum test the markers pregnancy-associated plasma protein A (PAPP-A) and free- beta-human chorionic gonadotropin (fβ-hCG) are measured between 9 and 14 weeks of gestation. The fetal nuchal translucency (NT) is a small translucent area in the posterior neck region and can be measured with ultrasound between 11 and 14 weeks gestational age. The test results of both, the serum test and the NT measurement, are combined with the age of the mother to calculate the risk on Down syndrome. The screening test has a sensitivity of 85-90% with a false-positive rate of 5-9%. When this calculation results in a risk-estimation on fetal trisomy 21 of 1 in 200 or higher, the woman is offered an invasive procedure to obtain fetal cells for analysis of the chromosomes. Since 2007 in the Netherlands the first trimester combination test must be offered to all pregnant women from 36 years and older. Younger women must be informed about the possibility of testing.

This screening test is based on measuring epiphenomena, associated with the chromosomal aneuploidy, trisomy 21 in particular. Although the test has a good test performance, it has no diagnostic potential. However, the most important disadvantage of the present diagnostic tests is that it is invasive and in that way holds a risk of miscarriage due to the procedure. This risk is small, but a threat to every tested pregnancy. The ratio of detection trisomy 21 on indication of advanced maternal age versus miscarriage is estimated to be 0.61.[7]

The availability of a non-invasive diagnostic test with the same potentials as the present diagnostic test would be ideal. Already for decades, development of such a diagnostic test has been a very challenging

research goal. Walknowska in 1969 published a landmark study showing the detection of cells bearing a Y-chromosome in blood of women carrying a male fetus. [8] Fetal cells in maternal plasma and its possibilities for clinical use have extensively been studied [9-14]. Detection of aneuploidy was obviously a part of that. Despite its high specificity, the sensitivity remains low. In a large multicentre trial fetal gender could be correctly identified in 41.4% of the cases with a false- positive rate of 11%. In cases of a known fetal aneuploidy, the aneuploidy was detected in 74.4% by using circulating fetal cells.[15] The rarity of fetal cells in the maternal circulation and the elaborate techniques necessary to enrich and identify these cells made a break-through to clinical implementation impossible. Since the finding of cell free fetal DNA (cffDNA) in maternal plasma and serum in 1997 a new era begun.[16] A few years later in 2000 the presence of placenta mRNA in maternal plasma was demonstrated.[17] The presence of these fetal nucleic acids in maternal circulation is a source for further research and development of clinical tests. The genetic differences between mother and fetus cffDNA can be used for prenatal tests. Research on determination of fetal gender has led to a reliable non-invasive test which can be used in clinical practice, for example in cases of sex-linked disease[18] or congenital adrenal hyperplasia[19]. Non-invasive determination of fetal RhD status in pregnancies of RhD-negative women also found its way to clinical practice[20]. For aneuploidy detection the situation is more complicated.

In this thesis the studies are directed toward development of a robust non-invasive prenatal test for trisomy 21 in the late first- or early second trimester of pregnancy, preferable easy to implement and at reasonable costs.

In the **second chapter** cellular origin, biological features and clinical potentials of cell-free fetal nucleic acids, both DNA and mRNA present in maternal plasma and serum during pregnancy is described. A general overview is given. **Chapter 3** is focussed on the non-invasive detection of aneuploidy, especially trisomy 21. Recent developments and techniques using fetal DNA and placental mRNA in maternal plasma are outlined. Their pros and con's are discussed.

Chapters 4 to 10 contain studies focussing on several aspects of the development of a clinical test for detection of trisomy 21 using mRNA in maternal plasma. We defined criteria markers for trisomy 21 detection: the gene analysed should be

1. encoded by chromosome 21.
2. located within the Down syndrome critical region (DSCR).
3. expressed in first trimester normal placenta tissue.

4. overexpressed by the placenta in trisomy 21 pregnancies.
5. detectable in maternal plasma during early pregnancy.
6. absent in plasma of non pregnant women.

In **chapter 4** detection of chromosome-21 encoded mRNA of placental origin in early placenta tissue and in first trimester maternal plasma was studied. The aim was to identify possible markers for prenatal testing on trisomy 21. A large panel of RNA targets, distributed over all chromosomes except for the Y chromosome, known or expected to be present in extra embryonic tissues, were tested for their presence in early placental tissue, presence in pregnant plasma and absence in non-pregnant plasma. These results are described in **chapter 5**. Among these were genes coding for transcription factors, meaning for gene products not accessible by conventional antibody-based assays. This can be expected to greatly increase the number of markers that become available for non-invasive prenatal diagnosis. The search for possible markers is extended in **chapter 6.** A novel method was tested to identify syncytiotrophoblast-derived RNA products in vitro. RNA was obtained selectively by controlled denudation from syncytiotrophoblast cells of an early second trimester placenta with confirmed trisomy 21. The RNA isolation can be used for cDNA synthesis including adequate recovery of small sized RNA s.

The mRNA LOC90625, nowadays called C21orf105, was the most promising candidate found as marker for a non-invasive test for trisomy 21. Using quantitative RT-PCR we tested this marker in plasma, obtained between 9 and 15 weeks gestational age, from pregnancies carrying a fetus with trisomy 21 and controls. The results are described in **chapter 7**. In the mean time another strategy was described, the RNA single-nucleotide polymorphism (SNP) allelic ratio strategy. In this strategy quantitative comparison of the allelic expression ratio of a chromosome 21 encoded gene (meeting the above mentioned criteria for a trisomy 21 marker) enables the detection of the differences between 2 and 3 copies of chromosome 21. Due to the fact that polymorphism is the crux for discrimination, the RNA-SNP ratio strategy is limited to a subset of the population with a heterozygosis of the SNP used. Theoretically an increase in population coverage can be obtained by combining the results of several markers. So the availability of useful SNP's was studied. In **chapter 8** we tested 44 SNP's expressed by 7 chromosome 21 encoded, placenta expressed genes. Heterozygote frequencies are described.

In the RNA-SNP allelic ratio strategy study from Lo et al, the assay used is based on extension of the polymorphic site to generate small but very specific allele-dependent differences in size. This approach requires highly specialized equipment, which might limit its widespread implementation in routine diagnostic setting. We tested and validated two

methodological adaptations: the quencher extension (QEXT) and the WAVE System (Transgenomic). **Chapter 9** covers both methods tested in a model system measuring allelic-expression ratios in trisomy 21 and control placentas. The WAVE System permits rapid identification of informative heterozygous samples. The QEXT reaction is a novel single-step, real-time method to quantify SNPs and is directly adaptable to current real-time PCR equipment. Finally in **chapter 10** the quencher extension technique is tested in clinical samples to discriminate trisomy 21 plasma samples from controls.

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Fetal DNA and mRNA in maternal plasma

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FETAL NUCLEIC ACIDS IN MATERNAL CIRCULATION

Introduction

For prenatal diagnosis of genetic disorders of the fetus like aneuploidy or inherited genetic diseases, invasive procedures are required to obtain informative fetal cells. As these invasive procedures, i.e. amniocentesis and chorionic villus sampling, have an intrinsic risk of fetal loss by the technique being invasive, it is considered a big challenge to develop a *non*-invasive test with identical diagnostic possibilities and specificities. The presence and detectability of fetal cells in maternal blood was seen as the entrance to a new era of 'non invasive' prenatal diagnosis. For instance, trophoblast studies demonstrated fetal DNA to be present amongst maternal cells in early pregnancy in the cellular fraction with densities characteristic for trophoblast. After analysis of the cellular fraction enriched for fetal cells it was possible to predict fetal sex correctly in 91.7% of the cases in weeks 9 to 13 of pregnancy by the polymerase chain reaction for X- and Y-chromosome specific sequences[1]. In addition, immunostaining (HLA-G) combined with fluorescent in situ hybridization (XY) confirmed the existence and detectability of trophoblast cells in the maternal blood [2]. However, the rarity of fetal cells, both of extraembryonic (trophoblast) and embryonic origin (nucleated red blood cells) in the maternal circulation and the elaborate techniques necessary to enrich and to identify these cells, prevented a break-through with widespread clinical use [3]. Despite its high specificity (when fetal cells are identified, their fetal origin can be scored reliably), the sensitivity remains low. Fetal cells cannot be found in all pregnant samples or found in numbers too low to permit reliable clinical implementation. The latter features prevented their introduction and use in a routine clinical setting. The finding of cell-free fetal DNA in plasma and serum of pregnant women as first demonstrated by Lo et al.[4] revitalized and redirected the pursue of non-invasive prenatal diagnosis. Circulating fetal nucleic acids, both genomic DNA and messenger RNA , can be obtained from pregnant plasma and serum in amounts much higher and by methods less elaborate than the amounts obtained and methods used for fetal DNA from the cellular fractions. Moreover, fetal mRNA can be obtained from maternal plasma as well.

This review focuses on the cellular origin, biological features and clinical potentials of the cell-free fetal nucleic acids, both DNA and mRNA present in maternal plasma and serum during pregnancy.

Biology of circulating DNA

Circulating fetal DNA during pregnancy

Fetal DNA is present in maternal blood in a high background of maternal DNA. Lo et al. started with the use of the most obvious difference between maternally and paternally derived genetic material, the Y chromosome. The Y chromosome is present if the fetus is male. With the polymerase chain reaction (PCR) technique, amplification of a single-gene copy sequence (DYS 14) from the Y chromosome was performed. By this technique, 80 % of the male pregnancies were correctly identified [4]. All the female bearing pregnancies were negative for the Y sequence. These findings were confirmed by others [5-7]. The mean concentration of fetal DNA in plasma is about 11 times higher in late gestation then in early gestation. In early pregnancy, fetal DNA in maternal plasma constitutes a mean of 3.4% (range 0.39%-11.9%) of the total amount of DNA which includes maternal DNA. In late pregnancy the mean is 6.2% (range 2.33%-11.4%). This is about 21 times higher than the amount of fetal DNA found in the cellular fraction in maternal blood at the same gestational age [8], even despite enrichment of these fractions for fetal cells. This clearly indicated one important advantage of cell free fetal DNA over cellular DNA. Fetal i.e. male DNA can be detected in maternal plasma as early as 32 days of gestation and can be detected consistently after 52 days of gestation, both by quantitative (real-time) or semiquantitative (conventional) PCR amplification [6; 9]. This demonstrates the second advantage if fetal DNA is to be used for clinical diagnosis: detection becomes possible within or before the same time window as currently employed for invasive prenatal diagnosis. By analysis of serial blood samples from women pregnant after in vitro fertilization or intra uterine insemination, the earliest detection of Y-chromosome specific SRY sequences was at 5 weeks and 2 days. By 10 weeks of gestational age, the correct fetal sex can be detected in all patients. In pregnancies ending in a miscarriage, SRY can not be detected [10]. In the first trimester, the calculated increase of the amount of fetal DNA is about 4.2 genome equivalents/ml/week [9]. In the late third trimester, there is an inter-individual variation in fetal DNA concentration. The median concentration is 74 genome-equivalents/mL, range 53-141. Serial sampling in late trimester shows a mean increase of 29.3% with each week. There is no statistically significant correlation between β-globin concentration and gestational age. β-Globin sequences in maternal plasma represent the total amount of extracted DNA, both maternal and fetal [11].

Information about fluctuation of DNA and fetal DNA levels within an individual is scarce. Zhong et al. analyzed blood samples taken on 3 consecutive days from healthy males, healthy females and 16 healthy preg-

nant women with uncomplicated pregnancies between 9 to 42 weeks of gestation. The total amount of free circulatory DNA in pregnant and non-pregnant individuals fluctuates with a range of 1.3 to 130 fold. The concentration of male fetal DNA in the 10 pregnant women carrying a male fetus is less with a mean difference of 2.2 fold (range 1.4-4.5). The variation in the amount of circulatory fetal DNA occurred independently of the variation in the quantity of maternal DNA. It can be concluded therefore that the release of fetal versus maternal DNA is not coupled[12]. This reflects their different origin, biological clearance or other factors.

Size distribution of DNA and fetal DNA has also been studied. The median percentage of plasma DNA with sizes > 201 bp was 57% for pregnant women and 14 % for non-pregnant women. The median percentage of fetal-derived DNA (SRY gene) with sizes >193 bp and > 313 bp was 20 and 0%, respectively, in maternal plasma. Fetal DNA molecules are therefore shorter than maternal DNA molecules. On the other hand, the DNA fragments in the plasma of pregnant women are significantly longer than those in the plasma of non-pregnant women[13]. Similar results were found in another study, where the potential advantage of size separation was explored. Theoretically, selection or enrichment could permit easier analysis of both paternally and maternally inherited DNA polymorphisms. But a solid determination of fetal ploidy by determination of paternally and maternally inherited polymorphic markers was not yet possible with the method used[14].

Methods to increase the percentage of free fetal DNA in maternal serum and plasma have been described. The addition of formaldehyde to maternal blood samples has been claimed to increase the yield of fetal DNA in samples shipped overnight[15]. By stabilization of the cell membranes, the percentage of maternal cell lysis is decreased, and thereby the amount of free maternal DNA leaking into the plasma. As such, the relative percentage of free fetal DNA increases. The absolute percentage remains the same. Another study done with samples shipped overnight, supports this hypothesis. No difference in fetal DNA concentration was observed in the sample groups with or without addition of EDTA and with or without addition of formaldehyde. The total DNA concentration decreases dramatically when the sample was treated with formaldehyde ($P<0.0001$). So the proportion of fetal DNA increased in samples collected in tubes containing formaldehyde[16]. This dramatic difference between samples with and without formaldehyde could not be confirmed in a systematic study of plasma samples taken in the second and third trimester of pregnancy and centrifuged immediately or after 6 or 24 hours. The authors also investigated the cause of the discrepancy. They conclude that this could have resulted from the imprecise estimation when the previously reported serial dilution method was used[17].

Circulating fetal DNA after pregnancy

If to be used reliably for clinical applications, it is crucial that fetal DNA disappears from the circulation after delivery with no carry over and persistence into subsequent pregnancies. In the first publication, female bearing pregnancies were tested negative although these women gave birth to sons in the past. This was a good indication. Detailed analysis on the clearance of fetal DNA from maternal plasma showed that the calculated mean half-life was 16.3 minutes (range 4-30 min). One day after delivery, no fetal DNA can be detected in the samples from 12 women[18]. This was confirmed by Rijnders et al. who studied 120 women who had either delivered previously or were childless. All women were non-pregnant at the time of analysis. Of these, sixty-four women had previously given birth to one or more sons, 43 were childless. From the latter, 5 had abortions or extra-uterine pregnancies. The rest only gave birth to daughters. This cohort therefore consisted of a representative study set of females. In all cases, no SRY sequences were found by PCR in the DNA isolated from plasma[19]. These results were confirmed by Smid et al. and Benachi et al.[20; 21].

Therefore, the majority of studies show absence of long time persistence of fetal DNA in maternal plasma, except for one study. Invernizzi et al. showed the presence of fetal DNA in maternal plasma decades after pregnancy[22]. Lambert et al. reported the presence of fetal DNA in 36% of the non-pregnant women who gave previously birth to a son, but filtration studies showed that this DNA was not cell-free [23]. Smid hypothesizes that the centrifugation protocol of Invernizzi et al. led to fetal SRY amplification originating from fetal cells remaining in the supernatant after centrifugation. For fetal nucleated cells, it is known that specific subsets can be detected years after delivery[24] and can settle and survive in specific maternal tissues. These persistent cells (pregnancy associated progenitor cells) are unique in nature, possess stem cell properties and share phenotypic characteristics with lymphoid cells. Details on these intriguiging cells are explained in another chapter.

Clearance from the circulation

The mechanism underlying clearance of fetal DNA from plasma is unknown. The kidney and liver are logical candidates to play a role in this process. Studies on renal clearance show conflicting results. In two studies, Y-chromosome specific sequences could be detected in a number of urine samples (first trimester) of pregnant women carrying a male fetus. Botezatu et al. could detect Y-chromosome specific sequences in urine of 8 of 10 pregnant women carrying a male fetus. Controls, i.e. pregnant women carrying a female fetus were all negative[25]. Al-Yatama et al. found in 38% of the samples a positive result[26]. Li et al. studied urine of normal

pregnant women and of women with preeclampsia and HELLP syndrome in the third trimester and could not detect fetal DNA[27]. Maternal plasma and urine samples were collected in the third trimester from 20 pregnant women. Eighteen of them had an unremarkable pregnant history and delivered healthy babies. Two had manifest HELLP syndrome with proteinuria of at least 5 g on a 24-hour urine collection. Urinary DNA was examined by Y-chromosome-specific nested polymerase chain reaction (PCR) or real-time PCR. No fetal DNA could be detected in maternal urine. Even not in those pregnancies affected by HELLP syndrome although copious quantities of cell-free DNA were present in maternal plasma. Total cell-free DNA in maternal urine was measured using real-time PCR assay for GAPDH (glyceraldehydes-3-phosphate dehydrogenase) gene, no differences in quantity was found between normal pregnancies and pregnancies complicated by HELLP syndrome.

Clearance of fetal DNA after delivery has been found impaired in patients with pre-eclampsia. Median half-life was 114 minutes in patients with pre-eclampsia and 28 minutes in controls. Six hours after delivery fetal DNA was not detectable anymore in the majority of the controls but still present (median 208 genome-equivalent/ml) in all of the preeclampsia cases. Preeclampsia is a multisystem disorder associated with damage and dysfunction of many organ systems, including liver and kidney. Organ damage is likely to induce the observed abnormalities in fetal DNA clearance[28]. The liver as a detoxification organ might play a role, but there is no direct proof for this hypothesis. Indirect evidence of the involvement of the liver is indicated by the study of Nelson who reported an impaired fetal DNA clearance in a case with acute fatty liver disease in pregnancy. Fetal DNA remained detectable until 11 days after delivery[29].

Source and mechanism of release

It is still an enigma where fetal DNA in maternal plasma comes from. After delivery, fetal DNA from maternal plasma is cleared rapidly, mean half-life of 16.3 minutes[18]. This result together with the fact that fetal DNA can be detected in all stages of pregnancy indicates that fetal DNA is liberated in large quantities into the maternal circulation as long as the placenta and/or fetus is present, while clearance by the maternal system is a constant process[30].

Because the fetal DNA is not immediately degraded and can be readily amplified from plasma and serum, it has been suggested that the circulating DNA is protected within apoptotic bodies. As possible sources haematopoietic cells, trophoblast and direct feto-maternal transfer of DNA molecules have been suggested. Of these, the placenta is the most logical source for several reasons. The placenta is large, has abundant cellular activity, is the first organ formed and is in direct contact with maternal

blood. In a case of placenta increta a small part of the placenta remained adherent to the uterus. Post partum monitoring showed that fetal DNA was detectable until 10 weeks after delivery[31]. Recently, an interesting case has been published. In a pregnant woman carrying a male pregnancy, the SRY gene was absent in maternal serum despite male genitalia at ultrasound. The karyotype was 45,X after direct trophoblast culture and 45,X/46,Xidic (Yp) after culture and in all fetal tissues studied. This confined placenta mosaicism with Y chromosomal sequences present in all fetal tissues, but absent in cytotrophoblast and maternal serum indicates that free fetal DNA originates from trophoblast cells[32]. Secondly, an interesting study on the time of appearance of fetal DNA in maternal plasma has recently been published. Fetal SRY gene sequences were measured in serum samples of 22 women who conceived using artificial reproductive technology. In male pregnancies, the SRY gene was found as early as day 18 following embryo transfer in a twin gestation, day 22 following embryo transfer in a singleton gestation and by day 37 in all ten women pregnant from a male fetus.[33] The definitive fetoplacental circulation is not established until days 28-30 post-conception. In another study SRY gene was detectable from 35 days of gestation (about 23 days after conception). Eighty percent of the study subjects had detectable fetal DNA in their blood at a gestational age of 49 days. The appearance of fetal DNA in the maternal circulation prior to the establishment of fetoplacental circulation, once again indicates that the trophoblast is the most important source and in first trimester probably the only source[10]. The finding of placental specific mRNA molecules in maternal plasma further support the hypothesis that the placenta is the main source [34] as is discussed below. Fetal nucleated red blood cells might contribute at least after the first trimester but the amount of DNA from these cells in maternal blood is less then the amount of cell-free fetal DNA[8]. There are about 19 nucleated fetal cells in 16 ml of maternal blood [35]. Apoptosis of fetal nucleated erythrocytes (NRBC) has been studied using a technique called TdT-mediated dUTP nick end labelling (TUNEL). A significant number, 42.7%, of nucleated erythrocytes were undergoing apoptosis in maternal blood at the time of sampling[36]. On the contrary fetal DNA levels were significantly elevated in preterm labour [37] without any incremental increase in fetal NRBCS[38]. So fetal haematopoietic cells add to the total amount of DNA, but they do not form the main source.

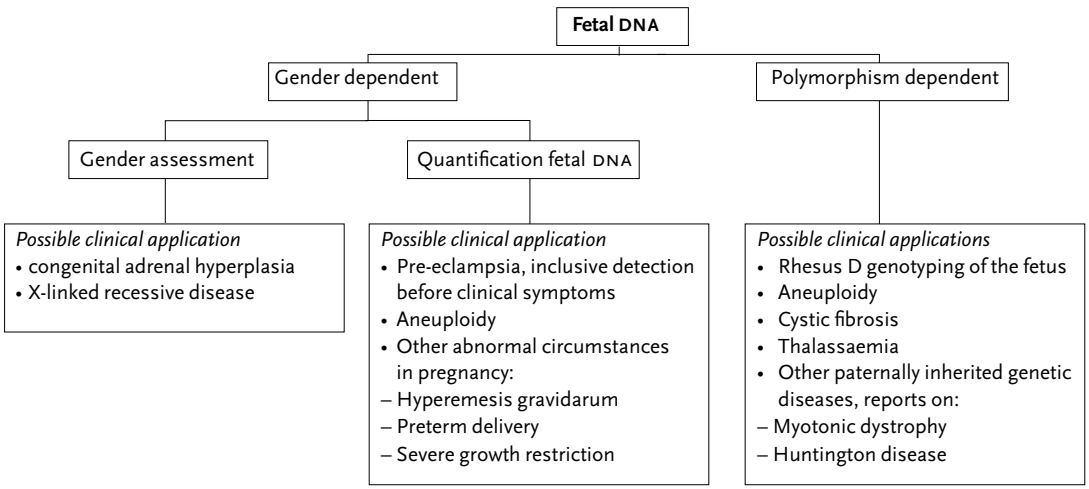
CLINICAL APPLICATIONS

Gender dependent approach

Fetal gender assessment

In pregnancies at risk for congenital adrenal hyperplasia or X-linked recessive diseases, like haemophilia, determination of the sex of the fetus is a common indication to perform an invasive procedure. In congenital adrenal hyperplasia, a female fetus is at risk for virilization. By giving the mother dexamethasone as early as possible, virilization of an eventually affected fetus can be prevented. If the pregnancy concerns a male fetus, dexamethasone can be stopped, because male fetuses are not at risk in utero. So in 50 % of the cases dexamethasone is started unnecessarily and an invasive procedure can be avoided in male fetus carrying pregnancies. In X-linked recessive diseases, it is the other way around. Female fetus carrying pregnancies are not at risk [39]. Using the Y single-copy gene sequence DYS 14 to identify fetal DNA in maternal plasma and serum[4] for fetal sex determination, 95-100% of male and 100% of female fetuses were correctly identified [5-7; 40-42]. These good results justify clinical application, especially for congenital adrenal hyperplasia. Rijnders et al. propose starting treatment with dexamethason and testing from 5 weeks gestational age onward with serial testing up to 11 weeks or until male DNA is detected. In male fetuses, dexamethasone treatment can be stopped with invasive tests becoming unnecessary. As long as no male DNA is detected, dexamethasone treatment should be continued except

Overview of clinical applications of fetal DNA in maternal plasma, grouped by fetal DNA characteristics. **Tabel 1**



when karyoptype analysis of fetal chorionic villi shows the fetus is an unaffected female[40]. Unnecessary invasive procedures and the related risk on miscarriage can be avoided by reliable identification of the fetus not at risk, in this case the males.

Quantification of fetal DNA

1. *Pre-eclampsia*

In pregnancies complicated by pre-eclampsia, levels of circulating fetal DNA are significantly (five to ten times) higher then in normotensive pregnancies[43; 44]. This increase is correlated with severity, the amounts are higher if the pre-eclampsia is more severe or complicated by the HELLP (Hemolysis, Elevated Liver enzymes, Low Platelets) syndrome [44; 45]. Total DNA is also elevated in plasma of pregnant women with pre-eclampsia[44].

Elevation of cell-free fetal DNA before the onset of preeclampsia has also been studied. Prior to the onset of clinical presentation of pre-eclampsia, an increased concentration of maternal plasma fetal DNA can be detected in mid-trimester, in one study even in early second trimester in susceptible subjects[46]. However, there was an overlap in fetal DNA concentrations between the pre-eclamptic and control groups [46-49]. Levine et al. reported a two-stage elevation in a group of 120 preeclampsia cases and 120 matched controls. At stage 1 (17-28 weeks of gestation) and stage 2 (within three weeks of the onset of preeclampsia), fetal DNA was elevated compared to controls. Before 17 weeks of gestation, no difference could be found between both groups. Amongst cases there were differences in the amount of fetal DNA according to the disease severity, gestational age and the presence of a small-for-gestational age infant [50]. Early identification of women at risk for developing severe preeclampsia is important for close monitoring and if preventive treatments are available. But as long as the overlap between normal pregnancies and pregnancies complicated by pre-eclampsia is high, neither early detection nor prediction is feasible in individual patients.

In patients with pre-eclampsia clearance is impaired[28]. The underlying mechanism, like the origin of fetal DNA is unclear, but it can be due to increased liberation of fetal DNA in the maternal circulation and/or reduced clearance of circulating DNA from maternal blood. Increased liberation can be a result of increased degradation of cells, by necrosis or apoptosis. The amount of fetal DNA can increase because of placental hypoxia, the amount of maternal DNA and as such total DNA because of endothelial damage of the maternal vessels. Both conditions are present in preeclampsia. However, placental dysfunction is not unique for preeclampsia.

2. Aneuploidy

Although not found in all studies, there seems to be an indication that fetal DNA levels in maternal plasma and serum are higher in trisomy 21 pregnancies compared to euploid pregnancies. Experienced research groups found significant higher concentrations fetal DNA in plasma of pregnant women carrying a male fetus with trisomy 21 fetal DNA. In the study of Lo et al., the median cell-free fetal DNA in women carrying trisomy 21 fetuses was 2.96-fold higher than that of women carrying euploid fetuses in the Hong Kong group. In samples collected in Boston, but measured in Hong Kong, a 1.97-fold increase was found [51]. The group of Zhong et al. confirmed these findings. In this study trisomy 13 and 18 were also studied. In the trisomy 13 cases (3) the concentration was also significant higher, but in the trisomy 18 group (6) no significant difference was found between cases and controls [52]. If archived maternal serum was analysed a 1.7 fold higher level of cell-free fetal DNA compared with matched controls were found. [53] It is remarkable that clinical used markers for Down syndrome of placental origin such as hCG and inhibin-A are also elevated about twice the value of euploid pregnancies. It is possible that the observed elevation of f-DNA levels reflects a similar biologic pathway. Like hCG and inhibin-A the median concentration of fetal DNA seems to be higher, but there is an important overlap with the normal range. If the analysed values were expressed as multiples of the median, test performance can be determined as a screenings marker for Down syndrome. Fetal DNA alone was calculated to give a 21% detection rate at a 5% false-positive rate[54]. When added to second trimester quadruple marker screening, fetal DNA increased the estimated detection rate from 81% to 86% at a 5% false-positive rate[54]. For comparison total hCG alone in maternal serum is calculated to gave a detection rate of 53% at a false positive rate of 5%, inhibin-A 59% at a detection rate of 5%. [55] Yet the calculated test performance of fetal DNA is not very impressive.

In two studies no difference in fetal DNA concentration could be found between cases and controls. Hromadnikova measured DNA levels in maternal plasma in the early second trimester using real-time PCR using SRY and β-globin genes as markers. The median fetal DNA levels in women carrying Down syndrome fetus (n=11) and the controls (n=13) were 23.3 and 24.5 genome-equivalents/ml. Total median DNA levels in cases and controls were also not significantly different[56]. Ohashi used maternal serum in the early second trimester. He included 5 male Down syndrome pregnancies and 55 male controls with normal karyotype. Mean concentration of fetal DNA in maternal serum of the controls was 31.5 copies/ml and 23.5 copies/ml in the

cases. No significant differences in the concentration of fetal DNA were found[57].

3. Others abnormal circumstances in pregnancy

In several pregnancy associated complications, like hyperemesis gravidarum, preterm delivery, severe growth restriction, a case of polyhydramnios[58] and invasive placenta, increased levels of fetal DNA in maternal plasma compared to normal pregnancies have been described. In patients with hyperemesis gravidarum the median concentration of fetal DNA in plasma of cases was higher then in controls [59]. If subdivided according to severity a relationship was found. The more severe the hyperemesis gravidarum, the higher the concentration of fetal DNA[60]. In patients with preterm delivery not reacting on tocolytic therapy the median concentration of fetal DNA was significantly higher compared to normal pregnancies. In cases of threatened preterm labour, for which tocolytical therapy was started and the delivery was postponed to term, no difference was found in fetal DNA between cases and controles[37]. Reports on the levels of cell-free fetal DNA in the plasma of pregnant women with severe fetal growth restriction are conflicting. Sekizawa et al. could not demonstrate an increase in fetal DNA level[61]. Caramelli et al. found in the (sub) group with an abnormal Doppler WAVEform in the uterine artery and growth restriction significantly higher levels of fetal DNA, with a Multiple of the Median (MoM) of 2.16 [62]. In a few cases with placenta praevia a significant higher concentration of fetal DNA was observed, in two cases of placenta increta the concentrations were even higher. In one case of placenta increta, a piece of placenta could not be removed. The concentration of fetal DNA during delivery was very high, 1104 genome-equivalent/ml [63]. Fetal DNA was detected until 10 weeks after delivery, whereas plasma hCGß could not be detected by 11 days post partum[31].

In special circumstances like twin pregnancies and external cephalic version near term, higher levels of fetal DNA were also reported[64]. In twin pregnancies with two male fetuses, higher levels of SRY-gen were measured compared to twin pregnancies with one male and one female. Chorionicity in male-male pregnancies did not result in different levels[65].

Considerations

In the above-mentioned approach, real-time quantitative PCR for the SRY- or DYS14 gene on the Y-chromosome was used as a model system representative of fetal DNA. In earlier studies, this approach proved to be reliable and technically feasible [7; 8; 10; 41; 42]. In pathological circum-

stances, like pre-eclampsia, or special circumstances, like twin pregnancies, the measured levels of fetal DNA were higher in cases then in controls, often with a considerable overlap. Elevated levels of fetal DNA in maternal plasma are a non-specific indication for pathological processes in pregnancy. Most of these pathological conditions are known to be placenta-related. The overlap of fetal DNA levels between uncomplicated pregnancies and pregnancies with pathological circumstances are considerable in nearly all studies. This makes discrimination between normal and abnormal situation difficult. Especially if fetal DNA is considered as a marker for aneuploidy the calculated test performance is poor.

Another important disadvantage is that the use of SRY- or DYS14-gen is gender dependent and so only applicable to 50% of the pregnancies. This limitation makes this method unsuitable for clinical application.

Polymorphism dependent approach

In contrast, detection of fetal-derived paternally inherited X-chromosome polymorphisms in maternal plasma can be used as gender-independent marker allowing positive identification of fetal DNA in all pregnancies. The technique applied for this purpose was detection of highly polymorphic short tandem repeats (STR). The use of five STR polymorphisms increased the change that a fetomaternal pair would be informative for sequences unique to the fetus by being derived from the father. Among the 25 pairs of samples of women carrying a female fetus, 76% were informative for at least one STR marker[66]. In this feasibility study, the possibility of this approach was clearly demonstrated. Possible other applications like rhesus D genotyping and genetic disorders such as recessive autosomal single-gene disorders are studied. The studies on several specific genetic disorders (later in the text mentioned) are case reports or contain small numbers of patients. They mainly demonstrate that identification in maternal plasma is possible, but their value in clinical practice must still be proven.

Rhesus D genotyping of the fetus

In this approach the genotypic difference between a Rhesus D negative pregnant women and her eventually Rhesus D positive fetus is used. The RhD gen is absent in a D negative individual and present in a rhesus D positive individual. If the RhD gene is detectable in maternal blood it must originate from the rhesus D positive fetus. A RhD negative woman with a heterozygote RhD partner has a 50% change of carrying a RhD positive fetus. If she develops antibodies the risk of haemolytic disease of the neonate increases with a possible adverse pregnancy outcome. The present strategy consists of screening RhD negative women for alloim-

munisation. If she has antibodies, the partner will be checked for the specific blood group genotype. If he is positive, the pregnancy will be monitored closely. Invasive procedures might be carried out to obtain fetal genetic material for prenatal testing of the blood group genotype to identify fetuses at risk. This invasive procedure carries a risk of miscarriage and has an increased risk of feto-maternal haemorrhage[67]. The great advantage of a non-invasive way to genotype the fetus is to avoid these risks. Another advantage is that immunoprophylaxis can be given on indication. Since there is a shortage of anti D, it would be better to give it only to pregnancies at risk. The detection of fetal RhD-specific sequences in maternal plasma has been studied extensively. With the use of real-time PCR technology the fetal rhesus D status can be determined correctly from early second to third trimester with a sensitivity of 100 % anda specificity of 96.6-100%[68-71]. The only exception concerned a false-positive case where the fetus had a variant rhesus-D gene, most likely a rhesus D φ gene[69]. In an early study 7 out of 9 first trimester samples were concordant to the outcome after birth. Two RhD positive fetuses were false negative.[68] Clinical implementation of fetal RhD genotyping in maternal plasma is clearly indicated, and the preferred method of choice, as demonstrated recently by screening of a large cohort in the Netherlands[72].

Aneuplodies

In cases of paternally inherited fetal aneuploidy, prenatal detection is possible using fetal DNA in maternal plasma. In this technique polymorphic markers outside the Y chromosome are used. This is only possible in the presence of a fully known paternal balanced translocation. This technique has been applied successfully in a case with fetal distal 3p trisomy and 7q36 deletion, resulting from a paternal t (3;7) reciprocal translocation [73]. In another case, three consecutive pregnancies of one mother were followed. The father had a balanced reciprocal translocation between the long arm of chromosome 10 and the short arm of chromosome 22. Analysis of fetal DNA in maternal plasma successfully demonstrated the presence of a balanced or unbalanced translocation in the fetuses [74].

Cystic Fibrosis

Cystic fibrosis is an autosomal recessive disorder and can be caused by different mutations in the CFTR gene. A child is affected if mutations are inherited from both parents. Detection of a paternally inherited cystic fibrosis mutation has been demonstrated successfully in maternal plasma at a gestational age of 13 weeks. Single PCR amplification of the Q890X mutation was used. In that case the fetus was carrier of the pater-

nal mutation [75]. In another study, the D1152H mutation was identified correctly by allele-specific PRC despite the presence of an excess of the corresponding wild-type sequence. Reliability of the assay was tested up to 300ng of DNA. From the ten controls nine were tested negative, one positive. This false-positive sample stood six days before processing. The amount of wild-type genomic DNA in the PCR exceeds 300ng, which is abnormal high and seems to be the result of lysis of intact cells. It appears that 300 ng is closely to the limit of the assay and analysis of samples containing amounts of DNA exceeding this limit is not reliable. The quantity of the other samples was < 300ng [76]. Population based studies are necessary to study test performances, like sensibility and specificity. If the mutation of the father is found in maternal plasma, an invasive procedure is still necessary to be sure if the fetus is affected or not. In case of a negative result the invasive procedure can be cancelled. The fetus can be a carrier of the maternal mutation, but will not be affected itself.

Thalassaemia

B Thalassaemia is a common autosomal recessive single-gene disorder and can cause severe anaemia. The disease is caused by mutations in the β globin gene. One of these mutations, namely the deletion of four nucleotides (-CTTT) at codons 41/42, has been studied. First the specificity and sensitivity of the allele-specific assay was investigated and confirmed by subjecting plasma, buffy coat and amniotic fluid samples from 100 pregnancies to screening for the mutation. Subsequently, the assay was applied for prenatal testing of eight fetuses at risk for β thalassaemia major. The aim was to exclude fetal inheritance of the paternally transmitted codon 41/42. The fetal genotype was completely concordant with conventional analysis and β thalassaemia major could be excluded in two of the pregnancies non-invasively [77]. New techniques appear very useful for this and related purposes. MassARRAY system was used to develop a new protocol, termed single allele base extension reaction (SABER).The approach was applied to exclude the fetal inheritance of the four most common South Asian β-thalassemia mutations in at risk pregnancies between 7 and 21 weeks of gestation. Fetal genotypes were correctly predicted in all cases studied. Fetal haploid analysis based on a single-nucleotide polymorphism linked to the β-globin locus, HBB, in maternal plasma was also achieved [78]. Development of such systems might help clinical implimentation.

Detection of fetal β^{E}-globin gene in maternal plasma was evaluated as a non-invasive strategy for the most common β-thalassemic condition among the South Asian population. Nested PCR method followed by the MnL I restriction analysis. From the five pregnant women examined, three were correctly identified [79].

Other paternally inherited genetic diseases

Prenatal diagnosis of myotonic dystrophy, an autosomal dominant disorder, was reported using fetal DNA in maternal plasma. The blood was sampled at 10 weeks gestational age [80]. In another case a Huntington disease unaffected fetus was successfully diagnosed by testing fetal DNA in maternal plasma. The blood was sampled at 13 weeks gestational age. In both cases it is essential to perform an analysis of parental DNA to know the size of paternal and maternal alleles [81].

mRNA OF PLACENTAL ORIGIN IN MATERNAL PLASMA

The presence of fetal RNA in maternal plasma was first demonstrated in 2000 by Poon et al. using Y-chromosome-specific zinc finger protein mRNA (ZFY). The detection rates of plasma fetal RNA in early and late pregnancies were 22% and 63%, respectively. This finding suggests that the concentration of fetal RNA in early pregnancy is lower than that in late pregnancy[82]. Ng et al. took it further and studied mRNA of two placenta-expressed genes, human placental lactogen (hPL) and the β subunit of human placental chorionic gonadotropin (βhCG) in maternal plasma by real-time quantitative RT-PCR. Placental hPL mRNA was detected in pregnant women at all gestational stages with concentration levels increasing with gestational age. βhCG mRNA was only detected in all women during the first trimester decreasing in concentration towards term. Detection rates were respectively 100, 42 and 7.7 % during first, second and third trimester. These results mirror the protein plasma levels of hPL and βhCG [34]. A smaller study on βhCG mRNA in whole blood and serum of pregnant women also reported on concentrations in the first compared to the second trimester. In whole blood data suggested a 30-fold higher concentration of hCGβ mRNA in the early samples compared to the second trimester. No hCGβ mRNA was detected in the corresponding sera [83].

Biology of circulating RNA

Circulating mRNA during pregnancy

The existence of RNA species in the circulation is a rather surprising finding, as free RNA is rapidly degraded in blood. After incubations with plasma as short as 15 seconds, 99% of free added RNA can no longer be amplified. RNA in plasma of healthy volunteers obtained from uncentrifuged EDTA blood and stored in 4° C is surprisingly stable even after 24 hours. The stability of RNA in serum is less. To obtain stable RNA from serum, uncentrifuged clotted blood should be stored at 4° C and processed within 6 hours [84]. Placental mRNA in maternal plasma has also

been demonstrated to be very stable; Human placental lactogen (hPL) and the β subunit of human placental chorionic gonadotropin (βhCG) mRNA are stable for up to 24 hours at room temperature[34]. An explanation can be found in the fact that plasma RNA is particle-associated. Filtration studies have been performed to study this possibility. Plasma samples from pregnant women were filtered through 5-µm filter. No significant change in maternal plasma hPL, β-hCG and GAPDH mRNA levels in pre- and post filtration samples were observed. But if these plasma samples were passed through 0.45 µm filters, a clear reduction in mRNA levels was observed. This difference in levels after filtration through 5 or 0,45 µm filter was statistical significant[34]. Filtration of plasma samples of healthy individuals and cancer patients was also studied. Unfiltered and filtered through 5, 0.45 and 0.22 µm pores GADPH mRNA and β-globin DNA concentrations were studied. GADPH mRNA levels were not different between unfiltered and filtered through 5 µm pores samples. But filtered through smaller pores, 0.45 and 0.22 µm, levels reduces significantly. These results indicate that a significant proportion of GADPH mRNA in plasma is particle associated. In contrast to these results β-globin DNA concentrations did not differ between the filtered (all three size pores) and unfiltered samples. Suggesting that most of the circulating β-globin DNA is non-particle-associated[85]. It is likely that this particle-association contribute to the stability of plasma RNA.

Circulating mRNA during after pregnancy

In blood of non-pregnant women who previously gave birth to at least one neonate, no hCGβ mRNA could be detected[83]. Clearance of hPL directly after delivery seems to be rapid[86]. In predelivery plasma samples collected at a gestational age of 38-42 weeks, the median levels of hPL mRNA was 50.004 copies per ml. Twenty-four hours after delivery, no hPL mRNA could be detected in any of these samples [34]. The clearance of mRNA of corticotropin-releasing hormone (CRH) was studied in uncomplicated pregnancies under controlled circumstances. Two hours post caesarean section no CRH mRNA was detectable anymore, indicating very rapid clearances under normal circumstances [87]. These findings are comparable with the postpartum clearance of fetal DNA in maternal plasma. The mechanism of clearance is still unsolved.

Source and mechanism of release

The main source of the studied mRNA in maternal plasma is the placenta. Most studies concentrate on mRNA of genes uniquely expressed by the placenta, for example human placental lactogen (hPL), β subunit of human placental chorionic gonadotropin (βhCG)[34], corticotrophin-releasing hormone (CRH)[87] and glia cell missing (GCM1) mRNA[88]. It was

expected that the placental mRNA demonstrated in maternal plasma would mainly originate from the extravillus trophoblast, because of its close contact with the maternal circulation. But surprisingly this was not the outcome of these studies. hPL, βhCG and GCM1 are synthesized by syncytiotrophoblast. There must be a regulation mechanism, yet unknown, releasing mRNA expressed by villus trophoblast into the maternal circulation.

The possibility that fetal hematopoietic cells contribute to the pool of circulating cell-free fetal nucleic acids during pregnancy was studied by quantification of plasma γ-globin mRNA in the first trimester. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as house-keeping sequence. The concentration of γ-globin mRNA was significantly higher in pregnant women compared to non-pregnant individuals. In plasma taken 8-20 minutes after first trimester termination of pregnancy the concentrations were significantly lower then in the pre-termination samples. GAPDH mRNA followed the same pattern. When termination of pregnancy was later than 9 weeks of gestation the concentration of γ-globin mRNA was increased. The authors suggest that increased post-termination γ-globin mRNA concentration can be an indication that the source of this message is fetal hematopoietic cells. They propose further evaluation of γ-globin mRNA in maternal plasma after 9 weeks in the perspective as being a marker for feto-maternal hemorrhage[89].

RNA from chromosome 21-encoded, placentally expressed gene, LOC90625, was detected in first-trimester maternal plasma [90]. It is very interesting that LOC90625, also called 21 ORF 105, turned out to be a member of the human endogenous retrovirus (HERV) -F family (91). The RNA expression of various HERV families has recently been studied in an extensive range of cell types, tissues and diseases. HERV -F is expressed in placental and fetal tissue [92;93]. It became apparent that the placenta is unique in both the diverse range of HERV transcripts and their high level of expression [94]. The transcriptional activity of HERV s has been studied. In the placenta three loci, syncytin and syncytin2 were active. Three different patterns were observed: constant expression through gestation, gradually decrease as pregnancy proceeded and a remarkable increases in term placentas [91]. Products of the expression of certain HERV proteins in the placenta may have a variety of physiological roles: the envelope of HERV -W seems to be involved in cell fusion to form the syncytium and ERV3 Env is associated with cytotrophoblast differentiation.

Possible clinical applications

The demonstration of placental mRNA in maternal plasma opens up the possibility of developing gender- and polymorphism independent non-invasive prenatal diagnostic tools. Studies have been started on this challenging subject. In plasma collected in the third trimester from pregnant women with pre-eclampsia the concentration of corticotrophin-releasing hormone (CRH) mRNA was 10 fold higher compared to the uncomplicated pregnancies [87]. A second study confirmed this finding, but a significant difference between mild and severe pre-eclampsia could not be demonstrated [95]. RNA from a chromosome 21-encoded, placentally expressed gene, LOC 90625, was present in first-trimester maternal plasma and could be detected in 100% of samples when 1600 µL of plasma was used [90]. This finding may allow development of plasma-RNA-based strategies for prenatal prediction of Down syndrome.

A more basic research field concerns the search for possible markers for clinical situations by systematic screening of placental mRNA in maternal plasma. Using RT-PCR a large number of RNA targets (n=80) known or expected to be present in extraembryonic tissues were screened in the first trimester of pregnancy in maternal plasma, plasma of non pregnant women and early placental tissue. With this strategy eight genes were found positive in early placenta tissue and plasma of pregnant women and were tested negative in non-pregnant women. This approach not only permits rapid screening of potential new markers, it allows the detection of markers not accessible by conventional antibody-based assays [88]. Using RT-PCR technique as method of screening the choice was made for a method with high sensitivity. Lower concentrations levels of mRNA can be detected. The possibility of a high abundancy screenings method was also studied. Oligonucleotide micro-array analysis has been applied as a systematic and high throughput strategy for the identification of new fetal RNA markers in maternal plasma. Gene expression profiles between placental tissues and corresponding peripheral blood from pregnant women in their first and third trimester were compared. Six transcripts were selected for further evaluation by RT-PCR [86]. So genes expressed uniquely in placenta, which can be detected in maternal plasma (and not in plasma of non-pregnant women) can be seen as possible markers for clinical situations. The important advantage of this approach is that the markers are gender and polymorphism independent and that may candidate for appliance in all pregnancies.

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Non-invasive aneuploidy detection in maternal plasma: a realistic possibility in the near future

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Submitted

Abstract

Recent advances made in the technical possibilities for non-invasive aneuploidy tests based on cffDNA and placental mRNA in maternal plasma are huge. In small studies and model systems strategies and techniques for non-invasive aneuploidy tests were demonstrated. The RNA-SNP allelic ratio strategy seems to be the most feasible test at this moment with the desirable quality that the result is based on fetal specific genetic material, but has the disadvantage of polymorphism dependence. Deep sequencing is promising, but at this moment too labour intensive for clinical implementation and at high costs. Of great advantage is the fact that the test is gender and polymorphism independent and several aneuploidies can be tested at the same time. Strategies and techniques have to be further tested on their diagnostic potential for aneuploidy detection. The time for population based, double blind, clinical trials has come.

Introduction

For the detection of aneuploidy or other chromosomal abnormalities of the fetus invasive procedures are necessary to obtain fetal cells. These procedures, like amniocentesis or chorionic villus sampling, carry a risk of miscarriage due to the procedure. Trisomy 21, related to advanced maternal age, is the most common reason for women to choose for prenatal diagnosis. Screening tests like the first trimester combination test are nowadays offered to pregnant women. Risk calculation is based on maternal age, the nuchal translucency measurement and two serum markers: namely pregnancy associated plasma protein A (PAPP-A) and free beta human chorionic gonadotropin (free β hCG). Both are proteins produced by the outer layer (syncytiotrophoblast) of the placenta i.e. the layer in direct contact with the maternal circulation. Although the test properties are rather good with a detection rate of about 85-90% with a false-positive rate of 5-9 %[1-4], the test lacks diagnostic power. Non-invasive tests that yield a definitive diagnosis rather than a probability score remain to be established. Such a test could potentially be developed by direct targeting for and analysis of fetal DNA and RNA in maternal plasma rather than through indirect analysis of placental proteins. This possibility emerged from the exciting discovery that the blood of pregnant women contains fetal DNA and placental mRNA from early pregnancy onwards, that appears in the maternal circulation as a normal consequence of placental physiology and can be isolated and subjected to molecular analysis[5;6]. This genetic material is representative of the genetic makeup of the fetus and of the transcriptional activity of the placenta and as such provides a reservoir of possible biomarkers for the development of non-invasive prenatal tests with diagnostic power[7]. Since decades the establishment of such a non-invasive test with clinical and practical applicability to detect fetal aneuploidy is a major research goal. The landmark discovery of cell-free fetal DNA in maternal plasma[5] and a view years later of fetal RNA in maternal plasma[6] was followed by technically challenging but accurate methods, allowing adequate correction of biological variation (based on allele ratio's) in addition to recent technology-driven breakthroughs allowing all-in one approaches (genome wide sequencing)[8;9]. Moreover, the notion that the biological information gained by targeting for fetal DNA and RNA , including non-

coding RNA (such as the large gene family of microRNAs) and regulatory DNA (such as differentially methylated DNA) exceeds that of conventional markers by a factor 4-10, has enormous implications and great potential for the future[10]. Most importantly, not the least for the pregnant women at risk, this includes the fact that the development of a robust non-invasive test for fetal aneuploidy now has become within reach. Tomorrow has started today.

Test requirements

Essential or desirable qualities of such a non-invasive diagnostic test are 1. specificity and sensitivity levels of (near) 100% each, 2. eligibility for and applicability in all pregnancies, 3. the possibility to perform from the first trimester onwards, 4. eligibility for population based implementation and large scale screening, 5. at reasonable costs, 6. practically executable in a clinical setting, while 7. meeting the technically demanding criteria allowing analysis in a routine laboratory setting. Obviously a very challenging goal.

Focus of review

This review summarizes the latest developments in the use of circulating cell-free nucleic acids in maternal plasma for non-invasive prenatal detection of fetal chromosomal aneuploidies, with emphasis on trisomy 21. Rather than being complete in coverage (excellent reviews have been published recently)[11], this focused review aims to provide the reader (pregnant female, clinicians, geneticists, decision makers) with a roadmap towards the destinies that lie ahead and those that have become within reach (see Table 1).

Selective targeting of fetal DNA in maternal plasma

The problem with circulating fetal DNA as discovered in 1997 to be present in maternal plasma [5] is that the proportion of cell free fetal DNA is only 3-6 % of the total amount of cell-free DNA (cf DNA). The main part of the total cf DNA is maternal of origin. This implies that either quantitative and/or qualitative corrections are needed or precautions taken to selectively enrich or target for fetal DNA. The notion that maternal DNA originates from normal or procedural leakage from peripheral blood cells led to the idea to reduce the procedural contribution by formaldehyde pre-treatment [12]. Although initially claimed to be successful, no enrichment by this pre-treatment was found by others[12-16]. The notion that fetal DNA is on AVErage smaller in size compared to maternal DNA has led to testing size fractionation as a way to enrich for fetal DNA[13-15].

Table 1

In this table an overview is given of properties of the different methods, based on selective targeting of fetal DNA and placental mRNA or discriminatory quantitative methods using cfDNA. Several aspects are compared: biological properties, clinical possibilities focussed on chromosomes (aneuploidy) and on feasibility for clinical use.

	mRNA	cffDNA		
	allelic ratio strategy	deep sequencing	methylation (MeDiP)	methylation, bisulphite conversion
copie number sample	≈	↓	≈	↓↓↓↓
gender independent	yes	yes	yes	yes
polymorphism independent	no	yes	yes	yes
amount of blood needed	≈	↑↑		
tris 21	yes	yes	yes	yes
tris18	yes	yes	yes	yes
tris 13	unknown	yes	yes	unknown
X/Y	unknown	yes	yes	unknown
technical demanding	yes	yes	yes	↑
costs	↑	↑↑	↑	↑
implementation	possible	not yet		
tested on clinical samples	yes	yes	no	yes
tested prospectively population based	no	no	no	no

Size fractionations, however, such as by conventional gel electrophoresis followed by isolation of the smaller-sized DNA fraction prior to analysis, is time-consuming and prone to contamination. This prevented its wide spread use.

Theoretically, the demand for enrichment is relative or absent, if the fetal nucleic acids targeted for are only found in or expressed by the fetus. This allows selective targeting and thereby selective analysis. The Y chromosome is a well known example and is still used as a target for validation of new methods[16-19]. However, straightforward discrimination between cell free fetal and maternal DNA in maternal plasma for quantification purposes of chromosomes other then the Y chromosome is not possible, since the fetus inherited half its DNA from the mother. Ways were found to solve this problem.

The epigenetic properties carried by certain DNA sequences (mostly CpG islands) appeared an interesting strategy. Epigenetics involve processes of heritable changes in gene expression that occur without changes in the DNA sequences[20]. Methylation is an example of epigenetic change. This approach is based on the presence of differences in methylated DNA between placenta and maternal cells. As first shown by Poon *et al.* to be possible[21], Chim *et al.* was the first to describe a marker with these discriminating properties[22]. He discovered that the maspin gene (SERPINB5), located in chromosome 18, is hypomethylated in the placenta

and hypermethylated in maternal blood cells. By using bisulphite DNA sequencing, the methylation status of the maspin gene promotor in placental tissues and paired maternal blood cells from pregnant women was analysed. Bisulphite converts unmethylated cytosine in uracil while leaving methylated cytosine unchanged[22]. Subsequently the feasibility of trisomy 18 detection was successfully tested in a model system using this bisulphite modification followed by methylation specific PCR and primer extension to asses the allelic ratio's. This generated a sensitivity of 100% and a false positive rate of 9.7%[23]. Subsequent systemic searches for placental DNA methylation markers on chromosome 21 with differential methylation differences between placenta and maternal blood cells resulted in the detection of multiple differentially methylated genes. Although the great advantage that the method is gender and polymorphism independent, while the low concentration of DNA can be overcome by PCR and genome wide screening is possible, one major drawback remains; bisulphite conversion, needed for the majority of methylated markers, destroys a large proportion of the input DNA[24]. The exception is the situation where the fetal marker is hypermethylated in placenta and hypomethylated in the maternal tissue[25]. Then bisulphite treatment would not harm the DNA of interest. The existence of chromosome 21 markers with that pattern (CpG islands of C21orf63 and C21orf29) has been described, but not yet tested in maternal plasma[26]. Recently an interesting new technique, not requiring bisulphite pre-treatment, has been tested to detect methylation differences between placenta tissue and whole blood by methylated DNA immunoprecipitation (MeDiP) coupled with high resolution tiling oligonucleotide array analysis. By this approach genome wide screening is theoretically possible. With this technique the DNA amount is not impaired. Methylation patterns of chromosomes 21, 18, 13, X and Y were analysed and led to the additional identification of previously unreported fetal epigenetic molecular markers[17]. So this selective targeting technique still has the advantage of being gender- and polymorphism independent, does not involve loss of input DNA and is discriminative for chromosomes 21, 18, 13, X and Y. Although the method has the right properties to target convenient biomarkers for aneuploidy detection, its clinical value remains to be proven.

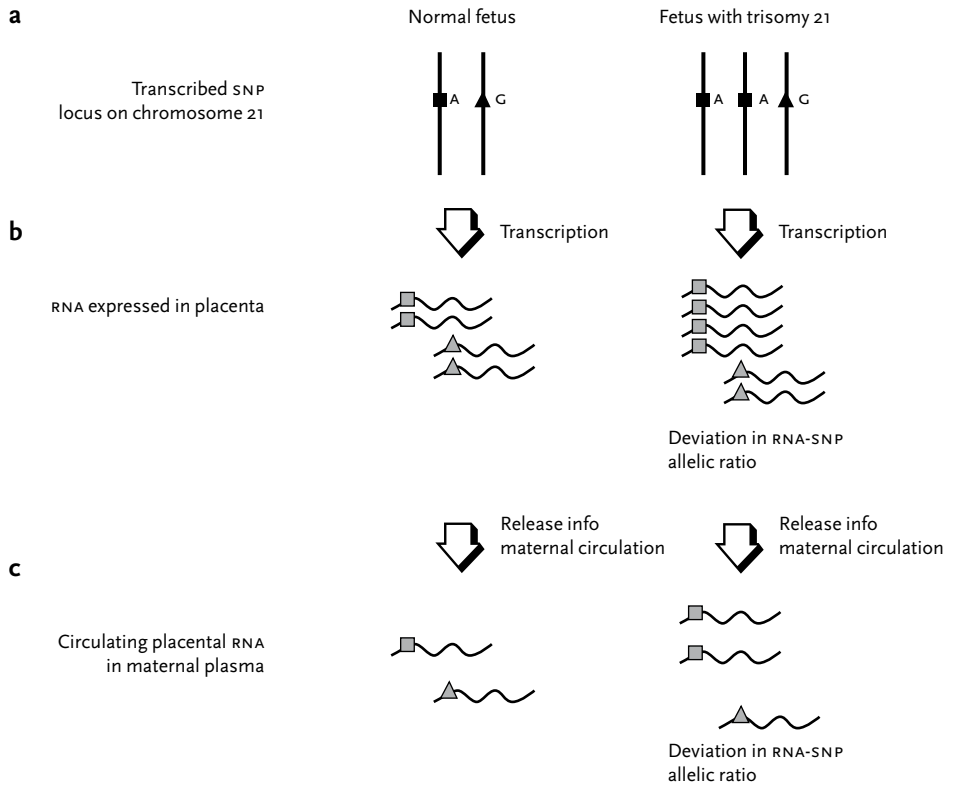
Selective targeting of placental mRNA in maternal plasma

The problem of discrimination between fetal and maternal genetic material was also addressed in a different way. It is known that each tissue in the body contains a characteristic profile of mRNA. If the presence of fetal specific mRNA in maternal plasma can be demonstrated, it might provide another source of potential fetal specific biomarkers. The pla-

centa is the most obvious organ that represents the fetus and is in close contact with the maternal circulation. Placental mRNA released in a tissue-specific or tissue-restricted manner in the maternal circulation, thereby being absent in the plasma of non-pregnant individuals, qualifies as fetal specific and therefore as potential biomarker for aneuploidy detection during pregnancy. The proof of principle of fetal RNA in maternal plasma was first demonstrated in pregnant women carrying a male child in 2000 by Poon *et al.* using Y-chromosome-specific zinc finger protein mRNA[6]. The presence and detectability of fetal i.e placental RNA in all pregnancies was subsequently demonstrated a few years later by Ng *et al.* through the analysis of placental specific mRNA, human placental lactogen (hPL) and the β subunit of human placental chorionic gonadotropin (βhCG) mRNA [27]. Placental mRNA in maternal plasma has been demonstrated to be stable. Filtration studies indicate that the particle associated nature of the RNA and DNA containing micro-particles circulating in the maternal blood is the secret of stability of plasma RNA that protect them from degradation by RNA se. Studies for systematic identification of placental mRNA markers, detectable in maternal plasma, followed. Gene expression profiles between placental tissues and peripheral blood from pregnant women were compared. In one study in the first and third trimesters by oligonucleotide micro-array followed by real time quantitative reverse transcriptase polymerase chain reaction assays[7]. In this study six genes with the pattern of interest could be identified: hPL, β-hCG, CRH, TFPI2, KISSI, PLACI. In another study a set of 80 genes was tested using RT-PCR for their presences in early placenta tissue, absence in non-pregnant plasma and presence in pregnant plasma[28]. In this study eight genes with the pattern of interest could be identified: GCMI, ZDHHCI, PAPPA, PSG9, PLACI, GCM, β-hCG, LOC90625. Not only was rapid screening of a large set of potential new markers demonstrated, it allowed the detection of markers not accessible by conventional antibody-based assays [7; 28].

The search also focused on potential markers for trisomy 21 detection. Oudejans *et al.* were the first to describe a chromosome 21 specific mRNA present in maternal plasma, LOC90625 (nowadays called C21orf105). Detection of chromosome 21–encoded markers is essential for selective testing of fetal specific genetic material for a non-invasive prenatal test for trisomy 21. Theoretically, as a consequence of the dosage-related difference in expression of chromosome 21-encoded genes between normal and Down syndrome pregnancies, message quantification could discriminate between trisomy 21 and normal pregnancies. Due to the large biological variation between and within individuals, this approach as analyzed by quantification of the chromosome 21 transcribed mRNA, C21orf105, in maternal plasma by quantitative RT-PCR appeared unsuc-

Figure 1 Schematic illustration of the allelic ratio strategy. Using the allelic ratio of a specific heterozygous SNP on cell-free fetal mRNA, aneuploidy of a fetus can be measured in plasma of pregnant women. Reprinted by permission from Macmillan Publishers Ltd: *Nature Medicine* 13(2):218-223 2007.



cessful despite correcting for variation using a housekeeping gene [29]. Lo *et al.* developed an as simple as intelligent strategy to correct for this: RNA allelic ratio assessment using single-nucleotide polymorphism (SNP) [30]. What is the principle? If a fetus is heterozygous for a certain single nucleotide polymorphism (SNP) on the chromosome of interest and expressed as placental transcripts, it possesses two different alleles that are distinguishable. If the fetus is euploid, containing two copies of the chromosome of interest, the ratio of these two SNP alleles would be 1:1. When the placenta releases its mRNA into maternal circulation, the ratio of placental mRNA in maternal plasma that is transcribed from each of these two alleles would also be 1:1. If a fetus has a trisomy 21 for example and is heterozygous for a SNP on a chromosome 21 transcribed mRNA, then the RNA-SNP allelic ratio would become 1:2 or 2:1 (see Figure 1). In Lo's study it was hypothesized that the difference in allelic ratio present in the

fetus could be measured in the plasma of the pregnant women by a placenta and chromosome 21 specific mRNA, PLAC4, using extension primers and mass spectometry detecting the extensions products. PLAC4 was choosen for its abundant expression. This strategy was tested on placenta tissue and plasma samples from euploid and trisomy 21 pregnancies. The sensitivity was 90% and the specificity 96.5%.[31] This RNA-SNP allelic ratio strategy has therefore great potential given its high accuracy. The placental mRNA tested is completely fetal specific and stable in maternal plasma. It was successful tested in trisomy 21 pregnancies and in a model system for trisomy 18 [32], and is theoretically applicable for trisomy 13 as well. However, although gender independent, the method is polymorphism dependent, which is an important disadvantage. The heterozygosity rate differs per SNP and between different populations. PLAC4 SNP (rs8130833) has a heterozygosity rate of 0.45 in the population (Chinese) studied. The population coverage can theoretically be broadened by combining several SNP's of genes transcribed from chromosome 21. The search for other convenient genes had lead to the finding of placental mRNA targets with potential different SNPs[33].

The methods tested require highly specialized equipment, which might prohibit their widespread implementation of this RNA-SNP strategy. An alternative technique was studied. The Transgenomic WAVE System and quencher extension techniques were adapted and applied for the allelic ratio strategy [34]. The quencher extension (QEXT) reaction is a single-step, real-time method to quantify SNP's and is directly adaptable to current real-time PCR equipment. These methods were tested in a model system with early second trimester placenta tissue[35]. A SNP (rs 2187247) located in exon 2 of C21orf105 was used as marker. Allelic ratio differences between bi-allelic and tri- allelic cases in the trisomy 21 model system could successfully be demonstrated. It has to be tested if this technique is feasible and discriminative in maternal plasma. But if so, combination of these techniques (WAVE System and QEXT) might facilitate implementation of a diagnostic test based on SNP-RNA allelic ratio differences, against lower costs and being less labour-intensive.

Discriminatory quantitative methods using cell free DNA

Several recent developments and breakthroughs are technology-driven. An example is digital PCR, a highly sensitive technique which uses limiting dilution to isolate single template DNA molecules to be amplified. Digital PCR was tested in a model system for molecular detection of fetal trisomy 21[36]. It is noteworthy that in this way fetal derived DNA is not specifically distinguished from maternal DNA. Chromosome dosage was determined by analysis of a non-polymorphic chromosome 21 locus rela-

tive to a non-polymorphic locus on a reference chromosome, in this study chromosome 1. Artificial mixtures containing placenta DNA in a background of maternal blood cell DNA were analysed. Based on these results the estimation was made that correct classification in 97% of the cases could be made with samples containing 25 % fetal DNA[37]. However, input of 15 ml maternal blood is needed for sufficient amount after DNA extraction. For implementation in a routine clinical test this would be unwanted. So although the method was demonstrated to be feasible, the technique is demanding and an enrichment step would be necessary.

A breakthrough with great potential is massively parallel sequencing. Millions of short sequence tags can be sequenced by high-throughput shotgun sequencing technology, enabling deep sequencing[38; 39]. Again discrimination between fetal and maternal DNA is not necessary. Fan *et al.* tested this technique on cell free DNA from plasma of pregnant women with a gestational age of 10-35 weeks [39]. Over- and under representation of chromosomes were measured. It was possible to discriminate all fetuses with trisomy 21 (n=6), trisomy 18 (n=1) and trisomy 13 (n=1) from the six normal cases. A drawback of this study was that the samples were collected 15-30 minutes after invasive procedures. Puncture, necessary to obtain fetal material, disrupt the barrier between maternal and placental circulation and artifially influences the amount of fetal DNA in the maternal circulation.

Chiu *et al.* tested the same technique, but followed a different strategy[18]. They tested this strategy for trisomy 21 and for the Y and X chromosome difference between male and female fetuses. They tested an algorithm to calculate the percentage unique sequences for the chromosome of interest in the test sample compared to the reference population of that same chromosome. They were able to discriminate trisomy 21 from disomy 21 samples. In this study 11 of the 14 trisomy 21 samples were collected after an invasive prenatal procedure (range 2-22 days). The other samples were collected before the invasive procedure. Nevertheless both studies demonstrated the feasibility of deep sequencing for non-invasive prenatal diagnosis. The important advantage of this technique is that it is gender and polymorphism independent; in potential applicable for all pregnancies and for testing several aneuploidies in the same test. At this moment the technique is demanding, the costs per tested samples are high and the throughput per instrument is low (16 samples per week). This prevents its use at this moment as a regular test for all pregnant women. The fact that selective targeting of fetal DNA is not obligatory might facilitate testing, but is also an element of attention. The concentrations of circulating cfDNA and cffDNA in maternal circulation are variable in normal pregnancies[40] while in women who developed pre-eclampsia increased fetal DNA concentrations were found in maternal plasma before the onset of

pre-eclampsia in the first half of the second trimester[41; 42]. Although a higher amount of fetal DNA is likely to facilitate early testing, a possible change in the composition of the cffDNA might interfere with the reliability of these quantitative tests. Although the deep sequencing technique is very promising, population based studies involving prospective studies in low risk populations i.e. normal populations have to indicate if the technique is robust.

New in the field: Placental microRNA

microRNAs are short, 19-25 nucleotides, single-stranded and non-coding RNA's [43; 44]. No protein is produced. Amongst other regulatory processes, they regulate gene expression by binding to the 3'untranslated region of the target mRNAs. Since 2001 microRNA has been described[43; 44] and studied because of their regulatory role in gene expression. For the same reasons as mentioned above, microRNA, if placental specific, qualifies as biomarker. The presence of placenta derived microRNA (miRNAs) in maternal plasma has recently been studied by Chim *et al.*[26] A systematic search of 157 well-established placental miRNAs in maternal plasma was performed by real-time quantitative RT-PCR and physical properties of the miRNAs were investigated[45]. Comparison was made between placental tissue obtained after a term delivery and maternal blood cells; samples collected in the first, second and third trimester and 24 hours post delivery into tubes containing EDTA. From the 157 placental miRNAs tested, 17 occurred at concentrations more then 10 fold higher in the placentas then in maternal blood cells. MiR-141, miR-149, miR-299-5p and miR-135b were the four most abundant of these placental miRNAs present in maternal plasma. They show a reduced detection rate 24 hours after delivery. For a useful placenta specific marker it is obligatory that the marker can not be detected after pregnancy in blood of non pregnant women. If a marker can be measured after pregnancy, the marker is not placenta specific or is not 'cleared' after pregnancy; both might interfere with results of in the same or the next pregnancy. MiR-149 and miR-149 showed a significant reduction, but not a reduction to zero like placental mRNA in maternal plasma. The presence of miRNAs was studied in serum as well; comparison was made between pregnant and non pregnant women. microRNAs associated with human placenta were significantly elevated in serum of pregnant women and their levels correlated with pregnancy stage[46]. Although levels of miR-141 and miR-149 were not as different between non-pregnant and pregnant women as found in the study of Chim *et al.* It can be stated that based on these studies microRNAs can be detected in maternal plasma and serum. This new class of circulating nucleic acids might provide a source of biomarkers for non-

invasive aneuploidy detection and pregnancy associated (pathological) processes. An additional advantage is that microRNAs appear extremely stable while methods are available allowing real-time quantification of all currently known microRNAs[47].

Summary

The last decade advances made in the technical possibilities for non-invasive aneuploidy tests based on cffDNA and placental mRNA in maternal plasma are huge.

In small studies and model systems strategies and techniques for potential non-invasive aneuploidy tests were demonstrated. For wide implementation a test has to be robust, preferable gender and polymorphism independent, possible to carry out from the first trimester on and at reasonable costs. The RNA-SNP allelic ratio strategy seems to be the most feasible test at this moment with the desirable quality that the result is based on fetal specific genetic material, but has the disadvantage of polymorphism dependence. Using several markers might help to enlarge population coverage. Deep sequencing is promising, but at this moment too labour intensive for clinical implementation and at high costs. Great advantage is the fact that the test is gender and polymorphism independent and several aneuploidies can be tested at the same time.

Strategies and techniques have to be further tested on their diagnostic potential for aneuploidy detection. The time for population based, double blind, clinical trails has come. Then the time might come that a promise will change into reality rather than a possibility.

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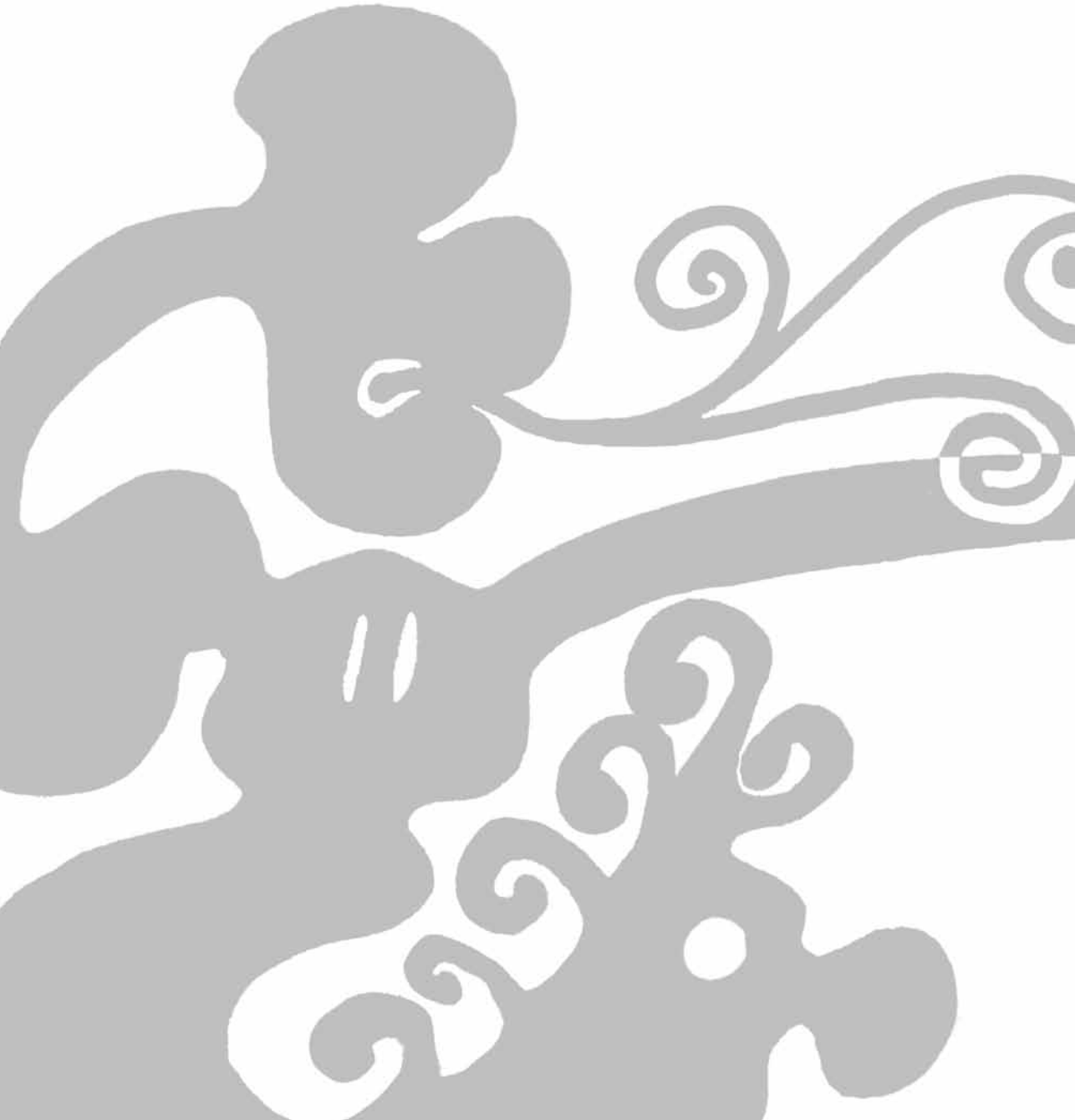

Detection of Chromosome 21-encoded mRNA of Placental Origin in Maternal Plasma

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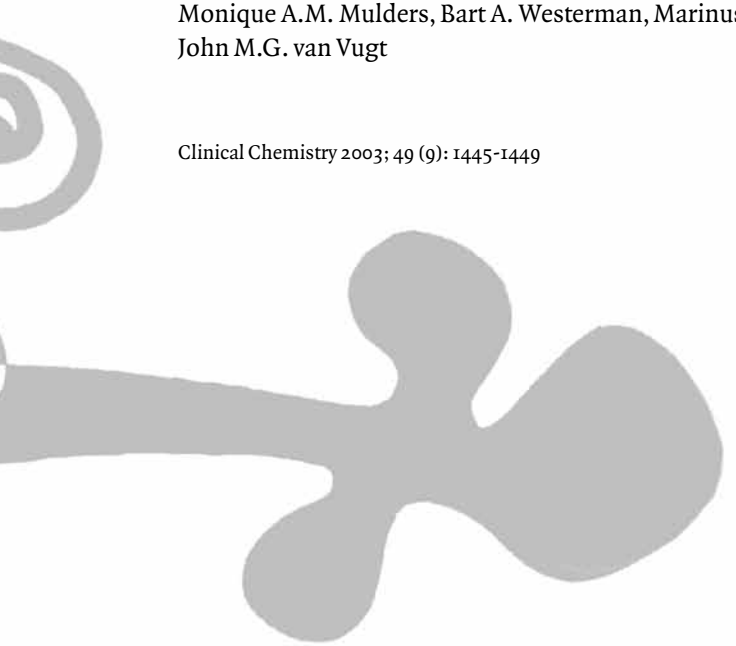
Clinical Chemistry 2003; 49 (9): 1445-1449



Abstract

Background mRNA of placental origin (i.e., human placental lactogen and -human chorionic gonadotropin) has been demonstrated to be easily detectable in maternal plasma. We tested whether detection of chromosome 21-encoded mRNA of placental origin is possible in maternal plasma obtained during the first trimester.

Methods Plasma samples were obtained from pregnant women between weeks 9 -13 of pregnancy. RNA was isolated from 800 or 1600 µL of plasma by silica-based affinity isolation and, after on-column DNase treatment, was subjected to two-step, one-tube reverse transcription-PCR with gene specific primers.

Results Three chromosome 21-encoded genes located within the Down syndrome critical region with overexpression in trisomy 21 placentas were screened for expression in early placental tissue to select their potential use for RNA based plasma screening. One of the chromosome 21-encoded genes (LOC90625) showed strong expression in first trimester placenta similar to CSH 1 (human placental lactogen) and was selected for plasma analysis. The RNA isolation assay was validated with CSH 1 mRNA, which could be detected in the plasma of all women tested in weeks 9-13 of pregnancy. RNA from the chromosome 21-encoded, placentally expressed gene, LOC90625, was present in maternal first-trimester plasma and could be detected in 60% of maternal plasma samples when 800 µL of plasma was used and in 100% of samples when 1600 µL of plasma was used.

Conclusion The detection of chromosome 21-encoded mRNA of placental origin in maternal plasma during the first trimester may allow development of plasma- RNA-based strategies for prenatal prediction of Down.

Recently, it has been demonstrated that mRNA of placental origin can be detected easily in maternal plasma [1]. Central to this breakthrough in the field of noninvasive prenatal diagnostics was the understanding that fetal RNA circulates in a protected form and is predominantly placental in origin[1–4] . The protected form of circulating fetal RNA not only allows its detection, but also necessitates adaptation of the RNA isolation methods used that require some form of denaturation[2]. The understanding that fetal RNA in maternal plasma is predominantly placental in origin with easy and reliable detection of human placental lactogen (hPL)[3] mRNA in all pregnancy stages is in accordance with the fact that the major cell type circulating in the maternal blood is of trophoblastic, in particular extravillus, origin[5]. The apparently physiologic manner by which components (i.e., nuclei and mRNA)[1,6,7] of these cells appear in the maternal plasma is unknown, although programmed cell death (apoptosis) seems to be involved[6, 7] .

We believe that given the predominantly placental origin of fetal RNA in maternal plasma, new markers could be developed for Down syndrome screening using the placental RNA in maternal plasma if they fulfill the following criteria: (a) the genes analyzed should be encoded by chromosome 21; (b) they should be located within the Down syndrome critical region (DSCR); (c) they should be expressed in healthy early placental tissue; (d) they should be overexpressed by the placenta in trisomy 21 pregnancies; and (e) they should be detectable in maternal plasma during early pregnancy. Gene expression profiling of trisomy 21 placentas has identified at least two genes that meet the first four criteria[8]. We therefore tested these genes and one additional gene from the DSCR[9] with placental expression to determine whether one or more of these genes is expressed in the first-trimester placenta and, if so, whether it or they also meet the last criterion, i.e., detection of the gene-encoded RNA in maternal plasma during the first trimester.

Patients and Methods

Patients

Peripheral blood samples (n=25) were collected from pregnant women attending the Prenatal Diagnostic Unit of the VU University Medical Center with informed consent and approval of the Ethics

Committee. EDTA blood was collected between weeks 9 and 13 of pregnancy. All blood samples were obtained before invasive diagnostic procedures, i.e., chorionic villus sampling.

Processing of blood samples

EDTA blood was stored at 4° C in an upright position and processed within 24 h after collection by two sequential centrifugation steps as described previously[10]. In brief, after centrifugation for 10 min at 2000g at 4° C in a Hettich Rotanta 96R centrifuge, plasma was subjected to a second centrifugation for 10 min at 25 000g at 4° C in a Hettich EBA12R centrifuge. Plasma was stored as aliquots at -70° C and thawed only once. Processing of blood was done within a laminar flow hood.

RNA extraction from maternal plasma

RNA was extracted from 800 or 1600 µL of maternal plasma by silica-based affinity isolation using the QIAamp MinElute Virus Spin or QIAamp MinElute Virus Vacuum system (Qiagen). Before isolation, plasma samples were thawed at room temperature, the heating block was preheated to 56° C, carrier RNA was added to AVE buffer (1 µg/µL), and the protease was thawed. All steps were done at room temperature unless stated otherwise. We added 50 µL of protease (Qiagen) to a 1.5-mL tube, followed by of 400 µL of plasma, and 400 µL of buffer AL (with 28 µg/mL carrier RNA). After vortex-mixing for 15 s, samples were incubated for 15 min at 56° C. After centrifugation, 500 µL of ethanol was added; samples were then vortex-mixed for 15 s and left at room temperature for 5 min. All centrifugation steps were done for 1 min at 8200g unless stated otherwise. After centrifugation,the lysate mixture was carefully loaded on a QIAamp MinElute column and centrifuged.

When the vacuum system was used, the columns were inserted into the QIAvac 24 vacuum manifold (Qiagen) according to the manufacturer's instructions. The vacuum conditions used were -80 to - 90 kPa with a 19 L/min vacuum pump (Biometra MP26). All centrifugations steps except for the final elution step described below were substituted by processing with the vacuum system. For plasma starting volumes of 800 or 1600 µL, the number of tubes needed was increased accordingly, although the total volume was loaded on a single column. After transfer of the column to a new tube, 500 µL of buffer AW1 was added, followed by centrifugation. For on-column DNase digestion, 70 µL of SDD buffer was added to 10 µL of DNase, loaded on the column, and left for 15 min at room temperature. After centrifugation, 500 µL of buffer AW2 was added, and the samples were recentrifuged. The column was placed in a new tube, 500 µL ethanol was added to the column, and the column was centrifuged. The column was then placed in a new tube and centrifuged for 3

min at 25 000g. The bound RNA was eluted by placing the column in a new tube, followed by application of 20 –150 µL of RNase-free MilliQ water, incubation for 5 min at room temperature, and centrifugation for 1 min at 25 000g. Finally, samples were concentrated by use of Microcon-PCR filters according to the manufacturer's instructions (Millipore). The samples were used all in one, i.e., the RNA obtained from 800 or 1600 µL of plasma was used for a single reverse transcription-PCR (RT-PCR) assay. For controls, plasma samples obtained from non- pregnant females of similar age and race were processed and used identically.

RT-PCR

The two-step, one-tube RT-PCR assay was performed as described previously[11] with the RNase H-negative Superscript II Platinum system (Life Technologies) in the presence of 1 M betaine. RT-PCR reactions were set up on ice within a PCR workstation (CBS Scientific). In brief, RNA was mixed with 50 pmol each of forward and reverse primers in a final volume of 10 µL in MicroAmp tubes and heated for 1 min at 95° C, followed by immediate cooling on ice. Forty microliters of master mixture was subsequently added, giving a final concentration of 1 × buffer; 1.25 mM magnesium sulfate; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 1 M betaine (Fluka); and 1 µL of enzyme mixture containing RNase H-negative Superscript II reverse transcriptase and Taq DNA polymerase (Life Technologies). After reverse transcription for 30 min at 50° C and denaturation for 1 min at 95° C, PCR was performed for 35 cycles (denaturation for 1 min at 95° C, annealing for 1 min, and extension for 2 min at 72° C), followed by a final extension for 10 min at 72° C and cooling. All reactions were performed identically except that the annealing temperature was set at the temperature predicted to be optimal for each target (Oligo 4.0). The gene-specific primers used were as follows:

DSCR4-F (5 ATC TTG ACG AGA GAT GAT GAA CCC C-3) and
DSCR4-R (5 -CTT TAC CTC CAT GGG CTC CAC A-3); $T_{annealing}$ = 57° C
PTTG1IP-F (5 -TGA AGA ACG TCT CCT GTC TT-3) and
PTTG1IP-R (5 -ACT ACC GAC ATG GTG ATG AT-3); $T_{annealing}$ = 53° C)
LOC90625-F (5 -ACA CCG CCT CGT GTT GTC TGT TGG-3 and
LOC90625-R (5 GGA TCC ATC GCA CCA GGG TCA A-3);
$T_{annealing}$ = 61° C
HPL-F (5 -GCA CCA GCT GGC CAT TGA CA-3) and
HPL-R (5 -CCG TGC GGT TCC TCA GGA GTA T-3); $T_{annealing}$ = 58° C

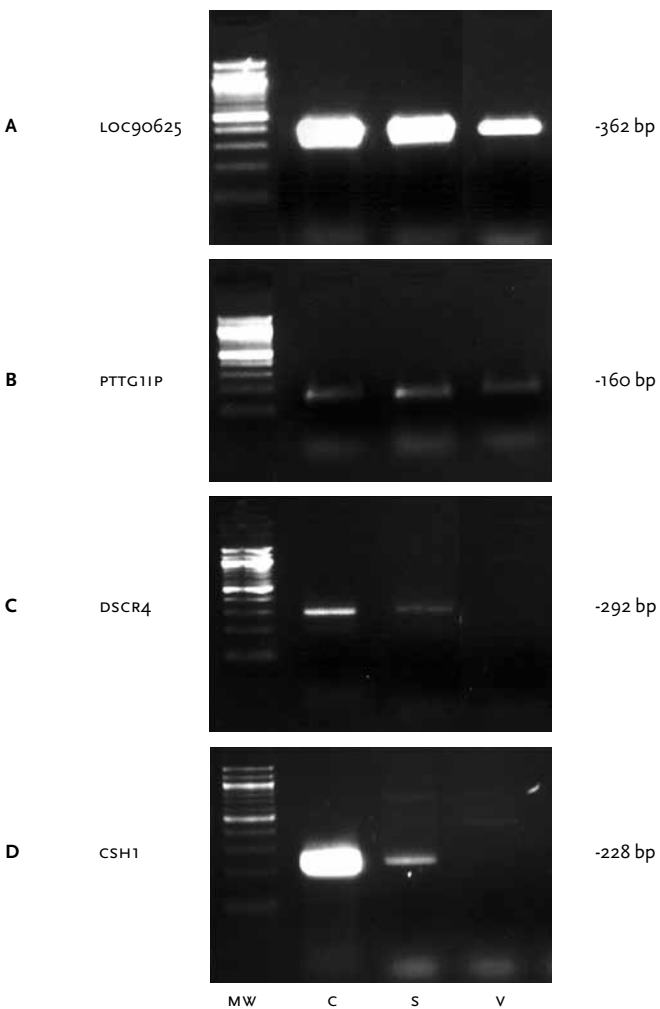
For expression analysis of early placental tissues and cells, RNA was obtained and isolated as described previously[11] . These samples were representative of total chorionic villi, villus fibroblast cells, and extravillus

trophoblast cells (SGHPL5)[12] . The latter cells were kindly provided by Dr. Judith Cartwright (St. George's Hospital Medical School, London, UK).

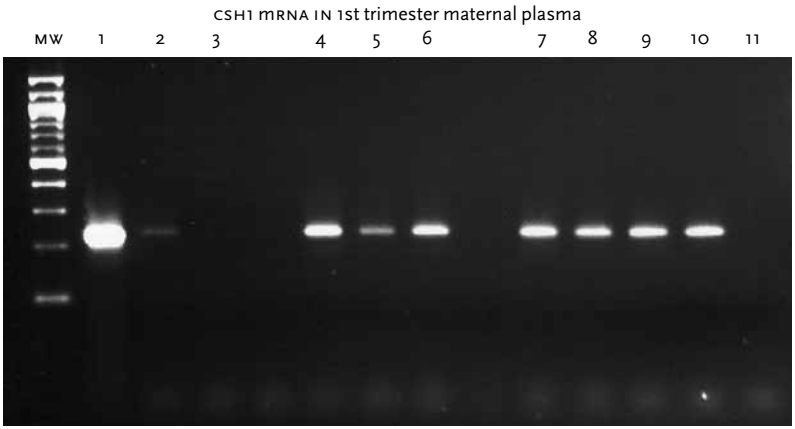
Sequence analysis

For sequence analysis of amplified cDNA fragments, PCR products were electrophoresed in agarose for size separation, purified by affinity-based isolation (Qiagen), subjected to cycle sequencing using BigDye terminators, and analyzed using an ABI Prism 3100 Genetic Analyzer.

Figuer 1 Expression of chromosome 21-encoded genes in early human placenta. High RNA expression in early placental tissues and cells can be seen for LOC 90625 (A) similar in intensity to the chromosome-17-encoded control gene CSH 1 (hPL; D). Signals for the other chromosome 21-encoded genes, PTTG1IP (B) and DSCR4 (C), are weak. Lane C, early placental tissue; lane S, extravillus trophoblast cell line; lane V, villus fibroblast cells; lane MW, molecular weight marker (100-bp ladder).



Detection of *CSH1* mRNA maternal plasma samples. **Figure 2**
CSH1 cDNA amplicons generated by RT-PCR from RNA isolated from maternal plasma (weeks 9 –13) can be seen in all pregnant samples analyzed (*lanes 4 –10*). All negative controls (n = 8) from nonpregnant females were negative (only one is shown, in *lane 11*). Positive controls consisting of early placental tissues and cells are shown in *lanes 1–3: lane 1*, early placental tissue; *lane 2*, extravillus trophoblast cell line; *lane 3*, villus fibroblast cells. *Lane* MW, molecular weight marker (100-bp ladder).



Results

Placental expression of chromosome 21-encoded genes

We tested three chromosome 21-encoded genes[8, 10] for their expression and cell type distribution in early placental tissues (Fig. 1). For comparison, expression analysis of CSH1 (hPL) was done identically. Of the three genes tested, LOC90625, PTTG1IP, and DSCR4, all located within or near the DSCR on chromosome 21q22, the strongest expression was observed for LOC 90625 (Fig. 1A), with expression in all major cell components of the early human placenta, i.e., trophoblast cells, both villus and extravillus, as well as villus fibroblast cells. Expression of LOC 90625 (Fig. 1A) was similar in intensity to that of CSH1, although expression of the latter was restricted to the trophoblast (Fig. 1D), whereas LOC 90625 was expressed in all placental cells. Expression of PTTG1IP (Fig. 1B) and DSCR4 (Fig. 1C) in early placenta was weak compared with that of LOC 90625 and CSH1 as analyzed in weeks 8-12 of pregnancy.

Detectability of chromosome 21-encoded RNA in maternal plasma

We subsequently tested whether chromosome 21-encoded RNA from LOC 90625 could be identified in maternal plasma samples obtained from pregnant women in a similar gestational age window (weeks 9-13). Before this, our RNA isolation procedure was validated using CSH1[1]. In all pregnant females tested (n = 7), CSH1 RNA was detected easily (Fig. 2) in 800 µL of maternal plasma between weeks 9 and 13 of gestation,

whereas plasma specimens from nonpregnant females (n =7) were negative. No false-positive or -negative results were obtained. Subsequently, RNA detection was performed identically for the chromosome 21-encoded gene LOC 90625. mRNA from this gene was successfully detected in maternal plasma, although with a lower intensity compared with CSH1. When 800 μL of plasma was used, detection was successful in 60% of samples; when 1600 μL of plasma was used, detection of LOC 90625 mRNA in first-trimester plasma from pregnant females (n=8) was 100% (Fig. 3). Moreover, sequence analysis of the LOC 90625 cDNA amplicons generated in this way confirmed the specificity of the products.

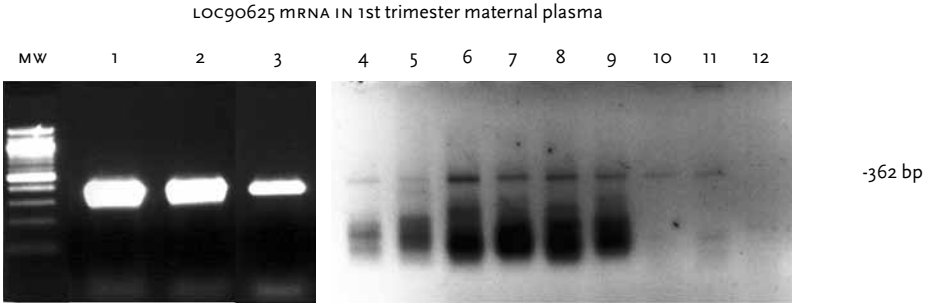
Discussion

In this report, we have shown the presence and detectability of chromosome 21-encoded mRNA of placental origin in maternal plasma during weeks 9 –13 of pregnancy. Given that the gene involved, LOC 90625, has up-regulated expression in trisomic placentas[8] and is located within the DSCR, our data, which extend the pioneering work of Dennis Lo's group[1], indicate that prenatal screening of Down syndrome becomes a realistic option with this plasma-RNA-based assay. To convert the experimentally validated strategy as described in this study to a clinical diagnostic application, quantitative plasma analysis of a placentally expressed, chromosome 21-encoded mRNA (target gene) must be complemented by correction for experimental and biological variability. This can be done through identical analysis of a second, placentally expressed gene from a different autosome (placenta-specific reference gene). We have shown that LOC 90625 is a good candidate for the target gene that meets the criteria presented above: it codes for placentally expressed, chromosome 21-encoded mRNA; it is located within the DSCR; it is overexpressed in trisomy 21 placentas[8]; and it is detectable in maternal plasma in the first trimester, as shown in this study.

Although CSH1 (hPL) is placentally expressed and CSH 1 mRNA can be detected easily in the maternal plasma [Ref. (1) and this study], we feel that CSH 1 is unsuitable for use as a placenta-specific reference gene. CSH 1 belongs to a large gene cluster on chromosome 17q24 with a complex genomic organization and tran- scription pattern. This cluster encompasses five genes (CSH 1, CSH 2, GH1, GH 2, CSH L1) with very similar organization and nearly identical sequences. In addition, each gene codes for at least four alternative transcripts (13). All of these genes except for GH 1 are expressed in the placenta, but expression differs according to time (gestational age), cellular origin, and the amounts expressed. The CSH primers used in our study react with eight transcripts, of which seven are produced by the placenta (CSH 1A, -B, CSH 2-A and -B,

Figure 3

Detection of chromosome 21-encoded mRNA of placental origin in maternal plasma samples. *LOC90625* cDNA generated by RT-PCR of amplicons isolated from maternal plasma (week 9 –13) are identified in all (n = 8) pregnant samples analyzed (*lanes 4 –12*). Note the absence of the nonspecific lower bands with retention of the specific 362-bp band when RNA isolation was performed under vacuum-controlled conditions (*lanes 11 and 12*). All negative controls from nonpregnant females (n = 7) were negative (only one is shown, in *lane 13*). Positive controls consisting of early placental tissues and cells are shown in *lanes 1–3: lane 1*, early placental tissue; *lane 2*, extravillus trophoblast cell line; *lane 3*, villus fibroblast cells. *Lane* MW, molecular weight marker (100-bp ladder).



and GH 2-A, -B, and -C). The same holds for the primers used previously by Ng et al.[1] This feature explains the clear difference we observe between the signal intensities for CSH 1 and LOC 90625 mRNA as detected in maternal plasma. Unfortunately, the obvious strategy of designing alternative primers reactive only with single CSH transcripts is not possible. Consequently, the mRNA copy number of placental mRNA molecules expected to circulate in maternal plasma during the first trimester is likely to be within the range observed for β-human chorionic gonadotropin, with an upper limit of 5000 copies/mL of maternal plasma, rather than the threefold higher amounts deter- mined for hPL[1] in the first trimester.

The RNA isolation procedure we used is similar in design to the method used by Ng et al.[1], i.e., it involves denaturation followed by silica-based affinity isolation. However, our procedure is less hazardous and simpler because it lacks Trizol and chloroform treatments. We noticed that the presence of nonspecific RT-PCR signals when low-abundancy targets were analyzed (see right hand panel in Fig. 3) was attributable to reactivity with the carrier RNA present in the isolation reagents. However, these nonspecific bands disappeared, whereas the specific bands were retained, when the vacuum manifold system was used. In addition, with the vacuum manifold system, up to 24 samples can be processed simultaneously. Using the same affinity-based isolation principle, the RNA isolation procedure can be automated and modified to a walk-away procedure for use with a robotic workstation (such as the BioRobot MDX).

In conclusion, we demonstrate the presence and detectability of chromosome 21-encoded mRNA (LOC90625) of placental origin in the plasma of pregnant women between weeks 9 and 13 of pregnancy. Because expression of the LOC 90625 gene is up-regulated in trisomy 21 placentas, this could permit the development of clinical diagnostic tests based on analysis of plasma RNA for Down syndrome screening during the first trimester.

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Detection of placental Transcription Factor mRNA in maternal plasma

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mRNA of placental origin, including chromosome 21-encoded mRNA, can be detected reliably in maternal plasma during the first trimester of pregnancy[1; 2] The presence and detectability of placental RNA in maternal plasma permits rapid screening of new markers to test their feasibility for the use in non-invasive prenatal diagnostic assays. In contrast to conventional protein-based assays, new markers can include gene products with intracellular localization and noncoding mRNA. We challenged these features by screening maternal plasma for large number of RNA targets (n=80) known or expected to be present in extraembryonic tissues. This set included genes coding for transcription factors, genes subject to genomic imprinting, genes coding for noncoding RNA and other genes with restricted or abundant expression in trophoblast cells. Target genes were distributed over all chromosomes except the Y chromosome.

Peripheral blood samples were collected from pregnant women attending the Prenatal Diagnostic Centre of the VU University Medical Center. All participants gave informed consent before being included in the study. The study was approved by the VU University Medical Center Ethics Committee. EDTA blood was collected between weeks 9 and 13 of pregnancy. All blood samples were obtained before invasive diagnostic procedures and processed as described previously[2]. RNA was extracted from 1.6 ml of maternal plasma by silica-based affinity isolation with use of the QIAamp MinElute Virus Vacuum system (Qiagen) with minor modifications[2]. The amount of carrier in buffer AL was reduced from 28 to 11.4 µg/ml. Elution of bound RNA was done with 150 µl of AVE buffer instead of MilliQ water. Finally, a 5 min incubation step before elution was introduced in the final step of RNA concentration by Microcon-PCR filters. The two-step, one-tube reverse transcription-PCR (RT-PCR) assay was performed as described except that for a selected set of genes the number of PCR cycles was increased to 50. The characteristics of the 80 genes selected for expression profiling and the PCR conditions used are listed in Table 1 of the data Supplement that accompanies the online version of this technical Brief at http://www.clinchem.org/content/vol50/issue8/. Using RT-PCR, we tested this set of 80 genes for their presence in early placenta tissue (positive control) and their absence in non-pregnant plasma (negative control) and pregnant plasma . Three patterns were observed. Pattern A consisted of no detectable amounts in pregnant as well as non-pregnant plasma (negative/negative). This was seen for 42

Figure 1 A and B Detection of *GCM1* mRNA in maternal plasma (A), and disappearance of *GCM1* mRNA from maternal plasma after delivery (B).

(A) GCM1 cDNA amplicons (232 bp) generated by RT-PCR from RNA isolated from maternal plasma (weeks 9-13) can be seen in all pregnant samples analyzed (lanes 1-6). Controls (non-pregnant females; lanes 7-12) are negative. MW, molecular marker (100-bp ladder).

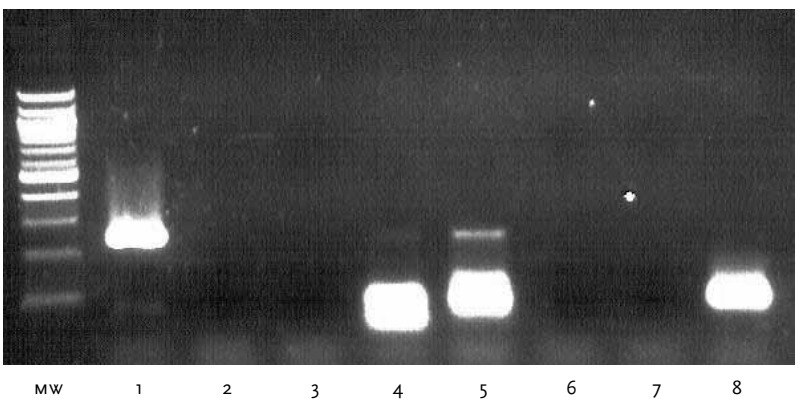
(B) In the first 24 h after delivery, GCM1 mRNA becomes undetectable in maternal plasma by ~ 14 h after delivery, as shown in *lanes 3-8*. Positive and negative controls are shown in *lanes 1 and 2*, respectively. Time intervals in hours between delivery and plasma isolation were 17 h (*lane 3*), 33h (*lane 4*), 13 h (*lane 5*), 16 h (*lane 6*), 15 h (*lane 7*) and 18 h (*lane 8*). The weak specific band in *lane 4* can be explained by the fact that in this pregnancy, delivery was followed by manual removal of the placenta.

Presence of GCM1 mRNA in 1st trimester maternal plasma

A

Disappearance of GCM1 mRNA from maternal plasma after delivery

B



genes (53%). Pattern B consisted of detectable amounts in both pregnant and non-pregnant plasma (positive/positive) and was observed for 30 of 80 genes (37%). Pattern C consisted of detectable amounts in pregnant plasma but no detectable amounts in nonpregnant controls (positive/negative). The latter pattern, the pattern of interest, was observed for eight genes (10%). For five genes, GCM1, PAPPA, ZDHHC1, PSG9 and PLAC1, detection in maternal plasma has never been described. Interestingly, two of these genes (GCM1 and ZDHHC11) code for transcriptional factors, that is, for gene products not accessible by conventional antibody-based assays. An overview of the RT-PCR results is given in Table 2 of the online Data Supplement. Two major conclusions can be drawn: (*a*) this expression profiling approach permits rapid screening of a large set of new fetal markers; and (*b*) the detection of CM1 is typical of the intrinsic power of the plasma RNA method, i.e., analysis of markers not accessible by conventional antibody-based assays becomes possible. In addition, given the hierarchical importance in genetic control and combinatorial and multiple actions of transcription factors on downstream effector genes, analysis of placental transcription factor RNA in maternal plasma is likely to yield (clinical and basic) information distinct from others. GCM1 mRNA codes for the placenta-specific transcription factor glial cells missing (GCM)[3;4] We detected GCM1 mRNA in the plasma of all pregnant women (n=6) tested between weeks 9-13 of pregnancy (Fig 1A, lanes 1-6). Negative controls consisting of identically processed plasma from nonpregnant females (n=6) were negative in all cases (Fig 1A, lanes 7-12). Moreover, after delivery, GCM1mRNA decreased to undetectable concentrations in maternal plasma after ≈ 14 h (Fig 1B, lanes 3-8). The latter demonstrated the first requirements (no persistence after pregnancy) if this factor is to be used for clinical applications.

The GCM1 gene codes for a transcription factor containing the conserved GCM domain[4]. The GCM domain is a zinc-coordinating, sequence-specific (A/G CCCGCAT) DNA binding domain[5]. In both mice and humans, the GCM1 gene is expressed in trophoblast cells [4-8]. In the mouse placenta, the Gcm-1 protein has been show to be essential for vascularization of the placenta by fetal vessels (branching morphogenesis) as well in the formation of multinuclear syncytiotrophoblast by fusion of uninuclear cytotrophoblast cells[9] .

The GCM1 gene can be expected to be dysregulated in pregnancies complicated by trisomy 21 or preeclampsia[10;11] Trisomy 21 placentas have a defect in villus trophoblast fusion as measured and demonstrated in vitro by the reduction in human chorionic gonadotropin βsubunit concentrations caused by delayed and reduced trophoblast fusion. In preeclampsia, decreased placental GCM1 gene expression has been observed[11]. The approach demonstrated in this report not only permits rapid screen-

Table 1 PCR characteristics of the genes (n=80) analyzed for their presence in maternal plasma and absence in non-pregnant plasma. T_{ann}: annealing temperature in °C,

	GENE	Tann	PCR	FORWARD	REVERSE
1	PLAC3	56.6	185	5'-ccg aga agc aga gac ctt taa c-3'	5'-caa aca cac ctg cga tga tg-3'
2	NEUROD1	58.0	540	5'-tcactg ctc agg acc tac taa-3'	5'-gag acc agg tct ggg ctt ttg-3'
3	LOC285173	55.3	391	5'-cgc att act aag ccc aga gca c-3'	5'-tcc ttt att ccc gtc tcc ctc a-3'
4	PREI3	53.1	266	5'-aac cac ctg aag gcc aag at-3'	5'-gct aac cct gct ggg aaa at-3'
5	EOMES	55.0	283	5'-ggc gtg gag gac ttg aat gag c-3'	5'-gga ggc cgt tgg tct gtg-3'
6	TLR3	53.7	355	5'-ttt tga acc tcc agc aca atg agc-3'	5'-cca att gcg tga aac acc ctg-3'
7	RASA1	53.0	260	5'-gac aca gtg gat ggc aag gaa atc-3'	5'-ggc ctg cca aag aga cta tca tga-3'
8	LOC254076	58.2	427	5'-ttc caa cct aga tga gga aag ccg-3'	5'-ttg ctg tcg cat ctt ctc ttc tgg-3'
9	GCM1	56.5	232	5'-gag gca cga cgg acg ctt tat att-3'	5'-caa ttg gac gcc ttc ctg gaa a-3'
10	FOXF2	57.9	232	5'-act cca gca tgt cct cct act cgc-3'	5'-cac atg acg cag ggc tta ata tcc-3'
11	TEF5	60.6	202	5'-cca ctt cct cgc ggt tct-3'	5'-gag gag gca atg gta cgg tc-3'
12	ZAC1	62.9	314	5'-gctg gac gga cgg acg gac-3'	5'-atg ttc ctg tca ctt gcc tgt ttg-3'
13	ERVWE1	56.4	212	5'-caa cca cga acg gac atc caa a-3'	5'-ttg aag agc agg cgc aaa tcc t-3'
14	SGCE	55.0	338	5'-gga atg tat acc cat cag cag gtg-'3	5'-tgt tgc ctt ctg agt tcc att ac-3'
15	PEG1	54.4	186	5'-ttt cac tta caa ggg act gcg tat-3'	5'-tca ctg aag cca aag cct aag a-3'
16	PEG10	59.6	248	5'-tgt ctt cgc aga gga gtc ctc g-3'	5'-ttg ggg aca cac gca ctc tta tg-3'
17	GDNFRB	61.5	384	5'-gaa gct gcg ctc ctc cta cat ct-3'	5'-aac cca atc atg cca gca taa gag-3'
18	EST-YD1	55.1	302	5'-agc atg aga cgg aat gag gtc a-3'	5'-gga tga gtc tgc ata cac cca ctt-3'
19	PAPPA	56.9	376	5'-aaa ctg ttg gct gtc agt ggg aag-3'	5'-gga tgt atg tga cag cag gtg atg-3'
20	LOC219738	59.2	147	5'-ggg gaa atc gtg cag gat ga-3'	5'-cct ggc acc tgc ctg ctg ct-3'
21	UNC5H2	54.2	138	5'-cag atc tac ttc aag tgc aa-3'	5'-aaa gag ctc ctc cac ctg ct-3'
22	KCNMA1	53.3	146	5'-atg ttt gcc agc tac gtc cct ga-3'	5'-tcc ttc agg aag ttg gaa aca ct-3'
23	PLAC9	52.8	325	5'-agg taa agg cca ccc ctc tt-3'	5'-ttc agg tta ctg aca cga ca-'3
24	HNRPH3	52.8	402	5'-atg acg cta gtg atg gga ca-3'	5'-cat cat atc cat cac ctc ct-3'
25	LCX	51.0	356	5'-ggcatc agt caa gac ttt aa-3'	5'-aat cat ttt ctg agt gct tt-3'
26	GU2	55.0	248	5'-cat gat tcc gac tgg ata ctc tc-3'	5'-ccc cca ctt ctt gat cga ttt ct-3'
27	DDX21	52.5	470	5'-cct tga tca act caa atg tg-3'	5'- tta ttg acc aaa tgc ttt ac-3'
28	SUPV3L1	53.5	141	5'-cat tca ggc ccc aca aac ag-3	5'-aca tgg cac gcc agc agc at-3'
29	MYST4	53.4	168	5'-gtg cag tgt gcc ctc cct ga-3'	5'-ggc gtg gaa gat aat tca gt-3'
30	DLG5	54.4	151	5'-gtg atg aag gcc tcc cag ca-3'	5'-caa tgt cca gga ggc agt gt-3'
31	SIRT1	50.3	141	5'-tct tgt ggc agt aac agt ga-3'	5'-tcc agc tcc tcc agc tct ct-3'
32	VR22	53.4	426	5'-tca gga tct gca ggt cca a-3'	5'-tga aaa gct gac aca tgt tg-3'
33	DKFZP564G092	51.9	333	5'-atg gct att gat cag gcc cac ag-3'	5'-tta gat ggt tgg aca ttt tac ct-3'
34	LRRTM3	52.5	194	5'-ccg gcg agg tgc tgc tga at-3'	5'-cat cgt atc ttc ctg tgc at-3'
35	MAWBP	57.6	338	5'-aac atg aac agc acg ctc acg tt-3'	5'-gtc tgc cca cca ggc tct cct tt-3'
36	RUFY2	53.6	315	5'-ttg gaa ctg gtg gag aag ctg ta-3'	5'-atc aat cac tcc aac ttg tga gt-3'
37	DNA2L	51.1	405	5'-tca ctg ctt cac agt cac tag aa-3'	5'-gac tta gat tta agc ggt aca tt-3'
38	SLC25A16	53.7	911	5'-ccc aca acc cgc aga gac ttc tac-3'	5'-agg tga aaa aac tgc ttc ata agt-3'
39	DNAJB12	54.4	157	5'-gat gcc tat cct cat cct ga-3'	5'-gga gaa agt gtc tcc cac at-3'
40	CBARA1	53.5	113	5'-ctt gag ttt gaa cgc cat ga-3'	5'-ctc tgc atg gcg gtc agc tt-3'

PCR : amplicon sizes in BP, Forward and reverse: gene specific primer sequences.

	GENE	Tann	PCR	FORWARD	REVERSE
41	DNAJC9	2.	359	5'-gat cct ggg aaa agt cta tt-3'	5'-tga caa agg cat tat agg at-3'
42	MRPS	55.6	186	5'-acc atg gtc cac ctc act ac-3'	5'-atg act gtt ggg caa tgg a-3'
43	PPP3CB	51.2	470	5'-aga gct cat gaa gct caa ga-3'	5'-cca cta ggc aac atc cct gt-3'
44	VCL	52.8	254	5'-tgg gtc aag ggg cat cct ct-3'	5'-tcg agt tca cca aca tca ct-3'
45	CDA017	55.1	335	5'- atc act tgg cag aag tga ca-3'	5'-aca ctt ccc cag gac acc tt-3'
46	TNCRNA	49.1	112	5'-tgt cgt tgg gat tta gag tgt att-3'	5'-caa cat acc agt act ttc aac cat-3'
47	TSSC3	64.6	331	5'-cct caa ggt gga ctg cgt gga c-3'	5'-cgg tcc gac tcg tcc agc gta t-3'
48	OVOL1	60.1	262	5'-ctc tga aga cat ggg cca ctt gac-3'	5'-gcc agt gtg agt tcg gac gtg t-3'
49	EED	53.5	205	5'-tca ttc aca agg aga aat ccg gtt-3'	5'-att gat agc att tcc atg gcc aac-3'
50	IGF2	67.2	272	5'-ctg gca gag gag tgt ccg gca gga-3'	5'-cgg ctt gcg ggc ctg ctg aag tag-3'
51	INS	65.7	290	5'-ccc agc cgc agc ctt tgt gaa c-3'	5'-tgc ctg cgg gct gcg tct agt t-3'
52	ATA3	57.6	259	5'-tga act tca tga tgg att aca ccc-3'	5'-ttt gac acc gtt tgc att ttt c-3'
53	EPSTI1	53.4	397	5'-ata aac cgg aga aat gag ata caa-3'	5'-cac ttc tgt ctg gcg att ctg-3'
54	DLK1	61.4	345	5'-aga tga ccg cga ccg aag c-3'	5'-agg agc att cat aga ggc cat cgt-3'
55	SNRPN	57.2	217	5'-ctg tct tcc ctc tgg agc tgt-3'	5'-tgt tgc ctt ctg agt tcc act tac-3'
56	SNURF	59.0	367	5'-ctg acg cat ctg tct gag gag c-3'	5'-gat aaa ggt atg acg cag gtt ctc-3'
57	RASGRF1	62.3	275	5'-gcg gag ttc gga caa cac aa-3'	5'-ctg cca ccc att cgt cac aat ctt-3'
58	ZDHHC1	62.2	273	5'-ttc agc aga tgc caa cgt gcg gg-3'	5'-agc acc agg agc agg acg ccc agt-3'
59	CSH1	57.8	228	5'-gca cca gct ggc cat tga ca-3'	5'-ata ctc ctg agg aac cgc acg g-3'
60	NEUROD2	62.0	695	5'-ggg aac aat gaa ata agc gag aag-3'	5'-gcg aca gac cct tgc aca gag-3'
61	IMPACT	51.6	429	5'-att agc gac gat ata gat gac c-3'	5'-tct tcg gtc tgt aat agg aat g-3'
62	BETA-HCG	59.4	135	5'-ccc gtg tgc atc acc gtc aac-3'	5'-ccg gat gga ctc gaa gc-3'
63	PSG9	53.4	261	5'-gaa tct tcc tgg cta ctt ctg gta c-3'	5'-gga gtc tcc aag tat aag gtg aag g-3'
64	CEACAM7	55.8	200	5'-ctg tcc ata cag agt gtg cat tcc-3'	5'-ccc ttt ccc ctt tgt acc agt t-3'
65	PP13	55.0	191	5'-gtg cca tac aaa ctg cct gtg tct-3'	5'-aac tca cgc ctg ttc atg acc a-3'
66	ZNF264	56.0	265	5'-ctg atc tgc cac cta gag cat g-3'	5'-act tct cct ggg gat cta ttc c-3'
67	NNAT	63.8	308	5'-aga cca gcg gat ctc ggc aaa ccc-3'	5'-ggg ttt gcc gag atc cgc tgg tct-3'
68	GNASXL	57.4	312	5'-aaa gcc cca gcg caa ctt ac-3'	5'-atc ctc atc tgc ttc aca atg g-3'
69	LOC90625	62.0	262	5'-gcg cgc tct ccg ggt tcc aac c-3'	5'-ggg gcc tgt cca ctt cgg tgg tag-3'
70	PTTG1IP	52.9	178	5'-ccc tca tca ttc ccc ata aa-3'	5'-aaa aag gtt tag cta ttc ccc a-3'
71	DSCR4	56.8	292	5'-atc ttg acg aga gat gat gaa ccc c-3'	5'-tgt gga gcc cat gga ggt aag-3'
72	LOC343766	58.9	378	5'-tta ttg tca acg cca gcc cc-3'	5'-cag tgt gca gca aac tcc cag t-3'
73	BACE2	58.3	375	5'-tat aac gca gac aag gcc atc g-3'	5'-gaa gat gac gta gaa gcc ctc cat-3'
74	CBR3	56.0	287	5'-gtc gcc ttc aag agt gat gat cca a-3'	5'-cct tcc ctc tca tgc acc tca ttt t-3'
75	ADAMTS5	57.4	299	5'-tct aag ccc tgg tcc aaa tg-3'	5'-cag att ctc ccc ttt cca ca-3'
76	CLDN8	48.6	137	5'-cac aac cca aaa aag tta tca c-3'	5'-atg cag tct tta gca atg tca t-3'
77	SYNGR3	63.3	300	5'-act ctg gtc ctt cct gtg gtt cgt-3'	5'-aag ggc ggg ctc tgg tag gt-3'
78	MAGEA4	60.2	225	5'-gag cag aca ggc caa ccg ga-3'	5'-agg agc ctg tgc acc cac ca-3'
79	CAPN6	56.4	293	5'-cag act ttt ctg tga tcc aac att-3'+K3	5'-aat gtt gga tca cag aaa agt ctg-3'
80	PLAC1	52.8	325	5'-agg taa agg cca ccc ctc tt-3'	5'-aca acg cag tca ttg gac tt-3'

Table 2 Schematic representation of RT-PCR results obtained for 80 genes (I-III) as analyzed in positive controls (IV) (first trimester placenta tissue, weeks 9-14), negative controls (non-pregnant females) (V) and pregnant females (VI). Results are presented as boxed PCR fragments,

	I	II	III	IV	V	VI	VII
	GENE	**DESCRIPTION**	**CHR**	**PLACENTA**	**NON-PREGN**	**PREGN**	**PATTERN**
1	PLAC3	placenta protein 3	1				A
2	NEUROD1	bHLH transcr factor	2				A
3	LOC285173		2				A
4	PREI3	cell-cyle protein	2				B
5	EOMES	T-box transcr factor	3				A
6	TLR3	toll-like receptor	4				A
7	RASA1	ras GTP-ase	5				B
8	LOC254076	C6orf63	6				A
9	GCM1	GCM transcr factor	6				C
10	FOXF2	forkhead transcr factor	6				A
11	TEF5	TEF1 transcr factor	6				A
12	ZAC1	ZnF transcr factor	6				A
13	ERVWE1	syncytin	7				A
14	SGCE	sarcoglycan	7				B
15	PEG1	hydrolase	7				B
16	PEG10	retrotransps gag prot	7				B
17	GDNFRB	GDNF receptor	8				A
18	EST-YD1	C9orf27	9				A
19	PAPPA	metalloproteinase	9				C
20	LOC219738	C10orf35	10				A
21	UNC5H2	unc-5 homolog	10				A
22	KCNMA1	potassium channel	10				A
23	PLAC9	placenta protein 9	10				A
24	HNRPH3	ribonucleoprotein	10				B
25	LCX	cxxc ZnF	10				A
26	GU2	RNA helicase	10				A
27	DDX21	RNA helicase	10				A
28	SUPV3L1	RNA helicase	10				A
29	MYST4	hist acetyltransferase	10				B
30	DLG5	discs, large homolog 5	10				A
31	SIRT1	ribosyltransferase	10				A
32	VR22	alpha-T-catenin	10				B
33	DKFZP564G092	ubiq prot ligase	10				B
34	LRRTM3	transmembr recept	10				B
35	MAWBP	oxireductase	10				B
36	RUFY2	FYVE ZnF	10				B
37	DNA2L	helicase	10				B
38	SLC25A16	solute carrier	10				B
39	DNAJB12	DNAJ chaperone	10				B
40	CBARA1	Ca sensor	10				B

either present or absent. Absent boxes indicate 'not tested'. Column VII shows that 3 patterns were observed. The specific pattern (C) was seen for eight genes highlighted in bold.

	I	II	III	IV	V	VI	VII
	GENE	**DESCRIPTION**	**CHR**	**PLACENTA**	**NON-PREGN**	**PREGN**	**PATTERN**
41	DNAJC9	DNAJ chaperone	10				B
42	MRPS16	mit ribosomal prot	10				B
43	PPP3CB	prot phosph	10				A
44	VCL	vinculin	10				A
45	CDAO17	ribonucleoprotein	10				A
46	TNCRNA	non coding RNA	11				B
47	TSSC3	tum suppr subtransf cand	11				B
48	OVOL1	OVO transcr factor	11				A
49	EED	PcG transcr factor	11				B
50	IGF2	growth factor	11				B
51	INS	growth factor	11				A
52	ATA3	solute carrier	12				A
53	EPSTI1	epith stromal interaction 1	13				B
54	DLK1	delta-like homolog	14				A
55	SNRPN	ribonucleoprotein	15				A
56	SNURF	SNRPN upstream RF	15				B
57	RASGRF1	exchange factor	15				A
58	ZDHHC1	ZnF transcr factor	16				C
59	CSH1	growth factor	17				C
60	NEUROD2	bHLH transcr factor	17				A
61	IMPACT	imprinted and ancient	18				B
62	BETA-HCG	growth factor	19				C
63	PSG9	pregnancy-specific prot	19				C
64	CEACAM7	adhesion molecule	19				A
65	PP13	lysophospholipase	19				A
66	ZNF264	C2H2 ZnF transcr factor	19				B
67	NNAT	neuronatin	20				A
68	GNASXL	GTPase	20				A
69	LOC90625	C21orf105	21				C
70	PTTG1IP	pit tum transf prot 1	21				B
71	DSCR4	down syndr crit region	21				A
72	LOC343766		21				A
73	BACE2	aspartyl protease	21				B
74	CBR3	carbonyl reductase	21				B
75	ADAMTS5	metalloprotease	21				B
76	CLDN8	claudin	21				A
77	SYNGR3	synaptogyrin	22				A
78	MAGEA4	melanoma antigen	X				A
79	CAPN6	cysteine protease	X				A
80	PLAC1	placenta protein 1	X				C

ing of a large set of potential new markers, it allows the detection of markers not accessible by conventional antibody-based assays. This greatly increases the number of markers that become available for non-invasive prenatal diagnosis. Given the nature of these new markers, i.e., the genes coding for placental transcription factors, such as GCM1, the information obtained by use of these markers could, given their hierarchical importance in genetic control and combinatorial and multiple actions, be distinct from the information provided by other markers and could be important for study of both normal pregnancy biology and pregnancy-associated diseases. The same arguments hold when searching for plasma RNA markers informative for active disease status in cancer.

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A novel method to identify syncytiotrophoblast-derived RNA products representative of trisomy 21 placental RNA in maternal plasma

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Summary

A novel in vitro method is described wherein gene expression profiling is reflective and informative for the way how syncytiotrophoblast cells shed RNA products in vivo in maternal plasma. After controlled denudation, RNA is obtained selectively from the syncytiotrophoblast cells of a trisomy 21 placentae. cDNA copies are subsequently analyzed by microarray profiling and cDNA cloning with sequencing. Given the preponderance of 5' mRNA fragments lacking a poly A tail, the placental RNA products are amplified after polymerase A-mediated tailing by using a method originally designed for small-sized microRNAs. This approach, when combined with cDNA library or microarray expression screening, is a novel in vitro method to screen for syncytiotrophoblast-derived RNA products representative of trisomy 21 placental RNA as present in vivo in maternal plasma.

Keywords: placenta, maternal plasma, RNA , syncytiotrophoblast, trisomy 21, denudation

1. INTRODUCTION

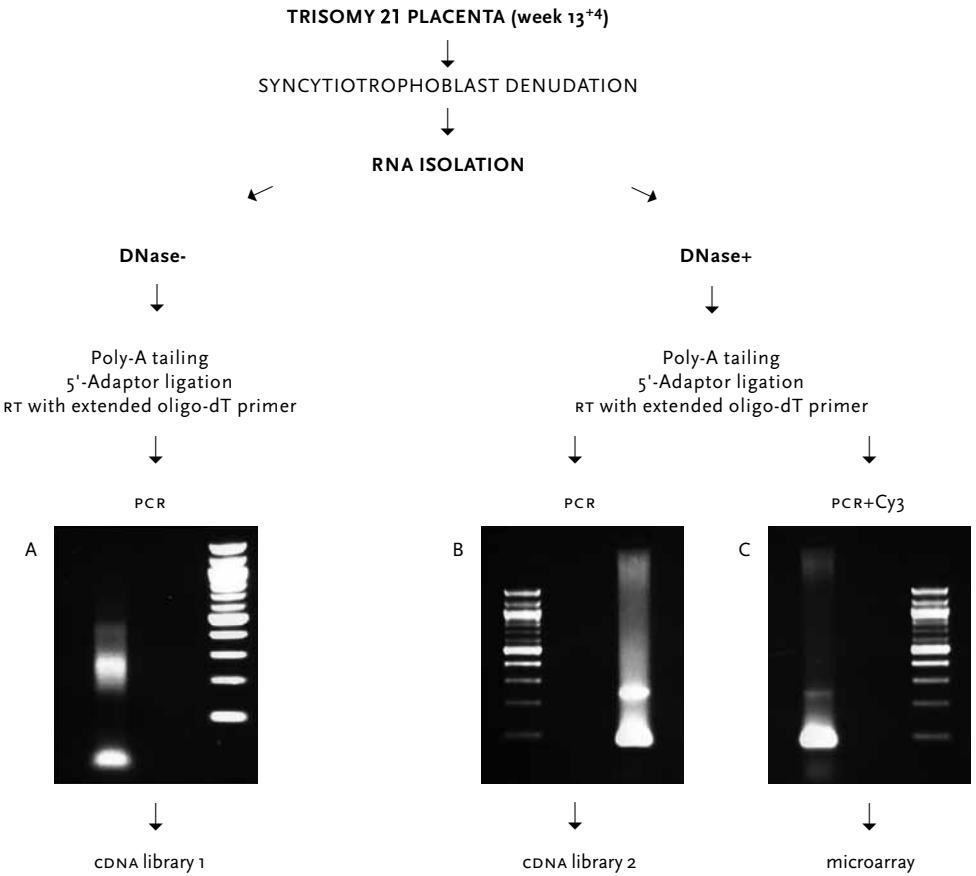
Non-invasive prenatal diagnosis of Down syndrome by quantitative analysis of placental RNA or DNA isolated from maternal plasma should be feasible when three conditions are met: (1) detectable in maternal plasma, (2) informative for chromosome 21, and (3) changed in copy number when a third copy of chromosome 21 is present.

RNA expressed from genes within the Down syndrome critical region on chromosome 21 and overexpressed in the syncytiotrophoblast of trisomy 21 placentas are candidate markers for screening by using RNA[1]. DNA sequences (i.e. CpG islands) from genes on chromosome 21 with differential modification (such as methylation) being restricted to the placenta are candidate markers for screening using DNA[2]. Both approaches have intrinsic merits, but critically depends on the identification of markers on chromosome 21, that fulfill all criteria of detectability, informativity and predictive power.

We showed chromosome 21-encoded RNA (LOC90625, now called C21orf105) to be detectable in maternal plasma during normal pregnancies[1]. By gene expression profiling using microarray, this gene was found to be up-regulated in placentas with trisomy 21 as compared to placentas with a normal phenotype[3]. However, marker profiling of affected tissues using whole tissue fragments does not correct for the fact that the predominant, if not exclusive, source of placental RNA shed into the maternal circulation is derived from the syncytiotrophoblast[4]. Genes with additional or restricted placental expression in other than syncytiotrophoblast cells such as villous stromal cells or cytotrophoblast stem cells, will generate a false-positive signal in expression profiling experiments when whole tissue fragments are used. In vitro methods should be designed and tested, where gene expression profiling is reflective and informative for the syncytiotrophoblastic origin of the placental RNA products found in maternal plasma. Baczyk et al. recently developed a floating villous explant culture model in which denuded first trimester villi spontaneously regenerate syncytiotrophoblast after 48 hours of culture [5]. Besides the biological importance of their data, showing the existence of bipotential trophoblast progenitor cells in the first trimester placenta, their method of controlled selective removal of syncytiotrophoblast can be of great use for plasma marker profiling. The in vitro

manipulation method allows comparison to the way the syncytiotrophoblasts shed DNA/RNA microparticles in vivo. After controlled denudation, we selectively obtained RNA from the syncytiotrophoblast of trisomy 21 placentae, that was subsequently analyzed by microarray expression profiling and sequencing after cDNA cloning (see figure 1). Given that placental RNA in maternal plasma is associated with a preponderance of 5' mRNA fragments lacking a poly A tail [6], the placental RNA products were subjected to polymerase A-mediated tailing, 5'-adapter ligation and reverse transcription-polymerase chain reaction (RT-PCR) with extended oligo(dT) primers using a method originally designed for microRNAs [7].

Figure 1 Schematic representation of the strategy used to identify syncytiotrophoblast-derived RNA products representative of trisomy 21 placental RNA in maternal plasma.



This novel approach, for several reasons, is clinical useful and biologically relevant: (1) The RNA recovered after denudation is representative of the RNA expressed by and released from the placental syncytiotropoblast as indicated by the presence of both high- and low-abundancy targets, i.e hPL and LOC90625, recovered reliably before and after amplification. (2) The RNA isolated can be used for cDNA synthesis, including adequate recovery of small-sized RNA s. The 95-bp microRNA precursor of hsa-miR-141[8] was correctly and consistently identified following cDNA synthesis and cloning.

(3) The amplicons generated after cyanine (Cy)3 labeling can be used for genome wide expression screening using microarray approaches. To ensure complete genome wide coverage, future screening strategies should correct for the fact that placental RNA is enriched for 5'-mRNA fragments [9], whereas most expression libraries including the library used in the present study, are enriched for 3'-mRNA fragments. (4) Application of this method to placental RNA isolated from maternal plasma during first trimester could permit a genome-wide expression strategy for fetal RNA marker screening in clinical samples.

2. MATERIALS

2.1. *Cell Culture and Lysis*

1. Hanks' Balanced Salt Solution (HBSS) (Invitrogen, Carlsbad, CA).
2. F-12 Nutrient Mixture (Ham's) with GlutaMAX I (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS) and penicillin/streptomycin (100 µg/ml).
3. Trypsin-EDTA (10X): 0.5% (w/v) trypsin in EDTA (2 g/L) (Invitrogen).

2.2. RNA *isolation (Virus Min-Elute system)*

1. Carrier RNA : 1 µg/µl in AVE buffer (Qiagen, Valencia,CA). Store diluted carrier RNA as aliquots at -20°C.
2. Lysis buffer: 9.04 mg/l carrier RNA in AL buffer (Qiagen). Prepare fresh and mix gently before use. Store AL buffer at room temperature and do not shake before use.
3. Protease solution: Dilute dry protease (Qiagen) in 4.4 ml Protease Resuspension buffer (Qiagen). Store Protease solution as aliquots at -20°C.
4. QIAvac 24 (Qiagen) connected to a vacuum pump producing –800 to –900 mBar. Connect the MinElute columns (stored at 4°C)(Qiagen) to the QIAvac by using the VacConnectors (Qiagen). Apply the extension tube on top of the column.

5. AW1 Washing buffer (Qiagen): Store at room temperature and shake before use.
6. AW2 Washing buffer (Qiagen): Store at room temperature and shake before use.
7. AVE Elution buffer (Qiagen): Store at room temperature.
8. DNAse: Dissolve dry DNAse (1500 Kunitz units) (Qiagen) in 550 µl RNA se-free water and mix gently. Do not vortex. Divide in aliquots of 10 µl and store at -20°C.
9. RDD buffer (Qiagen): Store at 4°C.

2.3. Q-RT-PCR (EZ RTth system)

1. TaqMan EZ buffer (5X): 250 mM Bicine, 575 mM potassium acetate, 0.05 mM EDTA, 300 nM Passive Reference, 1.40% (w/v) glycerol, pH 8.2 (Applied Biosystems, Foster City, CA).
2. Manganese acetate: 25 mM (Applied Biosystems).
3. Deoxynucleotides: 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 20 mM dUTP.
4. 2.5 U/µl RTth DNA polymerase: (Applied Biosystems).
5. 1 U/µl AmpErase uracil- N-glycosylase (UNG) (Applied Biosystems).
6. Taqman probes (50 µM; all from Eurogentec) : hPL 5'-FAM- TTC TGT TGC GTT TCC TCC ATG TTG G-TAMRA-3', C21orf105 5'-FAM-CGC CTA CTG GCA CAG ACG TG-TAMRA-3', 50 µM (Eurogentec).
7. Primers (50 µM; all from Eurogentec) : hPL-F 5'-CAT GAC TCC CAG ACC TCC TTC- 3'; hPL-R 5'-TGC GGA GCA GCT CTA GAT TG-3'; C21orf105-F 5'-tGC ACA TCG GTC ACT GAT C-3'; C21orf105-R 5'-GGG TCA GTT TGG CCG ATA-3'.
8. MicroAmp Optical 96-well reaction plates (Applied Biosystems).
9. ABI Prism Optical Adhesive Covers (Applied Biosystems).
10. ABI 7300 Real-Time PCR System (Applied Biosystems).

2.4. cDNA Synthesis

1. 1.5U/µl Poly(A) polymerase (Takara, Gennevilliers, France).
2. 10 mM ATP (Roche, Mannheim Germany).
3. 40 U/µl RNA sin: (Promega, Madison, WI).
4. 10X Reaction buffer: 0.5 M Tris-HCl (pH8.0), 2.5 M NaCl and 100 mM $MgCl_2$.
5. RNABee (Tel-Test Inc., Friendswood, TX).
6. Precipitation mix: Prepare a mix of Ethanol/NaOAc by mixing 95 ml of 100% ethanol with 4 ml of 3 M NaOAc (pH 4.8) and 1 ml sterile water and store at -20°C.
7. 20 mg/ml Glycogen: (Roche).

8. 10X T4 RNA ligase Buffer (Promega).
9. 10 U/µl T4 RNA ligase (Promega).
10. 40 U/µl RNA sin: (Promega).
11. 40% (w/v) Polyethylene glycol: (Sigma-Aldrich, St-Louis, MO).
12. 5'-Adapter RNA : (5'-CGA CUG GAG CAC GAG GAC ACU GAC AUG GAC UGA AGG AGU AGA AA'3, 100 ng/µl; Eurogentec).

2.5. *Reverse transcription*

1. RT- primer: 5'- ATT CTA GAG GCC GAG GCG GCC GAC ATG-$d(T)_{30}$ (A, G, or C) (A, G, C, or T)- 3' (2 µM ; Eurogentec).
2. Deoxynucleotides (all 10 mM; dATP, dCTP, dGTP, dTTP.
3. 10X Reverse transcriptase buffer (200 mM Tris-HCL (pH 8.4) and 500 mM KCl).
4. 25 mM.$MgCl_2$.
5. 0.1 M Dithiothreitol (DTT).
6. 40 U/µl RNA seOUT.
7. 200 U/µl SuperScript III RT (Invitrogen).

2.6. *Polymerase Chain Reaction*

1. Taq DNA polymerase.
2. 10X PCR-buffer II: (100 mM; Tris-HCL, pH 8.3, 500 mM KCl;. Applied Biosystems).
3. Deoxynucleotides (all 2 mM; dATP, dCTP, dGT, dTTP).
4. 25 mM $MgCl_{2.}$
5. 5 M (5X) Betaine (Fluka, Buchs, Switserland). Dilute 33.79 g Betaine in 50 ml MilliQ (Millipore Corporation, Billerica, MA) water. Store as aliquots at –20°C.
6. Forward PCR primer: 5'-ATT CTA GAG GCC GAG GCG GCC GAC ATG t-3' (50 pmol/µl; Eurogentec).
7. Reverse PCR primer: 5'-GGA CAC TGA CAT GGA CTG AAG GAG TA-3' (50 pmol/µl Eurogentec).

2.7. *Cloning*

1. QIAquick Gel extraction Kit (QIAGEN).
2. TOPO TA Cloning kit (Invitrogen).
3. TOPO vector (Invitrogen).
4. Transformaton SOC medium [2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5 mM kcl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose].
5. Competent cells F10' (Invitrogen).

6. Luria Bertani (LB) plates containing 1.0% (w/v) tryptone, 0.5% yeast extract and 1.0% NaCl, 50 μg/ml Ampicillin, 40 mg/ml 5-bromo-4-chloro-3-indolyn-β-D-galactoside (X-gal) and 100 mM isopropyl β-D-thiogalactoside (IPTG).

2.8. Sequencing

1. Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems)
2. Primers (Eurogentec): M13-F: 5'-GTAAAACGACGGCCAGT-3'; M13 -R: 5'-CAGGAAACAGCTATGAC-3'.
3. Primers (Eurogentec): PCR -F: 5'-ATT CTA GAG GCC GAG GCG GCC GAC ATG T-3' ; PCR-R: 5'-GGA CAC TGA CAT GGA CTG AAG GAG TA-3'.

2.9. Microarray

2.9.1. Preparation of labeled cDNA

All technical details can be found at http://www.vumc.nl/microarrays.

1. Fluorolink Cy3 Monofunctional Dye 5-pack (GE Healthcare, Chalfont, St. Giles, UK). Dissolve dry pellet in 20 μl of DMSO (Sigma). Aliquot 2 μl into 10 single use tubes that are then dried in speed vac. Store desiccated at 4°C. nhs-ester conjugated Cy-dye is rapidly hydrolysed in water, therefore, do not store in dimethyl sulfoxide (DMSO) or water.
2. 1 M $NaHCO_3/Na_2CO_3$, pH 9.0
3. Deoxynucleotides: dATP (10 mM), dCTP (8 mM), dGTP (10 mM), dTTP (10 mM), Cy3-dCTP (2mM).
4. Qiaquick PCR purification kit (Qiagen).

2.9.2. Hybridization of labeled cDNA tot oligonucleotide-DNA slides.

1. 10 μg/μl $pd(A)_{40-60}$, (GE, healthcare).
2. 9.2 μg/μl tRNA (Sigma-Aldrich).
3. 1.0 μg/μ human Cot-1 DNA l (Invitrogen).
4. 3 M NaAc, pH 5.2 (Sigma).
5. 10% sodium dodecyl (SDS) (Sigma-Aldrich).
6. 20 x standard saline citrate (SSC) (Sigma-Aldrich).
7. Dextran sulphate (USB).
8. 100% formamide.
9. Compugen Hum Librnr 2 slides containing 29,134 oligo's of 28,830 genes.

Culture media were obtained from InVitroGen. Primers and probes were obtained from Eurogentec. RNA isolation systems were purchased from QIAgen. Reagents for RT-PCR and sequencing were from InVitroGen and Applied Biosystems. TOPO-TA vector cloning system was from InVitroGen.

3. METHODS

3.1. Syncytial denudation of trisomy 21 placenta.

Placental tissue fragments (week 13^{+4}) are obtained by currettement of trisomy 21 pregnancies. The diagnosis is confirmed by karyotyping. Informed consent is obtained prior to use.

1. Within 10 minutes after removal, wash placental tissue fragments extensively in cold HBSS.
2. Perform syncytial denudation (see note1) by digestion for 5 min at 37°C with 0.125% trypsin in phosphate buffered saline. Use 2 ml per well. Immediately after this denudation, the medium containing DNA and RNA released selectively from the syncytiotrophoblast cells is subjected to RNA isolation.

3.2. RNA *isolation.*

1. Add to 75 ml protease, 400 ml of the medium containing shedded DNA/RNA and 400 ml of lysis buffer. Mix by pulse vortexing.
2. Incubate for 15 minutes at 56° C and centrifuge briefly.
3. Add 500 ml of 100% ethanol and mix by pulse vortexing.
4. Incubation of 5 min. at room temperature.
5. Apply the lysate to the extension tube of the QIAamp MinElute column and apply vacuum of -800 mbar.
6. Switch off the pump, when all lysate has passed the column. In the next washing steps the pump is also used at –800 mbar to get the fluid through the column.
7. Wash column with 600 ml AW1 buffer.
8. Apply a mix of 10 ml DNAse and 70 ml of RDD buffer (do no vortex) on column and incubate for 30 min.
9. Wash column with 750 ml AW2 buffer.
10. Wash with 750 ml ethanol 100%.
11. Remove the column from the QIAVac system and centrifuge for 3 min. at 20,000g.
12. Incubate the column for 5 min with 50 ml AVE buffer and centrifuge for 1 min. at 20,000g.
13. Store the eluate in aliquots of 10 ml at -80°C.

3.3. Quantitative RT-PCR.

1. Dilute a standard curve from 0 to 10^7 copies /10 µl in milliQ water for hPL or C21orf105.
2. Prepare the master mix for the number of samples needed by adding per sample: 1 x TaqMan EZ buffer, 3 mM manganese acetate; 300 µM each of dATP, dCTP, dGTP, 600 µM UTP; 0.1 U/µl RTth enzyme; 0.01 U/

µl AmpErase UNG; 300 nM each of forward and reverse primer (hPL or C21orf105); and 100 nM Taqman probe for hPL or 400 nM Taqman probe for C21orf105 in a volume of 40 µl.
3. Pipette the mix in the reaction plate and add 10 µl of sample. The total volume is 50 µl. Cover the plate.
4. Run the RT-PCR under the following conditions: 2 min. at 50°C, 30 min. at 60°C, 5 min. at 50°C followed by 50 cycles for 20 s at 94°C and 1 min at 56°C (hPL) or 59°C (C21orf105).

3.4. cDNA synthesis.

1. Prepare a mix of 10 µl RNA , 5 U poly(A)polymerase, 100 nM ATP, 40 U RNA sin, and 1 x reaction buffer.
2. Add MilliQ water to a total volume of 50 µl.
3. Incubate at 37 °C for 30 min.
4. The polyadenylation reaction is stopped by phenol/chloroform extraction.
5. Add 1 volume of RNA Bee and 0.1 volume chloroform.
6. Shake vigorously and incubate for 5 min. on ice.
7. Spin down for 15 min at maximum speed at 4°C.
8. Transfer the aqueous phase to a clean tube and add 2.5 volumes cold ethanol/NaOac mix and 2 µl glycogen.
9. Mix and incubate at -80°C for 30 min.
10. Centrifuge for 25 min at maximum speed at 4°C.
11. Wash twice with 80% cold ethanol.
12. Airdry the pellet and resuspend in 34.5 µl MilliQ water.
13. The 5' adapter RNA is ligated to poly(A)-tailed RNA in the reaction mix containing: 34.5 µl RNA , 1x ligase buffer, 120 U RNA sin, 20% polyethylene glycol (w/v), 30 U T4 RNA ligase and 750 ng 5'-adapter RNA .
14. Incubate at 37°C for 30 min.
15. The reaction is stopped by phenol/chloroform extraction and ethanol precipitation.
16. The pellet (see note 2) is diluted in 8 µl MilliQ water.

3.5. Reverse transcription

1. To the RNA pellet in 8 µl of Milli-Q water, add 2 nmol RT-primer and 1 nmol dNTP mix
2. Incubate the sample mix is for 5 min at 65° C and chilled subsequently on ice.
3. Mix the reverse transcription mix to contain 1x RT-buffer, 100 nM $MgCl_2$, 2 µM DTT, 40U RNA seOUT and 200 U of SuperScript III RT enzyme.
4. Add this mix to the sample mix and incubate for 1 h at 50°C.

5. Inactivate this reaction by 15 min at 70° C and store the cDNA at -20° C.

3.6. PCR

1. Amplify the cDNA with a PCR mix containing 10 µl cDNA, 1 x Buffer II, 0.2 mM dNTP, 10 µM forward PCR primer, 10 µM reverse PCR primer, 1.5 µM $MgCl_2$, 0.2 U/µl Taq polymerase and 1M Betaine in a reaction volume of 50 µl.
2. The PCR conditions are 4 min. at 95° C , 25 cycles of 1 min at 95°C, 1 min at 50°C, 2 min at 72°C followed by 10 min at 72°C.

3.7. Cloning

1. Separate the PCR-amplicon by running a 2% gel electrophoresis and extract the different fragments of the amplicon with the QIAquik Gel extraction kit.
2. The eluate volume is 30 µl.
3. Clone the isolated amplicons into the Topo-TA vector by performing the ligation and transformation reaction.
4. For this, mix 4 µl of isolated amplicon with 1 µl of salt solution and 1 µl TOPO vector.
5. Mix gently and incubate for 30 min. at room temperature. Store at -20°C.
6. The ligation mix is transformed into TOP 10 F' competent cells by adding 2 µl of ligation mix to 50 µl F10' competent cells.
7. Incubate for 30 min on ice.
8. Heat shock the mixture for 30 s at 42° C and chill on ice.
9. Add 250 µl SOC medium (room temperature) and incubate gently by shaking for 1 hour at 37° C.
10. Spread 100 µl of the mix on prewarmed (37° C) LB plates with Ampicillin, X-gal and IPTG.
11. Incubate overnight at 37° C.
12. The white colonies are screened by colony PCR. The colony is picked with a sterile toothpick and incubated for 5 hours at 37° C in 100 µl of LB media with ampicillin. PCR is done as described above with 10 µl of inoculated LB mix by using PCR or M13 primers.

3.8. Sequencing

1. The sequence reaction is performed on the amplicons by adding 1 µl terminator ready reaction mix, 1 µl amplicon, 3.2 pmol primer (M13 forward or M13 reverse) and 1x sequence buffer in a reaction volume of 20 µl MilliQ water.

2. The temperature profile is 25 cycles of 10 s at 96°C, 5 s at 50°C and 4 min. at 60°C.
3. Precipitation of the product is done by isopropanol.
4. Sequence analysis is performed on the ABI3100. (Applied Biosystems).

3.9. Microarray.

3.9.1. RT-PCR

For microarray screening, RT-PCR is done in the presence of allylamine-dCTP.

1. Amplify the cDNA with a PCR mix containing 10 µl cDNA, 1 x Buffer II, 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dTTP, 0.16 mM dCTP, 0.04 mM aminoallyl dCTP, 10 µM forward PCR primer, 10 µM reverse PCR primer, 1.5 µM $MgCl_2$, 0.2 U/µl Taq polymerase, and 1M Betaine in a reaction volume of 50 µl.
2. The PCR conditions are 4 min. at 95° C, 25 cycles of 1 min at 95°C, 1 min at 50°C, 2 min at 72°C followed by 10 min at 72°C.

3.9.2. Purification of RT-PCR product

The PCR product is purified with the QIAquick PCR purification kit protocol.

1. Add 500 µl buffer PB to the PCR product, mix and transfer it to a QIAquik column.
2. Spin at maximum speed for 1 min and discard flow through.
3. Wash the column with 750 µl buffer PE, spin at maximum speed for 1 min and discard flowthrough.
4. Spin again to completely dry the column.
5. Elute in a fresh 1.5 ml tube by adding 50 µl EB buffer directly onto the filter and incubate for 2 min.
6. Spin at maximum in the speed for 1 min.
7. Dry the eluate in the speed vac for 1 h.

3.9.3. Labeling and Purification of the RT-PCR *product*

1. Dissolve the pellet in 9 µl of 50 mM $NaHCO_3/Na_2CO_3$, pH 9.0 (prepared freshly from 1 M stock).
2. Incubate for 15 min at room temperature to dissolve.
3. Transfer the DNA to a tube containing 2 µl aliquots of Cy3 as dry pellet.
4. Mix well and transfer to original tube and incubate for 1 hour at room temperature in the dark.
5. Block the Cy3-dyes by adding 4.5 µl 4 M hydroxylamine and incubate for 15 min at room temperature in the dark.

6. Add 70 µl MilliQ water to the labelled DNA and remove uncoupled dye material by performing the QIAquik PCR purification kit protocol (see subheading 3.5.2.).
7. Elute in 30 µl buffer EB.

3.9.4. Hybridization of probe to microarray

1. Prepare 126.7 µl hybridisation mix by adding 90 µl MilliQ water, 2 µl pd(A) $_{40\text{-}60}$, 6.5 µl tRNA, and 24 µl Cot-1 DNA to 30µl of labeled DNA.
2. Precipitate this by using 1.2 volume of 3 M NaAc pH 5.2 and 2.5 volume Ethanol (100%), mix by inversion, and incubate on ice for 5 min.
3. Spin down for 10 min. at 12,000 g at 4° C.
4. Remove the supernatant with a pipette and air-dry the pellet for 5-10 minutes.
5. Carefully dissolve the pellet in 40.7 µl milliQ water and 2.5 µl of 10% SDS (avoid foam).
6. Incubate at room temperature for at least 15 min.
7. Prepare a master mix with 1 g dextran sulphate, 5.3 ml formamide 100%, 0.7 ml MilliQ water, and 1.0 ml 20 x SSC, pH of 7.0.
8. Add 83.5 µl mastermix to the dissolved pellet and mix gently.
9. Denature the hybridisation solution at 70° C for 10 min and incubate on ice/water for 1 min.
10. Subsequently, incubate for 1 hour at 37°C.
11. The mastermix with labeled DNA is ready to be hybridized on the hybridization station.
12. Scanning is done using the Agilent DNA microarray scanner.

4. NOTES

1. The completeness of the syncytiotrophoblast cell removal can be checked by using microscopic analysis with phase contrast. If necessary, optimize using immunohistochemistry as described in ref. 5.
2. The completeness and specificity of the cDNA synthesis reaction can be checked by the presence of sequences representative for hsa-miR-141 [8]. In addition, the presence of intronic sequences indicate incomplete DNA removal.

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C21ORF105, a chromosome 21-encoded mRNA, is not a discriminative marker gene for prediction of Down syndrome in maternal plasma

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Prenatal Diagnosis 2007; 27: 146-149

Abstract

Objectives The presence and detectability of placental mRNA in maternal plasma opens possibilities for the development of non-invasive prenatal diagnostic tests. In this study, we tested C21orf105, a chromosome 21-encoded, placentally expressed gene, in maternal plasma of women carrying a fetus with or without trisomy 21.

Methods Using real-time RT-PCR, we determined transcript levels of target (C21orf105) and reference (hPL) genes in first trimester plasma samples. Plasma was obtained from first trimester EDTA blood after two sequential centrifugation steps and stored at -70°C. After RNA extraction, quantitative RT-PCR was performed using Taqman probes.

Results From the 51 samples, 43 samples were conclusive. Comparison of transcript levels of C21orf105 in both groups showed no significant differences. When expressed as ratios of hPL/C21orf105, the differences between trisomy 21 and normal pregnancies remained non-significant.

Conclusions The amount of C21orf105 mRNA in maternal plasma, although situated in the Down syndrome critical region on chromosome 21 and up-regulated in trisomy 21 placentas, is not higher in women carrying a fetus with trisomy 21.

Introduction

For prenatal diagnosis of genetic disorders of the fetus, like aneuploidy, invasive procedures are required to obtain informative fetal cells. These invasive procedures, i.e. chorionic villus sampling and amniocentesis, have an intrinsic risk of fetal loss. It is therefore considered a big challenge to develop a non-invasive test with identical diagnostic possibilities and specificities using fetal genetic molecules circulating in maternal blood. The presence of fetal mRNA in maternal plasma was first demonstrated in 2000 by Poon et al. using Y-chromosome-specific zinc finger mRNA (ZFY)[1]. Ng et al. took it further and quantified transcript levels of two genes expressed in the placenta, human placental lactogen (hPL) and the ß subunit of human chorionic gonadotropin (ßhCG), in maternal plasma by real-time quantitative RT-PCR. hPL mRNA could be detected in pregnant women at all gestational stages with concentration levels increasing with gestational age[2]. The presence and detectability of placental RNA in maternal plasma permitted screening of new markers to test their feasibility for use in non-invasive prenatal diagnostic assays. We previously tested a set of 80 RNA targets, expressed in early placental tissue and distributed over all chromosomes except the Y chromosome, for their presence and absence, respectively, in plasma of pregnant and non-pregnant women. Eight genes met the criteria: they were absent in plasma of non-pregnant women, but present in first-trimester plasma of pregnant women[3]. One of these, C21orf105 (LOC90625) is a chromosome 21-encoded, placentally expressed gene[4]. As the expression of this gene, located within the Down syndrome critical region (DSCR), is increased in trisomy 21 placentas[5], C21orf105 is a promising candidate gene for the development of a screening or diagnostic test for Down syndrome.

In this study, using real-time quantitative RT-PCR, we determined transcript levels of target (C21orf105) and reference (hPL) genes in first-trimester plasma samples of pregnant women carrying a fetus with or without Down syndrome.

Materials and Methods

Patients

Peripheral blood samples (EDTA blood) were collected between 9-15 weeks of pregnancy from healthy pregnant women with singleton pregnancies attending the Prenatal Diagnostic Unit of the VU University Medical Centre. Samples were obtained with informed consent and approval of the Ethics Committee. Patients included were patients who came for a first trimester combination test or for an invasive procedure after a combination test result of a high risk for Down syndrome. They were asked for an extra blood sample. All blood samples were obtained before invasive diagnostic procedures, i.e. chorionic villus sampling or amniocentesis. The diagnosis trisomy 21 was based on the karyogram. The control or non-trisomy 21 group exists of patients with a normal karyogram of the fetus or patients who reported that the baby had no signs of chromosomal or congenital defects by returning our follow-up form (the majority was returned in the first weeks after birth). For each sample of a trisomy 21 pregnancy, two controls were matched on gestational age. The gestational age of the control differs less then one week from the gestational age of the index pregnancy. Primigravida's were matched to primigravida's.

Processing of blood samples

EDTA blood was stored at 4° C in an upright position and processed within 72 hour after collection by two sequential centrifugation steps as described previously[4; 6]. In brief, after centrifugation for 10 min at 2000g at 4° C, plasma was subjected to a second centrifugation for 10 min at 25000 g at 4° C . Plasma was stored as aliquots at -70° C and thawed only once. Processing of blood was done within a laminar flow hood.

RNA extraction from maternal plasma

The panel of samples was composed by the clinician and blinded. The pregnancy outcome was unknown to the person (AV) who performed the tests.

RNA was extracted from 1600 µL of maternal plasma by silica-based affinity isolation using QIAamp MinElute Virus Vacuum system (Qiagen). Before isolation, plasma samples were thawed at room temperature, the heating block was preheated to 56° C, carrier RNA was added to AVE buffer (1 µg/µL), and the protease was thawed. All steps were done at room temperature unless stated otherwise. We added 38 µL of protease (Qiagen) to a 1.5-mL tube, followed by 400 µL plasma, and 400 µL of buffer AL (with 28 µg/ml carrier RNA). After vortex mixing for 15 s, samples were incubated for 15 min at 56° C. After centrifugation, 500 µL of

ethanol was added; samples were then vortex-mixed for 15 s and left at room temperature for 5 min. All centrifugation steps were done for 1 min at 8200g unless stated otherwise. After centrifugation, the lysate mixture was carefully loaded on two QIAamp minute columns.

The columns were inserted into the QIAvac 24 vacuum manifold (Qiagen) according to the manifacturer's instructions. The vacuum conditions used were 40 kPa with a 19 L/min vacuum pump (Biometra MP26). Six hundred µL of buffer AW1 was added to the column and vacuum applied. For on-column DNAse digestion, 70 µL of SDD buffer (Qiagen) was added to 10 µL of DNAse, loaded on the column, and left for 30 min at room temperature. Then 750 µL of buffer AW2 was added and again vacuum applied. Seven hundred fifty µL ethanol was added on the column, and vacuum applied. The column was removed from the manifold and then placed in a new tube and centrifuged for 3 min at 25000 g. The bound RNA was eluted by placing the column in a new tube, followed by application of 50 µL of AVE buffer, incubation for 5 min at room temperature, and centrifugation for 1 min at 25000g. Finally, the eluate from the two columns were combined and concentrated by using Microcon-PCR filters according to the manufacturer's instruction (Millipore).

Quantitative RT-PCR

A two step, one tube RT-PCR assay was performed as described previously [4] with the RNA se H-negative Superscript II Platinum system (Invitrogen) in the presence of 1 M betaine. RT-PCR reactions were set up on ice. In brief, RNA was mixed with 50 pmol each of forward and reverse primers in a final volume of 13 µL in MicroAmp tubes and heated for 1 min at 95° C, followed by immediate cooling on ice. Thirty-seven microliters of master mixture was subsequently added, giving a final concentration of 1x buffer; 1.25 mM magnesium sulphate; 0.2 mM each of dATP, dCTP, dGTP and dTTP; 1M betaine (Fluka); 0.2 U RNA sin (Promega) and 1 µL of enzyme mixture containing RNA se H-negative Superscript II reverse transcriptase and Taq DNA polymerase (Life Technologies). After reverse transcription for 30 min in 55° C and denaturation for 2 min at 95° C, PCR was performed on 25 cycles (denaturation for 1 min at 95° C annealing for 1 min on 62° C, and extension for 2 min on 72° C followed by a final extension for 10 min at 72° C and cooling. All reactions were performed identically except that the annealing temperature was set at the temperature predicted to be optimal for each target (oligo4.0). The gene specific primers used were as follows:

LOC90625-F 5'- CCA CCC CTC CAT CTG GGA AAG CAG GCC ACA-3' and LOC90625-R 5'TGT GGC CTG CTT TCC CAG ATG GAG GGG TGG -3';
$T_{annealing} = 62°$ C

Quantitative procedure was performed in a ABI 7300 using Taqman probes. The QT-PCRs were set up according to the manufacturer's instruction in a reaction volume of 50 µL with Superscript III (Invitrogen). The fluorescent probes were used in a concentration of 400 nM with a primer concentration of 1000 nM. Template input of 2 µL of the RT-PCR product and a fixed amount of extracted placenta RNA run in parallel were used for amplification. A calibration curve consisting of 10-10^5 copies of purified PCR fragments was performed for each analysis. Negative controls consisting of water were also included in every analysis.

The termal profile used for the LOC90625 was as follows: The reaction was initiated at 95° C for 2 min, 1min 95° C, 1min 62° C, 2min 72° C for 50 cycli.

For the hPL Q-RT-PCR were performed as descripted by Ng et al.[2]

Statistics

For the data analysis the results of the diverse runs were corrected by the fixed amount of extracted placental RNA , which was in parallel for each run of samples. The Mann-Whitney-U test was used for the statistic analysis of the C21orf105 results between fetus with and without Down syndrome.

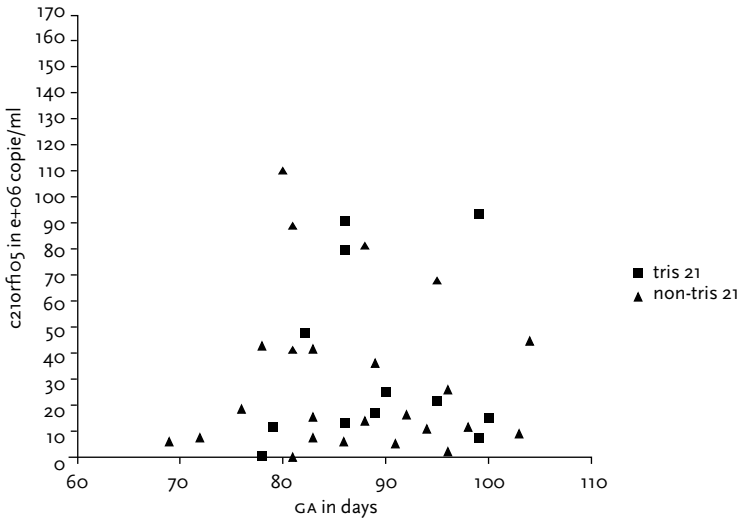
Results

Transcript levels of C21orf105 were quantified by real-time Q-RT-PCR in 51 plasma samples obtained from 46 pregnant females during first trimester (weeks 9-14). Four patients were tested twice. From these, one was inconclusive in the first test. In the three patients with two test results, the mean was used. Two sample was derived from two consecutive pregnancies of the same patient. From the 51 samples, 8 were undetermined. In this way, the final analysis involved twelve trisomy 21 samples that were compared with twenty-eight normal pregnancy samples matched for gestational age and parity. All water blanks were negative.

Comparison of C21orf105 transcript levels in both groups showed no significant differences, neither in absolute values (Figure 1) nor when expressed and compared as median values (Figure 2). The median in the trisomy 21 group was 19.20 in 10^6 copies/ml. The median in the non-trisomy 21 group was 16.08 in 10^6 copies/ml. The differences between the two groups were not significant, Mann-Whitney-U test $P = 0.6$.

Given the large range of absolute values between patients reflecting interpatient biological variation, we performed an additional analysis. Transcript levels of human placental lactogen (hPL) in maternal plasma were determined and used as reference gene to correct for biological variation. However, when expressed as ratios of hPL/C21orf105, the differ-

The C21orf105 results of trisomy 21 and non-trisomy 21 samples (in e+06 copies/ml) per gestational age (GA). **Figure 1**



Comparison of the trisomy 21 and the control group. The lines inside the boxes denote the median. The boxes mark the interval between 10 and 90 percentile. **Figure 2**

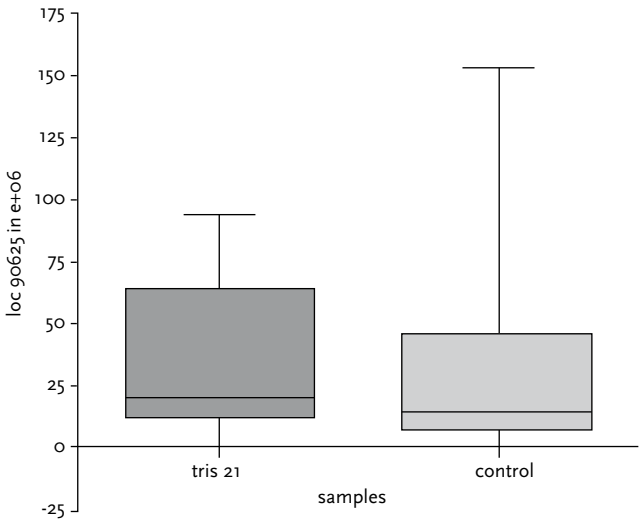
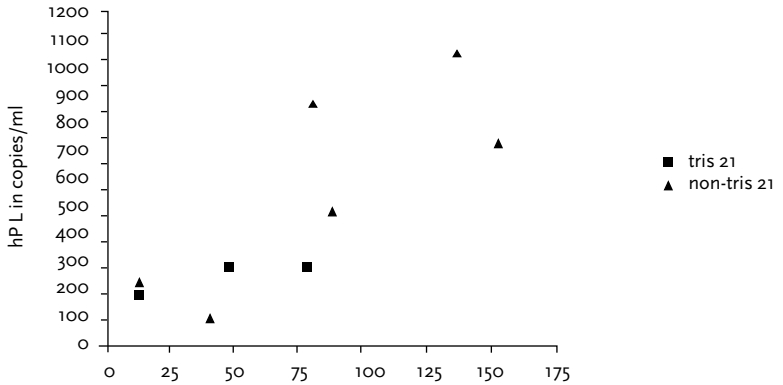


Figure 3 In this graph the ratios of LOC 90625 and hPL are compared. Note the scale difference between the axes.



ences between trisomy 21 and normal pregnancies remained non-significant. (Figure 3)

Discussion

In this study, we show that although the presence of C21orf105 mRNA in first trimester plasma of pregnant women is a consistent finding, no significant differences were found in plasma levels between normal pregnancies and pregnancies with a karyotypically confirmed trisomy 21. The copy number per ml turned out to be variable and in general low compared to hPL. The former is not unique for C21orf105, and seen for most if not all placental RNA markers tested so far. The latter is a reflection of the transcription level of C21orf105 being in the low abundancy range and necessitated the use of a sequential Q-RT-PCR method.

To correct for the inter-patient variation in transcript copy number, correction was implemented using hPL as reference gene. This assumes that the number of placental microparticles shed in each pregnant female varies greatly, yet that the distribution of RNA and DNA molecules per microparticle is relatively constant but changes in the event of pathology such as in the presence of an additional copy of chromosome 21. However, even after correction, the differences in transcript levels in pregnancies with and without trisomy 21 remained non-significant.

In our study, all patients included had a first trimester combination test. We tested if we could find an association with nuchal translucency measurement, levels of free β-hCG and PAPP-A. No association was found.

C21orf105 mRNA was expected to be a good candidate gene based on two qualities. C21orf105 is situated in the Down syndrome critical region (DSCR) on chromosome 21 and its expression is up-regulated in trisomy

21 placentas[5]. So we expected to find a higher concentration in maternal plasma of women carrying a fetus with trisomy 21. Although this was not the case, this does not disqualify the potential of the approach we followed in this paper, in particular as combined with correction for biological variation using a reference gene. We consider it a challenge to identify other markers on chromosome 21 within the DSCR, with baseline expression levels within the medium- to high-abundancy range.

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44 single-nucleotide polymorphisms expressed by placental RNA: Assessment for use in non-invasive prenatal diagnosis of trisomy 21

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To the Editor:

For non-invasive prenatal diagnosis, markers that directly reflect changes in chromosome dosage are preferred over indirect markers that are associated with epiphenomena[1;2]. The RNA- single-nucleotide polymorphism (SNP) allelic ratio strategy was described recently as a means to direct assessment of fetal chromosome dosage in maternal plasma[2]. Quantitive comparison of the allelic expression ratios of a placentally-expressed, chromosome 21-encoded gene (PLAC4), enabled detection in maternal plasma of the differences between two (normal) or three copies of chromosome 21.

The RNA-SNP ratio strategy is currently limited to a subset of the population with heterozygosity of the SNP used. Theoretically, increase in population coverage can be obtained by inclusion of additional SNPs within PLAC4 or other chromosome 21-encoded transcripts with placental expression and detectability in maternal plasma[2]. We therefore tested 44 SNPs expressed by 7 chromosome 21-encoded, placentally-expressed genes ([2]), i.e. PLAC4, collagen type VI alpha 2 (COL6A2), collagen type VI alpha 1 (COL6A1), BTG family member 3 (BTG3), ADAM metallopeptidase with thrombospondin type I motif 1 (ADAMTS1), chromosome 21 open reading frame 105 (C21orf105), and amyloid beta (A4) precursor protein (peptidase nexin-II, Altzheimer disease) (APP), for their potential use in non-invasive prenatal diagnosis. All SNP markers were tested for their presence in first trimester plasma and their absence in nonpregnant women.

Peripheral blood samples were collected from pregnant women attending the Prenatal Diagnostic Centre of the VU University Medical Center. All participants gave informed consent before study inclusion. The study was approved by the ethics committee at our institution. We collected EDTA blood samples between weeks 9 and 14 of pregnancy, before invasive diagnostic procedures were performed. Samples were processed and RNA extracted as described previously, with automated isolation (BioRobot MDX)[1]. RNA extraction from PAX gene tubes was performed using the BioRobot MDX with a standardized protocol (Qiagen). For selected genes, allele frequencies were determined by cycle sequencing with a Big Dye terminator, followed by capillary electrophoresis (ABI 3100XL).

Within the transcripts of the seven genes of interest, 44 SNPs were

Tabel 1 **SNPs expressed by placental RNA and present in maternal plasma but not in control plasma.**

No.	Gene	Exon	SNP[a]	HET FREQ[b]	A1[c]	A2[c]	Forward primer	Reverse primer
1	PLAC4	1	rs8130833	0.448	A	G	GGGACTCGCCGCTAGGGTGTCT	GGTGGGGATCCCTTATGCATGG
2	PLAC4	1	rs9977003	0.339	A	G	AACCGTGGGACCAGTGTAGAAGAATG	GGGCAAGTGGAAAACACGCAGT
3	COL6A2	28	rs11554667	<0.1	C	G	CACAGCAGGTGCGCAACATG	AAGCGCCGGGCCTTGTG
4	COL6A1	23	rs9637170	<0.1	A	C	CCTATCGGACCTAAAGGCTAC	TCCAAAATCTCGCATTCGTC
5	C21orf105	2	rs2187247	0.5	A	C	GCGCGCTCTCCGGGTTCCAACC	GGGGCCTGTCCACTTCGGTGGTAG

a Selected from among 44 tested SNPs of the 7 selected genes. The additional SNPs tested (n=39), their primer sequences are available on request.
b Heterozygote frequencies (HET FREQ) are given for white individuals only.
c A1 and A2, variant alleles.

identified (www.hapmap.org) (Table 1). Primers, flanking these SNPs, were designed with similar thermodynamic characteristics to permit RT-PCR analysis in single runs. All primers were intron-spanning, except for the primers of PLAC4. Using a sensitive, 2-step, 1-tube RT-PCR assay (Superscript II RT-PCR, Invitrogen) supplemented with 1 mol/L betaine to increase reverse transcriptase efficiency and enzyme stability[1], the marker set was tested in placental tissue (positive control), plasma of nonpregnant women (negative control) and pregnant women. During the initial screen, we used 3 chorionic villus samples (weeks 8, 11 and 12) to test markers for placental expression. Screening of pregnant and control plasma was done in triplicate. To minimize the effect of biological variation of marker levels in plasma, each of the 3 screens in plasma was performed on pooled RNA fractions isolated from individual females in series of 44. In practice, this process was performed by downstream pooling of the concentrated, individual RNA fractions isolated from plasma (10 μL each) after automated extraction of 44 different plasma samples. For the SNPs of most use, the final screen was done by individual analysis of 6 pregnant plasma and 6 control plasma samples. With the use of RNA isolated from EDTA-plasma, 5 of 44 SNP markers were detectable in maternal plasma and absence in non-pregnant plasma: rs8130833 (PLAC4), rs9977003 (PLAC4), rs11554667 (COL6A2), rs9637170 (COL6A1), and rs2187247 (C21orf105) (Table 1). In contrast, in RNA isolated from whole blood collected in Paxgene tubes, no SNP markers fulfilled the criterion of absence in non-pregnant blood. Identical analysis of *hPL* RNA [3] excluded false positivity, because in RNA recovered from whole blood in Paxgene tubes, this marker was clearly present and absent, respectively, in samples obtained from pregnant and nonpregnant females (data not shown).

We concluded the following: (*a*) Although the PAX gene tube reagent that stabilizes RNA may be beneficial for RNA isolation from whole blood, the large contribution of intracellular RNA from maternal periph-

eral blood cells prevents widespread prenatal use. Prenatal PAX gene tube use appears to be limited to genes with high relative expression differences between placental tissue and maternal blood cells, such as *hPL*. *(b)* Our data confirm the utility and high expression of PLAC4[2]. *(c)* The use of SNP markers is restricted to specific exons for genes with complex transcriptional organization, such as COL6A1 and COL6A2. *(d)* For the transcripts of COL6A2 and COLA1 with placental specificity (for example encompassing exon 23 in COL6A1), SNPs remain to be identified for use in RNA-SNP assays. The heterozygote frequencies of rs11554667 (COL6A2) and rs9637170 (COLA1) are <0.1% in the Caucasian population we tested. *(e)*Alternatively, for COL6A2 and COL6A1, the combined detection of exons with specificity (exons 28 and 23 for COL6A2 and -6A1, respectively) with additional exons carrying SNPs with high heterozygosity (rs2839114, rs1053312) might yield usefull combinations. *(f)*The predictive power of *C21orf105* [1;4] for prenatal diagnosis should be retested with the RNA-SNP allelic ratio strategy by use of rs2187247. *(g)* Our data permit an evidence-based selection of target genes and markers to increase the population coverage of the allelic ratio strategy for noninvasive prenatal diagnosis of trisomy 21.

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Financial disclosures None declared.
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Measuring of allelic expression ratios in trisomy 21 placentas by quencher extension of heterozygous samples identified by partially denaturing HPLC

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Background Measuring the allelic ratios of placental transcripts in maternal plasma permits non-invasive prenatal detection of chromosomal aneuploidy. Current methods, however, require highly specialized equipment (MALDI-TOF), limiting the widespread implementation of this powerful RNA single-nucleotide polymorphism (SNP) strategy in routine, diagnostic settings. We adapted and applied the Transgenomic WAVE system and the quencher extension reaction (QEXT) for this purpose.

Methods The expressed SNP (rs2187247) in exon 2 of the placentally-expressed, gene (chromosome 21 open reading frame 105) on chromosome 21 was tested in a trisomy 21 model system. In which we obtained RNA selectively released from the syncytiotrophoblast of normal and trisomy 21 placentas was obtained during first trimester.

Results In identifying heterozygous samples, we observed an exact correspondence between sequencing results and results obtained with the WAVE system. With respect to the analysis time required, the WAVE system was superior. In addition, the real-time QEXT assay (as optimized and validated with calibration standards consisting of 262 bp of cDNA amplicons) accurately measured allele ratios after we optimized fragment purification, concentrations of input DNA and quencher label, and calculation of reporter signal. Finally, the optimized and validated QEXT assay correctly distinguished normal placentas from trisomy 21 placentas in tests of the following clinically relevant combinations: diploid homozygous (CC), diploid heterozygous (AC), triploid homozygous (AAA), triploid heterozygous (AAC or ACC).

Conclusions The QEXT method, which is directly adaptable to current real- time PCR equipment, along with rapid identification of informative samples by the WAVE system, may facilitate routine implementation of the RNA-SNP assay for non-invasive aneuploidy diagnostics.

Methods of non-invasive prenatal screening for numeric chromosomal abnormalities that are based on indirect markers typically detect the associated epiphenomena, and therefore lack diagnostic power [1, 2]. Direct assessment of fetal chromosome dosage with maternal plasma should use molecular markers transcribed or derived from the chromosome of interest, i.e. placental RNA transcribed from chromosome 21 to detect Down syndrome[1, 2]. Lo and coworkers introduced a powerful, robust modification of this direct approach: the RNA- single-nucleotide polymorphism (SNP) allelic ratio strategy[2]. The relative quantification and comparison of allelic-expression ratios of a placentally expressed gene encoded by chromosome 21 enables the clear detection of differences in expression by two or three copies of the chromosome. The design of such comparisons dramatically increases the predictive power of this particular assay and other direct molecular assays. The use of a single marker (rs8130833) expressed by (placenta-specific 4) gene enables correct, non-invasive detection of fetal trisomy 21 in 90% of the cases (sensitivity) and excluded in 96.5% of controls (specificity)[2]. The RNA-SNP ratio strategy is currently limited to a population subset,the nature of which is dictated by the heterozygosity state of the SNP used and the ethnicity of the studied population (e.g. Caucasian, Asian, African) studied. We expect that population coverage can be increased by inclusion of other SNPs within or adding other chromosome 21-encoded transcripts that are expressed in the placenta and detectable in maternal plasma [i.e. (collagen, type VI, alpha 2), (collagen, type VI, alpha 1), (chromosome 21 open reading frame 105)][2, 3]. Technically, the RNA-SNP assay developed and used by Lo and coworkers is based on differential extension of the polymorphic site to generate small but very specific, allele-dependent differences in size. This approach, however, requires highly specialized equipment [matrix-assisted laser desorption/ionization time-to-flight (MALDI-TOF) analysis], limiting its widespread implementation in routine, diagnostic settings[2, 4].

We tested and validated two methodological adaptations: the quencher extension reaction (QEXT) and the WAVE system (Transgenomic). The QEXT reaction is a novel, single step real-time method to quantify SNPs and is directly adaptable to current real-time PCR equipment[5, 6]. In the QEXT assay, a probe with a 5'-reporter dye (FAM) is extended by a single base with a dideoxy nucleotide (ddNTP)

containing a quencher dye (TAMRA). Enzyme-mediated extension by Thermo Sequenase DNA polymerase (USB Corporation) takes place only if the target SNP allele is present. The extension is recorded in real time from the quenching (i.e., reducing the fluorescence) of the reporter dye. The relative amount of a specific SNP allele is determined by measuring the nucleotide incorporation rate in a thermo-cycling reaction. Because TAMRA can also serve as a fluorescence donor, depending on the 5'-fluorescent reporter, measuring by monitoring increases in fluorescence is also possible, as are multiplex reactions[5, 6].

The WAVE system allows rapid identification of informative (i.e. heterozygous samples) by partially denaturing high performance liquid chromatography (HPLC) of preformed homo- and heteroduplexes. At the temperature optimal for the formation and detection of heteroduplexes, these complexes, which are only formed if a SNP is present, will elute off the cartridge before the homoduplexes and will be visible as two additional peaks. If no SNP is present, all of the homoduplex DNA fragments elute as a single peak on the chromatogram. In routine clinical samples, the WAVE method detects DNA variations with a better detection limit, at lower cost, and in a shorter time than direct sequencing[7].

To verify if these methods could be implemented in the RNA-SNP ratio assay for placentally-expressed genes on chromosome 21[1, 4], we used an SNP (rs2187247) located in exon 2 of *C21orf105* as a model system. For this investigation, we isolated total RNA from normal placentas (n = 2) and trisomy 21 (n = 3) placentas, obtained from pregnancies terminated at a gestational age of about 13 weeks (after obtained informed consent). These placentas were denuded *in-vitro* by controlled digestion with trypsin[8, 9]. The nature of this treatment, which selectively releases the cellular contents, including RNA , from the syncytiotrophoblasts[8, 9], mimics the specificity seen *in vivo* for the cellular origin of the placental molecules released into the maternal plasma[10]. We confirmed the karyotypes of all placentas by G-banding analysis of metaphase spreads of cultured villi samples.

We started by testing wether the WAVE system could identify informative, heterozygous samples with a specificity equal to or superior to that of conventional DNA sequencing. We generated cDNA fragments (262 bp, intron-spanning, rs2187247 located at position 221) from all samples by reverse transcription-PCR, identified the variant alleles of rs2187247 by cycle-sequencing and compared the results with data obtained with the WAVE system (see the methods in the Supplemental Data Methods). Two of the three trisomy 21 placentas and one of the two normal controls were heterozygous (see Supplemental Data Figure 1). The frequency of rs2187247 heterozygotes in our population, as determined by sequencing of genomic DNA of Caucasian controls (n=104), was 0.5. The tempera-

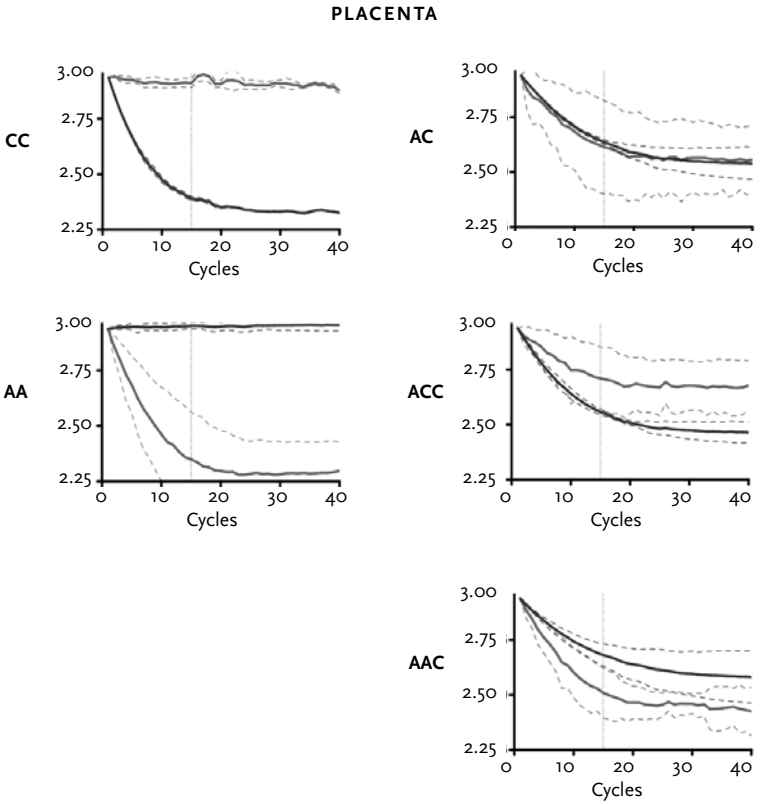
ture (62.7° C), predicted by the Navigator program (Transgenomic) corresponded exactly with the actual optimal temperature for distinguishing heteroduplexes and homoduplexes by partially denaturing HPLC with the WAVE system. In addition, we found an exact correspondence between the sequencing results and results obtained with the WAVE system. Finally, the WAVE system was superior with respect to analysis time: a single analysis takes only 2.5 minutes.

We subsequently generated calibration standards to optimize, calibrate and validate the QEXT assay (see Supplemental Data Methods). We cloned cDNA amplicons confirmed to contain either the A or C allele of rs2187247 into pTOPO vectors (Invitrogen), reamplified with the PCR and mixed the purified allele fragments in different A-to-C ratios (7:1, 6:1, 5:1, 4:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7). The total quantities tested ranged between 10 and 100 ng. Negative controls consisted of homozygous DNA containing the A or C allele and QEXT reactions performed with TAMRA-ddGTP or – ddUTP, respectively. The QEXT method of Rudi *et al*.[5, 6] accurately measured the allelic ratios (see Supplemental Data Figure 2), when the following modifications were implemented: (a) cDNA PCR-fragment purification with exonuclease I and Shrimp Alkaline Phosphatase (ExoSAP-IT; USB Corporation) was extended to 60 min to ensure the complete removal of unincorporated nucleotides and primers. (b) After QIAgen purification amplicons were eluted with Milli-Q-purified water (Millipore) to remove contaminants that interfere with downstream analysis (i.e., WAVE, QEXT). (c) A 50 ng DNA input was an optimal and practical compromise between specificity and cost effectiviness. (d) Correction for the observed difference in incorporation rate between TAMRA-labeled ddUTP and ddGTP, a 10 fold higher concentration of labeled ddUTP then the labeled ddGTP to generate identical thermocyclic efficiencies. (e) The FAM reporter signal corrected for assay imprecision with the ROX reference dye (normalized reporter) showed the best correspondence with the predicted outcome; inclusion of or correction for the quencher molecule, TAMRA, is unnecessary and in fact contraindicated. 6. Following thermocycling for 40 cycles, the labeling reaction, which is theoretically linear by the absence of amplification, was found to level off after about 15 cycles. (f) After 40 cycles, we found that the lbeling reaction, which is theoretically linear because of the absence of amplification, leveled off after about 15 cycles; therefore, calculating the slope ratios for the linear part of the trajectory allowed specific and reproducible measurement of allele ratios within the following required A:C windows: 1:1 (AC), 1:2 (ACC) and 2:1 (AAC).

Finally, to test the proof of principle that the QEXT assay is a specific method for the meauring allele expression ratios in clinical samples, we tested the optimized and validated QEXT assay in the trisomy 21 model

Figure 1

Representative results for measuring allelic-expression ratios for the placentally expressed C21orf105 gene by QEXT analysis of informative, heterozygous placental samples identified by partially denaturing HPLC. For allele determination and quantification, a probe with a 5'-reporter dye (FAM) is extended by a single base with a ddNTP containing a quencher dye (TAMRA). Enzyme-mediated extension by Thermo Sequenase DNA polymerase only takes place if the target SNP allele is present. The extension is recorded in real time by monitoring the quenching of the reporter dye. The relative amount of a specific SNP allele is measured from the nucleotide incorporation rate in a thermo-cycling reaction. Data are from placenta RNA obtained in the trisomy 21 placenta model system and are presented as the median (solid lines) and 95% confidence limits (dashes lines). The combinations tested (diploid, triploid, homo-, heterozygous) are indicated. The red and blue lines indicate determination of the A and C alleles, respectively. The point of transition at 15 cycles from a linear trajectory is indicated by a vertical line. Rn, normalized reporter signal.



system by evaluating all of the clinically important (i.e informative) combinations; diploid heterozygous (AC) and triploid heterozygous (AAC or ACC). We also analyzed diploid homozygous (CC) and triploid homozygous (AAA) samples as controls. We tested each combination in a double-double manner; that is, we measured each allele ratio identically in duplicate on two different occasions. We obtained first-order regression curves by polynomial regression by using reporter (FAM) signals normalized for assay imprecision with ROX reference dye and for calculation we used the initial slopes (15 cycles), which corresponded to the linear portions of the reaction trajectories. The measurements of the duplicates indicates that the reproducibility of the curves was good; the correlation coefficients for the A and C reactions were between 0.97 and 1.09. In addition, the profiles of the *in-vitro* (calibrator) and *in-vivo* (placenta model system) reactions were nearly identical for the tested combinations (diploid, triploid, homo-, heterozygous; Figure 1). Finally, the allelic ratios permitted clear differantiation between diploidy (Range 0.7-1.3) and triploidy (0.7<R > 1.3) in the trisomy 21 model system (see Supplemental Data to Figure 1).

We conclude that the WAVE and the QEXT methods, we have described and validated in our trisomy 21 model system with *C21orf105* as a representative direct molecular marker may facilitate routine implementation of the RNA-SNP assay for non-invasive aneuploidy diagnostics[2]. The WAVE system permits rapid identification of informative, heterozygous samples. Our QEXT method is directly adaptable to current real-time PCR equipment. Besides this practical advantage, the design permits real-time, multipoint measurements, multiplex reactions or the monitoring of increases in fluorescence. Preliminary observations indicate that, with minor modifications (use of Agilent BioAnalyzer; see Supplemental Data Figure 3A), both the WAVE and QEXT (see Supplemental Data Figure 3B and C) methods can be applied to clinical plasma samples (placental RNA from maternal plasma) and adapted easily to related placentally expressed genes on chromosome 21.

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SUPPLEMENTARY FILE 1

Identification by WAVE analysis of heteroduplex DNA containing rs2187247

a. Pretreatment. PCR fragments (262 bp of C21orf105 cDNA generated by RT-PCR) were treated using exonuclease I and shrimp alkaline phosphatase (ExoSAP-IT) (USB) followed by QIAgen column purification to remove deoxynucleotides and primers. Both procedures were done according to the manufacturers instructions, except that incubation with ExoSAP-IT was done for 60 min. RT-PCR was done as described elsewhere [1]. Purified DNA, eluted in MilliQ water, was heated at 95° C for 5 min and cooled to 25° C at a rate of 1.5° C/min to allow formation of heteroduplexes.

b. WAVE procedure. PCR product (10 µl) (100-500 ng DNA) was then loaded for partially denaturing HPLC analysis using the WAVE 3500 HT system (Transgenomic). Elution was done with a gradient mixture of buffer A (0.1 mol/L triethylammonium acetate) and buffer B (0.1 mol/L triethylammonium acetate, 250 ml/L acetonitrile) (Transgenomic). Running temperature and percentage of buffer B were 62.7° C and 55.5%, respectively. The temperature optimal for partially denaturing HPLC conditions was calculated using the NAVIGATOR Software. Initial tests included runs at 0.5° C above and below this temperature. Detailled running conditions are shown below.

c. Data analysis. DNA fragment elution profiles were captured online using UV detection at 260 nm and visually displayed using the Transgenomic WAVEMAKER software. Chromatograms were analyzed for the presence of rs2187247 and compared with the results obtained by DNA sequencing.

Running conditions **Table**

	Time	%A	%B
Loading	0	47.5	52.5
Start gradient	0.1	44.5	55.5
Stop gradient	2.1	34.5	65.5
Start clean	2.2	47.5	52.5
Stop clean	2.3	47.5	52.5
Start equilibration	2.4	47.5	52.5
Stop equilibration	2.5	47.5	52.5

Figure 1 Representative chromatograms obtained by WAVE analysis of DNA samples (calibration standards) with hetero- (upper panel) or homozygosity (lower panel) for rs2187247.

Supplemental Data Figure 1

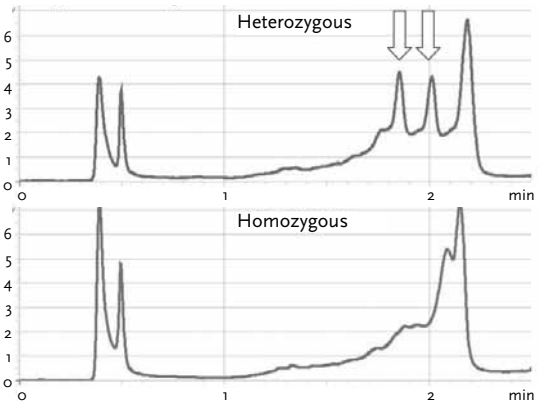
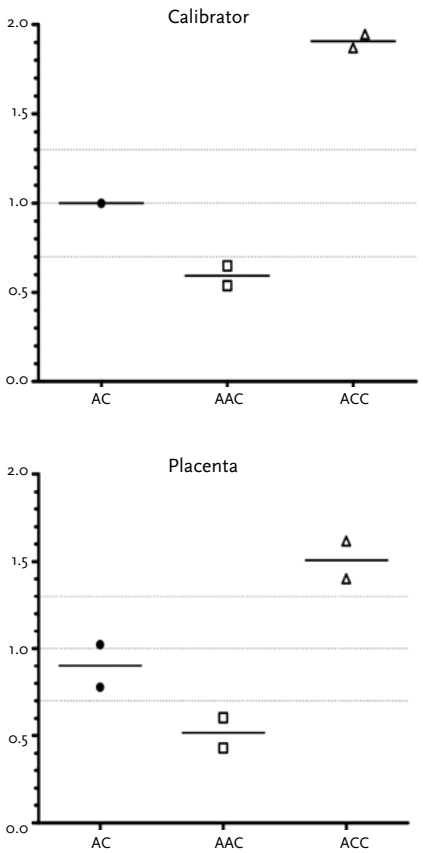
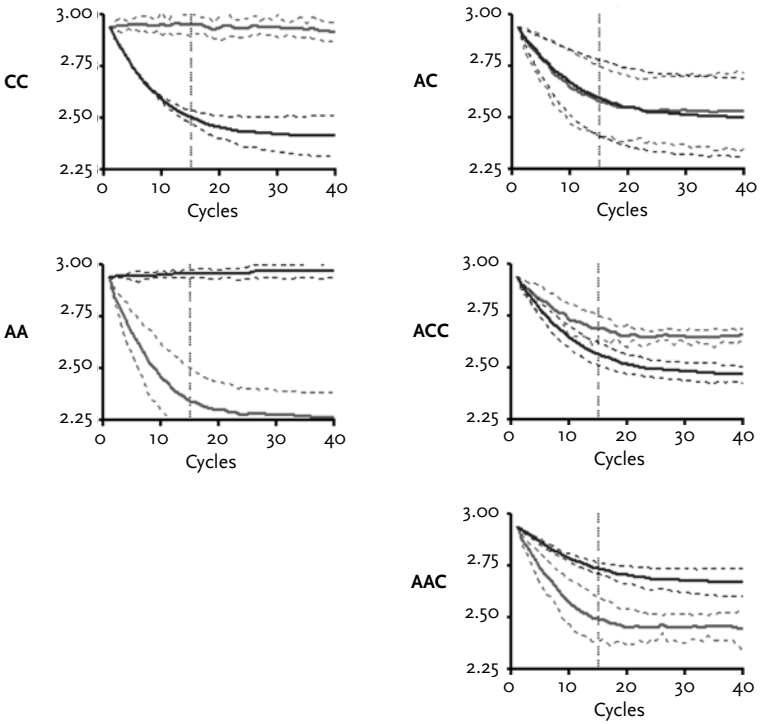


Figure 1 B



Representative results for determination of allelic expression ratios of the placentally-expressed, chromosome 21-encoded C21orf105 gene by quencher extension of informative, heterozygous DNA samples (calibration standards). **Figure 2**



SUPPLEMENTARY FILE 2

Quantification of allelic expression ratios of C21ORF105 by quencher extension

a. Pretreatment. PCR fragments (262 bp of *C21orf105* cDNA generated by RT-PCR) were treated using exonuclease I and shrimp alkaline phosphatase (ExoSAP-IT) (USB) followed by QIAgen column purification to remove deoxynucleotides and primers. Both procedures were done according to the manufacturers instructions, except that incubation with ExoSAP-IT was done for 60 min. RT-PCR was done as described elsewhere (1).

b. Standard curves. To generate standard curves, PCR fragments containing either the A or C allele of rs2187247 were cloned into pTOPO vectors. Plasmids containing either the A or C allele, as confirmed by sequencing, were purified by QIAgen column affinity purification and subjected to PCR. Amplicons were treated (ExoSAP-IT) and purified as above (Qiagen), quantified using A260/280 ratios (Nanodrop) and used to generate standard curves. Standard curves included 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1 mixtures of the respective alleles of rs2187247 with concentrations ranging between 10 and 100 ng DNA.

c. Quencher extension reaction. The treated, purified products were used as templates in the Quencher Extension reaction (QEXT) using a modification of the procedure of Rudi and coworkers[5, 6]. For this, 5 µl PCR product (50 ng) was added to 1 x Thermosequenase reaction buffer, 1 pmol of 5'-6-FAM labeled probe (5'-AGC TTT TTG TGA GGG TCA G-3') (Eurogentec), TAMRA-ddUTP (4 pmol/µL) or TAMRA-ddGTP (0.4 pmol/µl) (Perkin Elmer), ROX reference dye (1 µl) (Applied Biosystems) and 8 U Thermosequenase (Amersham Biosciences) in a total volume of 25 µl. The probe is complementary to the coding strand and directly flanks the single nucleotide polymorphism (rs2187247). Probe design (length, strand) were optimized using Oligo software with selection for absent or low frequencies of duplex-, hairpin- and 5'-overhang formation. Following incubation for 3 min at 95°C, sequence-specific extension was done by thermocycling for 40 cycles using the ABI 7300 Real-Time PCR system. Each cycle consisted of denaturation at 95°C for 30 sec and labeling at 55°C for 1 min. Positive controls consisted of 50 ng of AA or AC samples for QEXT reactions performed for the A allele using TAMRA-ddUTP. Positive controls consisted of 50 ng of CC or AC samples for QEXT reactions performed for the C allele using TAMRA-ddGTP. Negative controls consisted of homozygous DNA (50 ng) containing either the A or C allele for qext reactions performed with TAMRA-ddGTP or – ddUTP, respectively.

d. Data analysis. The signals were measured in relative fluorescence units (RFU) and expressed as Rn (normalized reporter signal) as measured for each cycle during the denaturation phase. The Rn value is the fluorescence emission intensity of the reporter dye (6-FAM at 541 nm) divided by the fluorescence emission intensity of the passive reference dye (ROX at 585 nm). For the combination used (FAM reporter, TAMRA quencher), the increase in extension, i.e. amount of SNP present, correlates with a decrease in fluorescence of the reporter (FAM) molecule. Regression curves were obtained using polynomial regression employing the normalized reporter (FAM) signals. The initial slopes (15 cycles) of the regression curves were used for quantification. For this, the raw data (component data), are exported to Excel. The FAM signal (probe) divided by the ROX signal (internal standard) generates Rn. The maximum (Rnmax) is calculated for all Rn values. Subtraction of the Rn value (first cycles) from the maximum generates the correction factor for each sample (Cfa). This is applied to the Rn value for each cycle. All these values are exported to Prism and used for calculation.

Calculation of slope ratios

For each sample the slope ratio (ddUTP 'curve': ddGTP 'curve' or A : C) of the regression curves were calculated. From the results of the duplicates the mean was calculated. This number was compared with the result of diploid calibration standard sample (AC) from the same run. The result of the AC was used as the median, by definition 1. The results of the ACC and AAC and the placenta samples were expressed as multiples of the median (MoM). All heterozygous triploid calibration standard samples and trisomy 21 placenta samples showed deviated allelic ratios from that of the diploid calibration standard sample and euploid placenta. We defined the interval between 0.7 and 1.3 MoM as the 'normal' or diploid range.

SUPPLEMENTARY FILE 3

Application of WAVE and QEXT on first trimester plasma samples

Placental RNA was isolated and subjected to RT-PCR as described previously (1) with modifications (Prenat. Diagn. 2007, 27:149). Prior to WAVE and QEXT analysis, the total amount of the specific ExoSap-treated, purified amplificons is calculated using the Angilent 2100 BioAnalyzer. For first trimester plasma samples (weeks 9-14), the AVErage total amount of specific product available at the start of the WAVE and QEXT procedures is about 5-10 fold higher than the minimal amount needed.

In 3A (BioAnalyzer Electropherogram), the specific amplicon concentrations of two samples are shown (bottom). Sample 1: 10.7 ng/ µl (total: 535 ng) and sample 2: 7.7 ng/ µl (total: 385 ng). The characteristics of the marker are shown as well (top).

In 3B (WAVE), the discrimination between non-informative (homozygous) and informative (heterozygous) samples is shown. Despite the presence of primer-dimer bands (visible at 1.3 min), the additional peak (arrow) indicates heterozygosity.

In 3C (QEXT), the correct prediction in all samples of the type and number of the allele (A or C) present in euploid samples is shown. Notice the correlation between the calibrator (left) and plasma samples (right).

These data demonstrate that, with minor modifications (BioAnalyzer), the WAVE and QEXT procedures work on first trimester plasma as demonstrated for (euploid) samples.

Determination of specific amplicon concentrations using the Agilent 2100 Bioanalyzer. In the Electropherogram, representative results are shown for the marker (top) and two patient (bottom) samples. Patient samples consisted of cDNA specific for C21orf105 generated by reverse transcription followed by PCR amplication of RNA isolated from first trimester plasma. By this, the concentration and total amount of specific amplicon in sample 1 was calculated to be 10.7 ng/ml and 535 ng, respectively. In sample 2, the concentration and total amount are 7.7 ng/ml and 385 ng, respectively.

Figure 3A

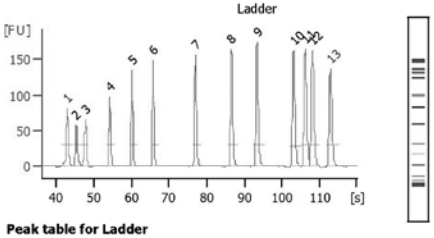
2100 expert_DNA 1000_DE54704321_2007-09-25_12-08-42.xad Page 1 of 1

Assay Class: DNA 1000
Data Path: C:\...-25\2100 expert_DNA 1000_DE54704321_2007-09-25_12-08-42.xad

Created: 9/25/2007 12:08:42 PM
Modified: 9/25/2007 12:50:38 PM

Electropherogram Summary

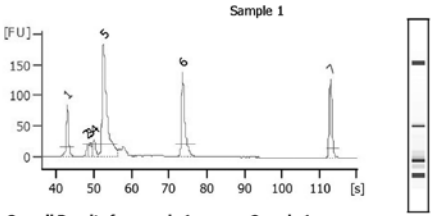
Ladder



Peak table for Ladder

Peak	Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations	Peak Height	Peak Width	% of Total	Area
1	15	4.20	424.2	Lower Marker	81.5	3.3	0.0	25.0
2	25	4.00	242.4	Ladder Peak	56.9	2.3	0.0	26.2
3	50	4.00	121.2	Ladder Peak	64.8	1.8	0.0	34.4
4	100	4.00	60.6	Ladder Peak	98.1	2.1	0.0	41.2
5	150	4.00	40.4	Ladder Peak	133.8	2.0	0.0	45.0
6	200	4.00	30.3	Ladder Peak	148.1	2.0	0.0	52.0
7	300	4.00	20.2	Ladder Peak	156.3	2.3	0.0	63.4
8	400	4.00	15.2	Ladder Peak	163.7	2.6	0.0	76.2
9	500	4.00	12.1	Ladder Peak	173.1	3.0	0.0	82.9
10	700	4.00	8.7	Ladder Peak	161.6	2.9	0.0	87.7
11	850	4.00	7.1	Ladder Peak	163.6	2.0	0.0	86.1
12	1,000	4.00	6.1	Ladder Peak	162.8	2.8	0.0	89.8
13	1,500	2.10	2.1	Upper Marker	137.1	3.4	0.0	52.0

Sample 1



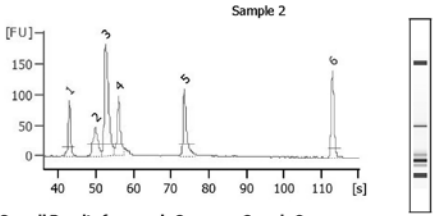
Overall Results for sample 1 : Sample 1

Number of peaks found: 5

Peak table for sample 1 : Sample 1

Peak	Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations	Peak Height	Peak Width	% of Total	Area
1	15	4.20	424.2	Lower Marker	85.5	3.8	0.0	58.6
2	54	0.69	19.4		20.4	1.7	2.8	12.1
3	60	0.92	23.3		22.7	0.9	3.8	16.5
4	67	1.25	28.1		28.6	1.6	5.3	23.1
5	87	12.95	225.0		186.0	5.1	58.7	257.2
6	270	4.29	24.0		139.7	5.3	29.4	129.4
7	1,500	2.10	2.1	Upper Marker	129.0	3.4	0.0	104.6

Sample 2



Overall Results for sample 2 : Sample 2

Number of peaks found: 4

Peak table for sample 2 : Sample 2

Peak	Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations	Peak Height	Peak Width	% of Total	Area
1	15	4.20	424.2	Lower Marker	90.0	3.4	0.0	62.8
2	67	3.21	73.2		46.2	3.6	13.4	64.3
3	87	10.62	184.8		182.8	3.5	47.6	228.8
4	116	3.67	48.1		96.5	2.5	17.6	84.9
5	270	3.14	17.7		108.6	4.3	21.4	102.8
6	1,500	2.10	2.1	Upper Marker	142.1	3.2	0.0	113.5

Figure 3B Representative chromatograms obtained by WAVE analysis of DNA samples with hetero- (upper panel) or homozygosity (lower panel) for rs2187247. DNA samples were generated by reverse transcription of RNA isolated from maternal plasma during the first trimester. Despite the presence of primer-dimer bands (visible at 1.3 min), the additional peak (arrow) indicates heterozygosity.

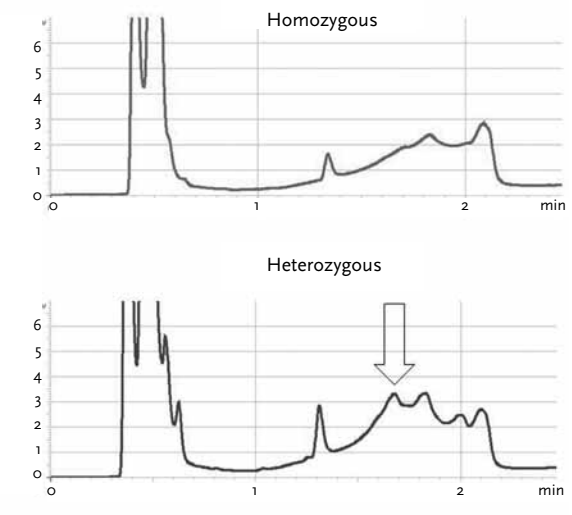
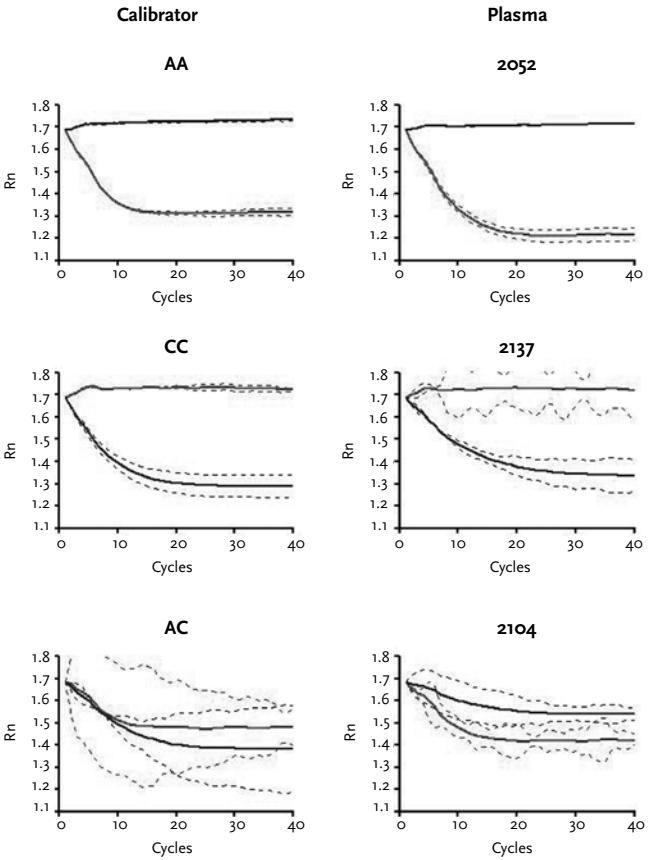


Figure 3C

Representative QEXT results of CDNA amplicons of *C21orf105* generated from first trimester plasma. In all samples, the type and number of the allele (A or C) present in euploid samples is predicted correctly. Notice the correlation between calibrator (left) and plasma samples (right). These data demonstrate that with minor modifications (BioAnalyzer), the WAVE and QEXT procedures permit allelic ratio determination of low abundancy targets in first trimester plasma samples.



Non-invasive detection of trisomy 21 in maternal plasma by allelic ratio determination using the Quencher Extension technique: a feasibility study

Go, ATJI; Visser A, Poutsma A, Spencer K, van Vugt JMG, Oudejans CBM

This thesis

Abstract

Objective Non-invasive diagnostic testing of fetal trisomy 21 using placental mRNA in maternal plasma is a research area of great interest. The RNA allelic ratio strategy, by determining the ratio between alleles expressed by a chromosome 21-encoded gene (PLAC4), was successful in identification of fetal trisomy 21 in maternal plasma in 90% of the cases. The quencher extension (QEXT) technique was adapted and applied for the allelic ratio strategy. In that first study the QEXT method was tested successfully in an in-vitro model system on RNA isolated from normal and trisomy 21 placentas collected at a gestational age of about 13 weeks. The aim of this study is to test the feasibility of this method on plasma samples.

Methods The RNA-SNP allelic ratio is determined with the quencher extension technique in plasma samples, collected during the late first and early second trimester from trisomy 21 and control pregnancies. The SNP used, rs2187247, is located in exon 2 of C21orf105. A RNA isolation method with high yield was used.

Results By QEXT the alleles of all tested samples were called correctly in correspondence with the sequencing results. Reproducibility and correlation coefficients were both 0.9. Although informative, i.e. heterozygous, samples were limited in number, normal and trisomy cases could clearly be distinguished.

Conclusion The feasibility to employ the QEXT technique on mRNA extracted from clinical plasma samples, collected in the late first and early second trimester, is demonstrated. Trisomy 21 and control cases could be distinguished.

Introduction

For pregnant women, trisomy 21 detection is the most common reason to choose for prenatal testing. Although in most developed countries the first trimester combination test is offered to pregnant women as screening test for Down syndrome[1-6], the diagnosis of chromosomal abnormalities still requires an invasive procedure with the risk of miscarriage due to the procedure.

Non-invasive diagnostic testing of fetal aneuploidy using fetal (i.e. placental) DNA and mRNA in maternal plasma is extensively studied the last decade. A variety of high standard techniques and strategies are tested in small studies with promising results.[7-10] In 2006 Lo and coworkers introduced the plasma placental RNA allelic ratio strategy.[11] By determining the ratio between alleles in informative samples, heterozygous for single-nucleotide polymorphisms (SNP) expressed by a chromosome 21 encoded gene such as PLAC 4, they could successfully identify fetal trisomy 21 in maternal plasma in 90% of the cases. The technique used was primer extension and mass spectrometry. This approach requires highly specialised equipment, limiting its wide spread implementation in a routine diagnostic setting. We studied an alternative technique. The quencher extension (QEXT) technique as described by Rudi et al[12] was adapted and applied for the allelic ratio strategy. The quencher extension (QEXT) reaction is a single-step, real-time method to quantify SNP's and is directly adaptable to current real-time PCR equipment.[13] This method was successfully tested in an in-vitro model system using a SNP located in exon 2 of C21orf105 and as tested on RNA isolated from normal and trisomy 21 placentas collected at a gestational age of about 13 weeks. By this, all combinations of heterozygous normal and trisomic samples were determined correctly[13]. In this study, we applied this method on plasma samples obtained during first trimester. As a proof of principle, our data indicate that allelic ratio determination of low abundancy targets using current real-time PCR equipment is technically feasible on clinical samples provided that a RNA isolation method with high yield is used[14].

Material and methods

Patients

Peripheral blood samples (EDTA) were collected between a gestational age of 10 to 15 weeks from pregnant women attending the Prenatal Diagnostic unit of the VU University Medical Centre, Amsterdam or attending the Harold Woods Hospital, London. Samples were obtained with informed consent and approval of the local Ethics Committees. Individuals included were pregnant women, who came for screening by the first trimester combined test or women who were scheduled for an invasive procedure as indicated by the result of the combination test (risk on Down syndrome ≥ 1: 200). All blood samples were obtained before invasive diagnostic procedures i.e. chorionic villus sampling or amniocentesis. The diagnosis trisomy 21 was based on the karyogram. The control group is composed of patients with a normal karyotype or patients who reported that the baby was healthy and had no signs of chromosomal or congenital malformation as indicated by the follow-up form.

Processing of blood samples

EDTA blood was stored at 4° C in an upright position and processed within 72 hour after collection by two sequential centrifugation steps as described previously.[15; 16] In brief, after centrifugation for 10 min at 2000g at 4° C in a Hettich Rotanta 96R centrifuge, plasma was subjected to a second centrifugation for 10 min at 25 000g at 4° C in a Hettich EBA12R centrifuge. Plasma was stored as aliquots at –70° C and thawed only once. Processing of blood was done within a laminar flow hood. London samples were processed identically, transported to Amsterdam on dry ice and stored at -70° C.

Fractionated RNA extraction from maternal plasma

Fractionated RNA isolation from plasma was performed with the mirVana PARIS kit (Ambion) as modified from Mitchell.[17] For this, 400 µl of plasma is thawed on ice and transferred to a 2 ml tube containing an equal amount of 2 × Denaturing Solution at room temperature and immediately mixed thoroughly. An equal volume (800 µl) of Acid-Phenol: Chloroform is added, vortexed for 30-60 s and centrifuged for 15 min at maximum speed (≥ 10,000 × g) at room temperature. The upper aqueous phase is re-extracted with a second volume of Acid-Phenol:Chloroform equal to the total volume recovered. One-third volume of room temperature 100% ethanol (ACS grade) is added to the aqueous phase recovered after the second extraction, mixed thoroughly and pipetted onto a filter cartridge (labelled with T). The cartridge is centrifuged for 30 s at RCF

10,000 x g or until the mixture had passed through the filter. The flow-through is mixed with 2/3 volume room temperature 100% ethanol, mixed thoroughly and passed through a second filter cartridge (labelled with S). The flow-through is discarded. Wash Solution 1 (700 µl) is added to both cartridges (T and S) and centrifuged for 15 s. DNAse I stock solution (10 µl) (Qiagen) is added to 70 µl Buffer RDD (supplied with the RNA se-free DNAse set), mixed gently by inverting the tube, and centrifuged briefly. The DNAse I incubation mix (80 µl) is added directly to the cartridges and placed on the benchtop (20-30° C) for 15 minutes. Wash Solution 2/3 (500 µl) is applied onto the column and centrifuged for 15 s. The assembly is spinned for 1 min to remove residual fluid from the filters. After transfer of the cartridges to a fresh collection tube, preheated (95° C) elution solution (105 µl) is applied to the centre of the filters, centrifuged for 30 s and the eluate (about 80 µl for each cartridge) collected and stored as aliquots at -80°C. The eluate from the cartridge labelled with T is enriched for total RNA; the eluate from the cartridge labelled with S is enriched for small RNA (< 200 bp).

Allelic ratio determination

Allelic ratio determination using the quencher extension (QEXT) method was performed as described previously. For this, rs 2187247 located in exon 2 of C21orf105 was used. As control, the cDNA samples were subjected to DNA sequencing and scored independently.

Calculations

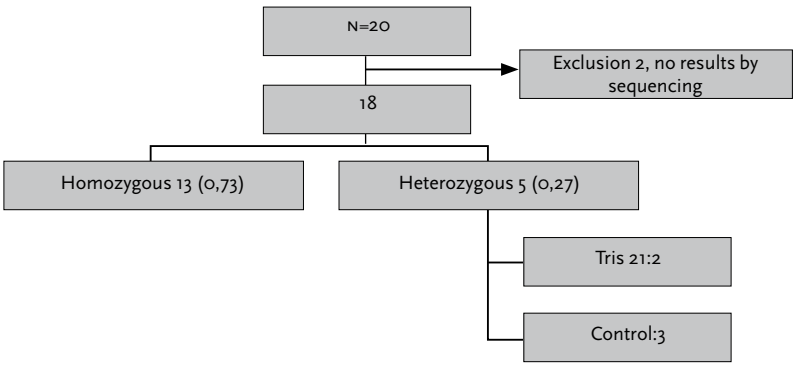
From each sample the allelic ratio per SNP was measured in duplicate. We obtained first order regression curves by polynomial regression by using reporter (FAM) signals normalized for assay imprecision with the ROX reference dye and for calculations was used the initial slopes (15 cycles), which correspond to the linear portion of the reaction curves. The ratio of these slopes (ddUTP : ddGTP) was calculated followed by calculation of the mean of the duplicates. The required A-C windows are 1:1 (AC), 1:2 (ACC) or 2:1 (AAC).

Results

We tested 10 trisomy 21 and 10 control samples (Figure 1). In two samples (no calls), no cDNA was obtained after RT-PCR. In the remaining 18 samples, 13 homozygous and 5 heterozygous samples were identified as classified by both sequencing and quencher extension. The five heterozygous i.e. informative samples contained two trisomy 21 and three normal samples. Reproducibility and correlation coefficients were both 0.9. All

blanks were negative. In the five informative samples, trisomy 21 (mean duplicates of the ratio of slopes: 1.29) could be clearly discriminated from the controls (mean duplicates of the ratio of slopes: 0.87) (Figure 2).

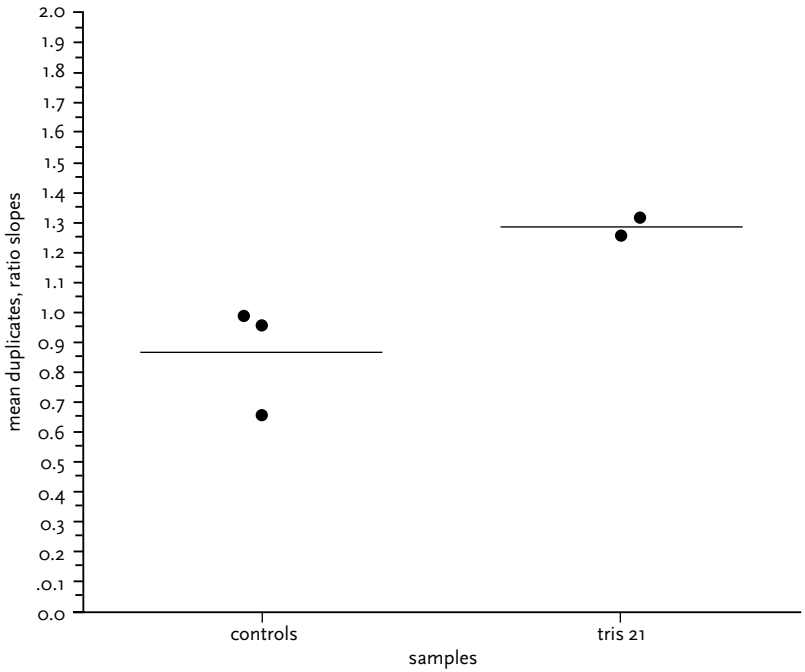
Figure 1 Flow-chart of the 20 samples included: 10 trisomy 21 and 10 control samples were tested.



Discussion

The RNA-SNP allelic ratio strategy for the purpose of fetal aneuploidy detection was first described in 2007 for PLAC4, a chromosome 21 encoded placentally expressed gene. Although highly informative -fetal trisomy 21 could be detected in 90% of the cases and excluded in 96.5% of controls- the assay requires the use of elaborate methods: primer extension and mass spectometry.[18] Using a less elaborate alternative. i.e the quencher extension reaction (QEXT), we previously tested the allelic ratio strategy in a model system of early second trimester placenta tissue.[19] The SNP chosen was situated on exon 2 on C21orf105, another chromosome 21 transcribed mRNA. In this model system the trisomy 21 and control placenta samples could be classified correctly. In the current study, we tested the clinical feasibility of the QEXT technique on mRNA extracted from maternal plasma, collected in the late first and early second trimester. The results were encouraging. By QEXT the alleles of all tested samples were called correctly and in correspondence with the sequencing results. In addition, in the samples tested, despite their limited number, normal and aneuploid cases could be clearly discriminated. As described by others[20], we noted a similar deviation in the reference intervals as theoretically predicted and as actually observed. As a rule of thumb, the actual values observed appear to be lower for all in addition to narrowing of the three intervals (windows). Confirmation of the trend observed in the present study will require testing of additional samples, both normal controls and aneuploid cases. The samples were currently tested for a SNP rs 2187247, on exon 2 of C21orf 105. The theoretical number of informative cases for this SNP is 50% (heterozygote rate 0.5

Figure 2 RNA-SNP allelic ratio determination in maternal plasma samples. Five samples, heterozygous for rs 2187247 were informative: 3 controls and 2 trisomy 21 samples. The ratio of the slopes found by the QEXT technique was calculated. Then the mean of the results of the duplicates was calculated and plotted. The controls (left) and the trisomy 21 results (right) are grouped. Mean of the controls and the mean of the trisomy 21 samples is indicated by the horizontal line.



in the Caucasian population).[21] In the current study, only 27% of the tested samples were heterozygous and probably the consequence of the limited number of samples analyzed. In future studies increase in population coverage can be accomplished by combining several SNP's, either in the same or other genes.[22]

Finally, our method involved a new RNA isolation method originally described for microRNA isolation. Instead of 1.6 ml of plasma, only 400 µL of plasma was needed. Moreover, rather than a single test, multiple analyses can be performed. It can be envisaged that increases in input or other modifications that were beyond the scope of the present study will overcome the fact that we had a no call for two samples.

In summary, we show that it is feasible to employ the QEXT technique for the RNA-SNP allelic ratio strategy in clinical plasma samples collected in the late first and early second trimester. Homozygous and heterozygous samples could be distinguished correctly. The technique is reliable while results can be reproduced in a second test with a correlation coefficient around 0.9. Controls and trisomy 21 samples can be distinguished.

The formerly defined normal range, based on calibrators and a model system, seems to be insufficient for clinical plasma samples. Definition of a normal range by testing a large group of normal samples at different gestational ages is necessary to be able to discriminate between trisomy and normal pregnancies. Since the QEXT technique has a much lower threshold, technically and financially, further development towards a reliable diagnostic test is justified.

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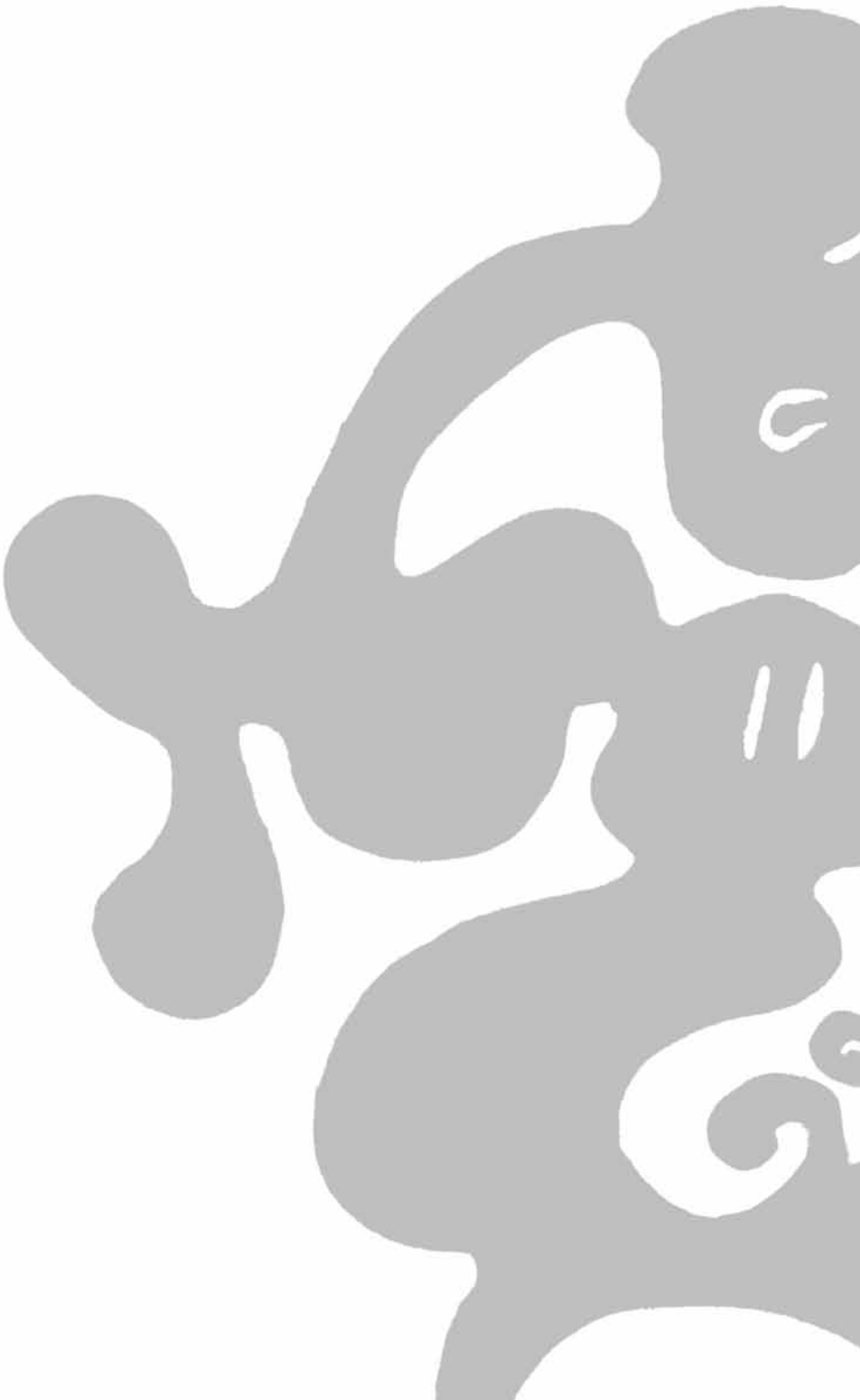

CHAPTER 11

General discussion

The process of developing a non-invasive, prenatal test for trisomy 21 and other aneuploidies can be compared with the quest of the Holy Grail. It is a long lasting journey. Enthusiasms, tenacity, optimism, perseverance, intelligence, creativity, capability to cooperate and to collect set-backs are desirable qualities of the travelers. And the Grail?

Since the 1970's it became possible to detect fetal cells in the maternal circulation.[1] The potential application of using circulating fetal cells for prenatal testing on chromosomal abnormalities has been studied extensively, but did not lead to a successful result.[2] A robust test suitable for population wide implementation could not be developed. But the awareness of the presence of fetal cells in maternal circulation became the offspring of very challenging research regarding fetal cell microchimerism and the role of these cells for the health of the mother. The publications about the presence of fetal DNA [3] and placental mRNA in maternal plasma[4;5] brought new life to the research of non-invasive prenatal testing. It became possible to detect and analyze fetal genetic material in maternal circulation. The enormous technical development is an essential part of this process. It gives the opportunity to detect and measure fetal DNA, mRNA and microRNA even in very low copy numbers. Research on fetal DNA has already leaded to prenatal clinical tests for example for noninvasive determination of fetal RhD status and sex determination.[6-10]

For trisomy 21 detection the situation is rather complicated. Identifying and subsequently measuring these chromosome 21-derived nucleic acids, between the abundant amount of maternal cell free DNA, has proven to be a true challenge. Finding proper markers is still an actual item. Our first studies have focussed on mRNA, on identification of possible markers for trisomy 21 detection and possible reference markers. After defining criteria for a proper marker, placental mRNA's were tested on early placenta tissue, on late-first trimester maternal plasma and for their absence in plasma of non pregnant women. The results showed mRNAs coding for proteins known from serum tests, for example pregnancy associated plasma protein A (PAPP-A). But interestingly also two genes were found coding for transcription factors, for gene products not accessible by conventional antibody-based assays. This means access to a larger pool of possible markers. We tested mRNAs for their presence in early placenta tissue. But this placenta tissue is a mix from various pla-

centa cell-types. Syncytiotrophoblast cells are the cells of interest because these cells are the source of placenta RNA shedding into the circulation. Mix with villus stromal cells and cytotrophoblast stem cells might generate false-positive signals, when exploration of placental gene expression profiling is done with whole tissue fragments. A novel method was tested to identify syncytiotrophoblast-derived RNA products in vitro. RNA was obtained selectively by controlled denudation from syncytiotrophoblast cells of an early second trimester placenta with confirmed trisomy 21. RNA of high and low abundancy targets, hPL and LOC90625, respectively could be recovered reliable. The RNA isolation can be used for cDNA synthesis including adequate recovery of small sized RNAs.

From the tested mRNAs several met the defined criteria. LOC90625, nowadays called C21orf105, was the most promising candidate: Chromosome 21 encoded, located within the Down critical region, expressed in early placenta tissue and presence confirmed in syncytiotrophoblast cells of a trisomy 21 placenta. Our group was the first to describe the detection of a chromosome 21-encoded mRNA in maternal plasma. Using quantitative RT-PCR we tested this marker on plasma of pregnant women collected between 9-15 weeks gestational age. The fact that the marker could not discriminate between trisomy 21 and control pregnancies was a great set-back. The marker was blamed. The low copy number per ml was thought to be the most important reason for not being discriminative. But the low abundancy of placenta mRNA's and fetal DNA in maternal plasma still is the universal problem in the field. Studies on enrichment and selective targeting fetal DNA are many.[11-18]

On the another hand high-standard techniques, like digital PCR, microfluidics digital PCR and deep sequencing are being tested to deal with this problem. Now it can be concluded that the method itself, quantitative RT-PCR was part of the reason for failure. Is C21orf105 a useless marker? The markers still meets the right criteria for trisomy 21 detection. The marker is intron-exon spanning, which is an advantage. After the introduction of the RNA-SNP allelic ratio strategy by Lo[19] *et al.* the C21orf105 marker made a come-back. Due to the fact that polymorphism is crucial for discrimination, only heterozygous samples are informative. Heterozygote rate in populations became important. Combining several SNP's might increase population coverage. SNP's are present in C21orf 105 and heterozygote rate of SNP rs 2187247 is tested 0.5 in Caucasian population. C21orf105 was used in a model system to test quencher extension for the allelic ratio strategy. Controls and trisomy 21 placenta tissue could successfully be discriminate. The results were encouraging. So C21orf105 can still be useful for non-invasive testing for trisomy 21.

The last years development of the test possibilities came in a rapid stream. In parallel several techniques are studied with promising results:

allelic ratio strategy using primer extension and mass spectrometry, digital PCR and massive parallel sequencing of fetal DNA in maternal plasma. The first two, used for measuring relative chromosome dosage, can test one chromosome at a time. So the test is informative for trisomy 21 if chromosome 21 is tested. Detection of other aneuploidies might be possible, but per chromosome of interest a separate test is needed. With massive parallel sequencing it seems to be possible to test more aneuploidies at the same time. That would be a major advantage compared to a 'single result' test. Another important advantage is that this technique is polymorphism independent. For both reasons a very promising candidate. But none of the techniques is ready for wide clinical implementation. The techniques are demanding and the costs are still very high. For a non-invasive test for trisomy 21 the throughput can't take weeks and costs should be reasonable.

With respect to costs and the fact that highly specialised equipment is necessary, we studied an alternative technique for the allelic ratio strategy. Instead of using primer extension and mass spectrometry we tested the quencher extension technique, a method directly adaptable to current real-time PCR equipment. In a model system this technique could correctly discriminate between control and trisomy 21 placentas. On clinical samples collected between 9 and 15 week of gestation it works well, but adapting the reference interval based on a large group of clinical samples seems to be necessary. Depending on the speed of development, improvements and availability of the other techniques there might be a place for the quencher extension technique. Implementation is much easier and costs are low.

All together it can be concluded that there is a lot of movement in this field. In the near future large scale clinical trials can be expected. Since samples need to be collected before an invasive procedure and the trisomy samples are relative scarce, it would be a recommendation to start collecting samples nation wide; for example collecting an extra EDTA tube during every venous withdrawal for the serum test (for the first trimester combination test). Patients who choose for a screening test on Down syndrome can be expected to be interested in a diagnostic test as well. In the Netherlands we have an organised program for implementation of the first trimester screening on Down syndrome. Availability of a sample bank can facilitate large-scale clinical trials and implementation of the test. It would give the Netherlands a lead in testing and implementation of a potential new diagnostic test.

And the grail?

What if this quest leads to the development of a reliable diagnostic non-invasive test for trisomy 21 (and other aneuploidies)? The main advantage is that the change of miscarriage due to invasive procedures like amniocentesis and chorionic villous biopsy can be avoided. Invasive procedures for reason of advanced maternal age can be replaced. It would be logical that screening tests on Down syndrome will be replaced as well, not only the first trimester combined test, but also the triple and integrated test. Because it can be expected that this diagnostic test to be will be independent of gestational age. This fact would be a great advantage for daily practice. A second advantage is the fact that one test can replace two others (nuchal translucency measurement and the serum test) and a calculation of the chance is redundant. A lot of time can be saved.

What definitely can not be replaced is the pre-test counselling. It is likely that explanation of a diagnostic test is easier then explanation of a screenings test. But the impact of the result has to be clearly discussed and thought over before the patient chooses for carrying out the test. The risk of an easy and low threshold test is that thinking starts after the test result. The result in this case can mean a serious prenatal diagnosis for the unborn child. In the Netherlands great effort has been taken towards a good implementation of the first trimester screenings test on Down syndrome and the 20 week ultrasound-scan. Counselling and informed choice is and must stay an important part of it. Under these conditions the test must be offered to all pregnant women. There is no reason for an age depended discrimination. A non-invasive diagnostic test for trisomy 21 is in direct line with the choice for the first trimester screenings test. Nowadays in the Netherlands an age dependent discrimination is made from financial perspective. Women younger then 36 years must pay the costs for the first trimester screening test themselves, for women 36 years and older the costs are paid by the insurance companies. An odd situation, women can take their own decision after being informed about the existence of prenatal tests and eventually further counselling and the process of informed choice. Financial considerations interfere with the freedom of this choice and are an undesirable cause for unequal treatment.

What to do with the nuchal translucency (NT) measurement? It is a time consuming test, not only to carry out, but a lot of education and continuous surveillance of the quality is needed. The continuous surveillance is mainly necessary for reasons of precise measurement in perspective of the risk calculation for Down syndrome. Since ultrasound-scan operators are working in their own (small) practices spread over the whole country, adequate quality checks are an enormous exertion. Can we abandon the

NT measurement? An increased or thickened nuchal translucency is not only associated with trisomy 21 but also with other chromosomal abnormalities, congenital malformations or syndromes of the fetus. If the non-invasive diagnostic test can diagnose aneuploidies (trisomy 18, 13, 45X0 as well), it has to be studied and discussed what the remaining reasons would be for carrying out screening by the nuchal translucency. At the moment a NT of 3.5mm or more is a reason for offering an invasive procedure with full karyotyping. Extra material is taken in storage for eventual additional testing, for example for testing on Noonan syndrome.[20] In the future formation of a screening panel forDNA mutations, microdeletions or duplications indicating syndromes or diseases associated with a thickened nuchal translucency is conceivable. Association with cardiac abnormalities or other structural abnormalities can be obviate by the 20 week ultrasound scan test or earlier [21]. It might be well possible that the NT measurement will be offered in parallel to a non invasive diagnostic test for aneuploidies. But it must be discussed what the place of this additional screening test would be. Last but not least the present law forbids screening on non treatable diseases. Termination of pregnancy is not classified as a treatment. So for screening on fetal abnormalities dispensation has to be asked with the ministry of Health.

What about the ideal timing to perform such a test? It has not been studied in this thesis. Until about 10 weeks of gestation miscarriage is, depending on maternal age, a regularly occurring phenomenon. It might be very well possible that concentration of circulating nucleic acids in maternal plasma is lower in cases of pregnancy demise. And that way might interfere with the feasibility of performing the test. Good timing seems to be between 10 and 15 weeks of gestational age.

What will happen to the numbers? In the Netherlands 59% of the invasive procedures is carried out for reason of advanced maternal age.[22] Participation to the first trimester screening for Down syndrome is estimated to be 25-30%. Of course the right answer to this question can not be given. But a wild guess can be made that the number of invasive procedures will be halved and the non-invasive testing for trisomy 21 detection doubled.

What about the other side of informed choice and the right of "not to know" in prenatal testing. A child with trisomy 21 or other chromosomal abnormalities, congenital malformation or syndromes is and has to be welcome in this world. They need love and care, medical and psychosocial support, education and integration. The estimated life expectance of persons with Down syndrome has increased from just 12 years in the 1940s to average ≈ 60 years in the present-days populations of developed countries. Per life stage different causes of death can be distinguished.[23] Right treatment improves survival and quality of life. For example cardiac

surgery in case of congenital heart defects, present in 45-50% of the newborns with Down syndrome, shows good results. [24] The other part of a genuine choice is that the society guarantees facilities and conditions for this support. The promotion and monitoring of this situation is an important responsibility of the government.[25] For a true freedom of informed choice the possibility of testing and termination of pregnancy and the choice for no testing and the right "not to know", must be equally respected.

What next...

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Summary

Prenatal diagnosis has become an integral part of obstetric care in the Western world. For the detection of chromosomal abnormalities of the fetus invasive procedures, like amniocentesis and chorionic villus sampling, are necessary to obtain fetal cells. Unfortunately these procedures carry a risk of iatrogenic miscarriage. Fetal trisomy 21, related to advanced maternal age, is the most common reason for women to choose for invasive prenatal tests. The availability of a non-invasive diagnostic test with the same potentials as the present diagnostic test would be ideal. Already for decades development of such a diagnostic test has been a very challenging research goal.

Circulating fetal cells in maternal circulation have been extensively studied but it was not possible to develop a test suitable for population wide implementation. In the blood of pregnant women fetal DNA and placental mRNA can also be detected. This genetic material provides a reservoir of possible biomarkers for the development of non-invasive prenatal tests with diagnostic potentials. In this thesis the studies are directed toward development of a robust non-invasive prenatal test for trisomy 21 using maternal plasma collected in the late first- or early second trimester of pregnancy, preferably easy to implement and at reasonable costs.

Chapter 1 contains a brief introduction on invasive and non-invasive prenatal testing, with the accent on trisomy 21 and describes the outline of the thesis.

In **chapter 2** the cellular origin, biological features and clinical potentials of cell-free fetal nucleic acids, both DNA and mRNA present in maternal plasma and serum during pregnancy are described. For clinical application of cell free fetal DNA in maternal circulation two approaches can be distinguished, a gender and a polymorphism dependent approach. Both already found their way to the clinic. By non-invasive fetal gender assessment an invasive procedure can be avoided and so the number of pregnancies at risk for miscarriage, for example in case of congenital adrenal hyperplasia, are decreased. An example of a polymorphism dependent approach is determination of fetal Rhesus D genotyping in Rhesus negative pregnant women.

In **chapter 3** the recent advancement of technical possibilities for non-invasive aneuploidy tests based on cffDNAand placental mRNA in mater-

nal plasma is reviewed. In small studies and model systems strategies and techniques for potential non-invasive aneuploidy tests are demonstrated. The RNA-SNP allelic ratio strategy seems to be the most feasible test at this moment with the desirable quality that the result is based on fetal specific genetic material, but has the disadvantage of polymorphism dependency. The combination of several markers might help to enlarge population coverage. Deep sequencing is promising, but at this moment too labour intensive for clinical implementation and at high costs. Great advantage is the fact that the test is gender and polymorphism independent and several aneuploidies can be tested at the same time.

Chapters 4 to 10 contain studies focussing on several aspects of the development of a clinical test for detection of trisomy 21 using mRNA in maternal plasma. At the start we defined criteria for markers for trisomy 21 detection: the gene analysed should be

1. encoded by chromosome 21.
2. located within the Down syndrome critical region (DSCR).
3. expressed in first trimester normal placenta tissue.
4. over expressed by the placenta in trisomy 21 pregnancies.
5. detectable in maternal plasma during early pregnancy.
6. absent in plasma of non pregnant women.

In **chapter 4** the detection of chromosome21-encoded mRNA of placental origin in early placenta tissue and in first trimester maternal plasma was studied. The aim was to identify possible markers for prenatal testing on trisomy 21. Plasma samples were obtained from pregnant women between weeks 9-13 of pregnancy. RNA was isolated from 800 and 1600 µL of plasma by silica-based affinity isolation and, after on-column DNAse treatment, subjected to two-step, one tube reverse transcription-PCR with gene specific primers. Three genes which met the above mentioned criteria were tested. One of these chromosome 21-encoded genes, LOC90625, showed strong expression in first trimester placenta and was selected for plasma analysis. RNA from LOC90625 was present in maternal first trimester plasma and could be detected in 60% of maternal plasma samples when 800 µL of plasma was used and in 100% of samples when 1600 µL of plasma was used.

The detection of chromosome 21-encoded mRNA of placental origin in maternal plasma during the first trimester may allow development of plasma-RNA based strategies for prenatal prediction of trisomy 21. LOC90625 is a good candidate gene for this purpose.

In **chapter 5** a large panel of RNA targets, distributed over all chromosomes except for the Y chromosome, known or expected to be present in extra embryonic tissues, was tested for their presence in early placental tissue, presence in pregnant plasma and absence in non-pregnant female plasma. This set included genes coding for transcription factors, genes

subject to genomic imprinting, genes coding for non-coding RNA, and other genes with restricted or abundant expression in trophoblast cells. RNA was extracted from 1.6 mL of maternal plasma by silica-based affinity isolation with use of the QIAamp MinElute Virus Vacuum system (Qiagen) with minor modifications. The two-step, one tube reverse transcription-PCR assay was performed as described previously, except that for a selected set of genes the number of PCR cycles was increased to 50. Three patterns could be distinguished. Pattern C consisted of detectable amounts in pregnant plasma and not detectable amounts in non-pregnant controls (positive/negative). This was the pattern of interest and was observed in eight genes. Two of these genes (GCM1 and ZDHHC1) code for transcription factors. It was demonstrated that this approach permits rapid screening of a large set of potential new markers, it allows the detection of markers not accessible by conventional antibody-based assays. This greatly increases the number of markers that become available for non-invasive prenatal diagnosis.

The search for possible markers is extended in **chapter 6**. A novel method was tested to identify syncytiotrophoblast-derived RNA products in vitro. RNA was obtained selectively by controlled denudation from syncytiotrophoblast cells of an early second trimester trisomy 21 placenta, that was subsequently analyzed by cDNA cloning and microarray profiling. Given the preponderance of 5' mRNA fragments lacking a poly A tail, the placental RNA products were amplified following polymerase A mediated tailing using a method originally designed for small-sized microRNAs. The RNA recovered following denudation is representative of the RNA expressed by and released from the placental syncytiotrophoblast as indicated by the presence of both high and low abundancy targets, i.e. hPL and LOC90625 were recovered reliable before and after amplification. The RNA isolated can be used for cDNA synthesis including cDNA synthesis of small sized RNAs. The 95 bp microRNA precursor of Hsa-Mir-141 was correctly and consistently identified following cDNA synthesis and cloning. So this approach when combined with cDNA library- or microarray expression screening is a novel *in vitro* method to screen for syncytiotrophoblast-derived RNA products representative of trisomy 21 placental RNA as present *in vivo* in maternal plasma.

The mRNA LOC90625, nowadays called C21orf105, was the most promising candidate found as marker for a non-invasive test for trisomy 21. In the study described in **chapter 7** C21orf105 was tested in maternal plasma of women carrying a fetus with or without trisomy 21. Using quantitative RT-PCR we determined transcript levels of target (C21orf105) and reference (hPL) genes in first-trimester plasma samples. Plasma was obtained from EDTA blood, sampled between 9-15 weeks of gestational age, after two sequential centrifugation steps and stored at -70 ˚C. After

RNA extraction, quantitative RT-PCR was performed using Taqman probes. From the 51 samples, 43 samples were conclusive. Comparison of transcript levels of C21orf105 in both groups showed no significant differences. When expressed as ratios of hPL/C21orf105, the differences between trisomy 21 and normal pregnancies remained non-significant. It was concluded that the amount of C21orf105 mRNA in maternal plasma, although situated in the Down syndrome critical region on chromosome 21 and up-regulated in trisomy 21 placentas, is not higher in women carrying a fetus with trisomy 21.

In the mean time another strategy was described, the RNA single-nucleotide polymorphism (SNP) allelic ratio strategy. In this strategy quantitative comparison of the allelic expression ratio of a chromosome 21-encoded gene (meeting the above mentioned criteria for a trisomy 21 marker) enable the detection of the differences between 2 and 3 copies of chromosome 21. Due to the fact that polymorphism is the crux for discrimination, the RNA-SNP allelic ratio strategy can only be employed to a subset of the population with a heterozygosity for the SNP used. Theoretically an increase in population coverage can be obtained by combining the results of several markers. So the availability of useful SNP's was studied. In **chapter 8** we tested 44 SNP's expressed by 7 chromosome 21-encoded, placenta expressed genes for their potential use in non-invasive prenatal diagnosis. Blood samples were collected in EDTA and PAX gene tubes. Within the transcripts of interest, 44 SNPs were identified. Primers flanking these SNPs were designed with similar thermodynamic characteristics to permit RT-PCR analysis in single runs. All primers were intron spanning, except for primers of PLAC4. Using a sensitive, 2-step, 1-tube RT-PCR assay the marker set was tested in placenta tissue, plasma from pregnant women and non-pregnant women. From RNA isolated from whole blood collected in PAX gene tubes, no SNP marker fulfilled the criterion of absence in non-pregnant blood. Identical analysis of hPL RNA excluded false positivity, because in RNA recovered from whole blood in PAX gene tubes, this marker was clearly present and absent, respectively in samples obtained from pregnant and non-pregnant females. Prenatal use of PAX gene tubes appears to be limited to genes with high relative expression differences between placental tissue and maternal blood cells. With the use of RNA isolated from EDTA plasma, 5 of 44 SNP markers were detectable in maternal plasma and absent in non-pregnant plasma. This result permits an evidence-based selection of target genes and markers to increase population coverage.

In the RNA-SNP allelic ratio strategy study from Lo *et al*, the assay used is based on extension of the polymorphic site to generate small but very specific allele-dependent differences in size. This approach requires

highly specialized equipment, which might limit its widespread implementation in routine diagnostic setting. In **chapter 9** we adapted and applied the Transgenomic WAVE System and the quencher extension (QEXT) for measuring heterozygosity and allelic ratio of placental transcripts. The expressed SNP (rs2187247) in exon 2 of the placentally-expressed, chromosome 21-encoded C21orf105 gene was tested in a trisomy 21 model system. For this, RNA selectively released from the syncytiotrophoblast of normal and trisomy 21 placentas, confirmed by karyotyping, was obtained during first trimester. An exact correlation was seen between the results observed by sequencing and the WAVE system used for the identification of heterozygous samples. With respect to the analysis time needed, the WAVE system was superior. Secondly, as optimized and validated with calibration standards consisting of cDNA amplicons (262 bp) of C21orf105, the real-time QEXT assay was highly accurate in determination of allele ratios following optimization of fragment purification, input DNA- and quencher label concentrations, and reporter signal calculation. Thirdly, the optimized and validated QEXT assay correctly discriminated normal and trisomy 21 placentas as tested in clinically relevant combinations: diploid homozygous (CC), diploid heterozygous (AC), triploid homozygous (AAA), triploid heterozygous (AAC or ACC). In conclusion: The QEXT method, which is directly adaptable to current real time PCR equipment, along with rapid identification of informative samples by the WAVE system, will facilitate routine implementation of the RNA-SNP assay for non-invasive aneuploidy diagnostics.

Finally in **chapter 10** the quencher extension technique is tested in clinical samples to discriminate trisomy 21 plasma samples from controls. Despite the numbers tested were low, the proof of principle was demonstrated that by the use of less elaborate assays the allelic ratio strategy can be used on clinical samples.

Samenvatting

In de Westerse wereld is prenatale diagnostiek een integraal onderdeel geworden van verloskundige zorg. Om chromosoom afwijkingen bij de foetus te kunnen vaststellen is een invasieve procedure, zoals vruchtwaterpunctie of vlokken test, nodig om foetale cellen te verkrijgen. Het nadeel van deze testen is dat er een kans bestaat op een iatrogene miskraam. Foetale trisomie 21, samenhangend met oudere leeftijd van de zwangere, is de meest gangbare reden voor vrouwen om voor een invasieve prenatale test te kiezen. Het zou ideaal zijn als een niet-invasieve diagnostische test met dezelfde mogelijkheden als de huidige diagnostische test beschikbaar zou zijn. Al tientallen jaren geldt de ontwikkeling van een dergelijke diagnostische test als een zeer uitdagend onderzoeksdoel.

Aanwezigheid van foetale cellen in de moederlijke circulatie is intensief bestudeerd, maar het is niet mogelijk gebleken een test te ontwikkelen die geschikt is voor grote groepen. In het bloed van zwangere vrouwen kan ook foetaal DNA en placenta mRNA gedetecteerd worden. Dit foetaal genetische materiaal blijkt een reservoir van mogelijke biomarkers, waarmee een niet-invasieve prenatale test ontwikkeld zou kunnen worden met diagnostisch potentieel. In dit proefschrift zijn de studies gericht op ontwikkeling van een robuuste, niet-invasieve prenatale test voor foetale trisomie 21, waarbij gebruik gemaakt wordt van moederlijk bloed (plasma) dat is afgenomen in het late eerste of vroege tweede trimester van de zwangerschap; bij voorkeur gemakkelijk te implementeren en tegen redelijke kosten.

Hoofdstuk 1 geeft een korte introductie over invasieve en niet-invasieve prenatale testen, met het accent op trisomie 21 en beschrijft de hoofdlijnen van het proefschrift.

In **hoofdstuk 2** worden de cellulaire oorsprong, biologische eigenschappen en klinische mogelijkheden van celvrije foetale nucleïne zuren, van zowel DNA als mRNA, aanwezig in moederlijk plasma en serum tijdens zwangerschap beschreven. Voor klinische toepassing van celvrij foetaal DNA in de moederlijke circulatie kunnen twee benaderingen worden onderscheiden, een geslacht afhankelijke en een polymorfisme afhankelijke benadering. Beide benaderingen hebben hun weg naar de kliniek reeds gevonden. Door gebruik te maken van niet-invasieve geslacht bepaling, bijvoorbeeld op indicatie van congenitaal bijnier

hypertrofie, kan een invasieve procedure vermeden worden en zo kan het aantal zwangerschappen dat risico op een miskraam loopt verminderd worden. Een voorbeeld van een polymorfisme afhankelijke benadering is de bepaling van foetaal Rhesus D genotypering bij Rhesus D negatieve zwangere vrouwen.

In **hoofdstuk 3** worden de meest recente technische mogelijkheden voor niet-invasieve aneuploidie testen, gebaseerd op celvrij foetaal DNA en placenta mRNA in moederlijk plasma, besproken. In kleine studies en model systemen zijn strategieën en technieken met hun resultaten beschreven. De RNA-SNP allel ratio strategie lijkt op dit moment de meest haalbare test met de gewenste eigenschap dat de uitslag gebaseerd is op specifiek foetaal genetisch materiaal, maar met het nadeel dat de test polymorfisme afhankelijk is. Het combineren van verschillende markers kan helpen om de populatie dekking te bevorderen. Deep sequencing is veel belovend, maar op dit moment te arbeidsintensief voor klinische implementatie en bovendien zijn de kosten zeer hoog. Een groot voordeel echter is dat de test geslacht en polymorfisme onafhankelijk is en dat verschillende aneuploidieën tegelijkertijd getest kunnen worden.

Hoofdstukken 4 tot 10 bevatten studies die gericht zijn op verschillende aspecten van het ontwikkelen van een klinische test voor de detectie van trisomie 21, gebruikmakend van mRNA in moederlijk plasma. Aan het begin van het onderzoek hebben we criteria gedefinieerd waaraan markers voor trisomie 21 detectie zouden moeten voldoen: een kandidaat gen moet:

1. Gelokaliseerd zijn op chromosoom 21,
2. gelokaliseerd zijn in de Down syndrome critical region (DSCR),
3. tot expressie komen in eerste trimester normaal placenta weefsel,
4. over-expressie vertonen in moederlijk plasma tijdens in de jonge zwangerschap,
5. afwezig zijn in plasma van niet-zwangere vrouwen.

In **hoofdstuk 4** wordt detectie van chromosoom-21 coderend mRNA afkomstig van placenta weefsel onderzocht in plasma, afgenomen in het eerste trimester van de zwangerschap. Het doel was om potentiële markers te vinden, bruikbaar voor prenatale testen gericht op trisomie 21. Plasma samples werden verkregen door middel van bloedafname bij zwangere vrouwen tussen 9 en 13 weken zwangerschapsduur. RNA werd geïsoleerd uit 800 en 1600 µL plasma met behulp van DNA/RNA bindende silica kolommen, behandeld met DNase en met behulp van een gevoelige methode omgezet in een DNA kopie en miljoenvoudig geamplificeerd met behulp van een PCR reactie. Drie genen die voldeden aan de bovengenoemd criteria werden verder getest. Eén van deze chromosoom-21 coderende genen, LOC90625, vertoonde sterke expressie in eerste trimester placenta weefsel en werd geselecteerd voor verdere analyse in plasma.

RNA van LOC90625 bleek aanwezig in eerste trimester plasma samples. Het kon worden gedetecteerd in 60% van de samples met moederlijk plasma indien 800 µL plasma werd gebruikt en in 100% van de samples indien 1600 µL plasma werd gebruikt.

Detectie van chromosoom-21 coderend mRNA afkomstig van placenta en aanwezig in moederlijk bloed tijdens het eerste trimester van de zwangerschap zou de ontwikkeling van een prenatale test voor foetale trisomie 21 detectie mogelijk kunnen maken. LOC90625 lijkt een goede kandidaat voor dit doel.

In **hoofdstuk 5** werd een groot panel van mogelijke markers met bewezen of verwachte expressie in de vroege placenta en verspreid gelokaliseerd op alle chromosomen behalve het Y chromosoom getest op aanwezigheid in vroeg placenta weefsel en detecteerbaarheid in plasma van zwangere en van niet-zwangere vrouwen. In deze set zijn genen opgenomen coderend voor transcriptie factoren, genen onderhevig aan genomic imprinting, genen die wel RNA maar geen eiwit maken (niet-coderend RNA) and andere genen met lage of sterke expressie in trofoblast cellen. RNA werd geëxtraheerd uit 1600 µL moederlijk plasma door isolatie via binding aan silica kolommen gebruikmakend van het QIAamp MinElute Virus Vacuum systeem (Qiagen) met enige aanpassingen. De detectie werd uitgevoerd zoals eerder beschreven; voor een aantal groepen genen echter werd het aantal PCR cycli opgehoogd naar 50. Drie patronen werden onderscheiden. Patroon C werd gedefinieerd als de detecteerbaarheid in plasma van zwangere vrouwen en het niet detecteerbaar zijn in plasma van niet-zwangere vrouwen (positief/negatief). Dit was het gewenste patroon en werd bij acht genen gevonden. Twee van deze genen (GCM1 en ZDHHC1) coderen voor transcriptie factoren. In deze studie kon worden aangetoond dat met deze benadering snelle screening van een grote set van potentieel nieuwe markers mogelijk is en dat het tevens mogelijk is om markers op te sporen die niet zouden worden gedetecteerd met conventionele op antilichaam gebaseerde assays. Op deze wijze kan het aantal markers dat beschikbaar is voor niet-invasieve prenatale testen enorm worden uitgebreid. De zoektocht naar mogelijke markers wordt verder uitgebreid in **hoofdstuk 6**. Een nieuwe methode werd getest om syncytiotrophoblast afkomstige RNA producten in vitro te identificeren. RNA werd verkregen door middel van selectieve en gecontroleerde denudatie (chemisch afpellen) van syncytiotrophoblast cellen van vroeg tweede trimester placenta weefsel en vervolgens geanalyseerd door cDNA klonering en 'microarray profiling'. Wegens het overschot aan 5'mRNA fragmenten zonder poly A staart, werden de placenta RNA producten geamplificeerd middels polymerase gemedieerde 'tailing' waarbij een methode gebruikt werd die oorspronkelijk ontwikkeld was voor kleine microRNAs. Het RNA verkregen via denudatie is

representatief voor het RNA dat tot expressie komt door en vrij wordt gemaakt uit placenta syncytiotrofoblast cellen zoals kan worden afgeleid uit de aanwezigheid van transcripten met hoge en lage expressie; zoals hPL en LOC90625 die betrouwbaar voor en na amplificatie verkregen konden worden. Het geïsoleerde RNA kan toegepast worden voor cDNA synthese inclusief cDNA synthese van kleine RNAs. De 95bp microRNA voorloper van hsa-MiR-141 kan correct en consistent worden geïdentificeerd na cDNA synthese en klonering. Geconcludeerd kan worden dat deze opzet indien gecombineerd met brede screening middels de daarvoor beschikbare DNA technieken een nieuwe in vitro methode is om syncytiotrophoblast-afgeleide RNA producten representatief voor trisomie 21 placenta RNA, zoals aanwezig in vivo in moederlijk plasma, te screenen.

Het mRNA van LOC90625, tegenwoordig C21orf105 geheten, was als marker voor een niet-invasieve test voor trisomie 21 de meest veelbelovende kandidaat. In de studie die in **hoofdstuk 7** wordt beschreven werd C21orf105 getest in moederlijk plasma van vrouwen die zwanger waren van foetus met en zonder trisomy 21. Gebruikmakend van kwantitatieve RT-PCR werden de expressie en het niveau daarvan van het doelwit gen (C21orf105) en van het referentie gen (hPL) in eerste trimester plasma samples bepaald. Plasma werd verkregen uit EDTA bloed dat bij een zwangerschapsduur tussen 9 en 15 weken was afgenomen en na twee opeenvolgende centrifugatie stappen werd opgeslagen bij -70°C. Na RNA extractie werd kwantitatieve RT-PCR uitgevoerd gebruikmakend van Taqman probes. Van de 51 samples waren 43 samples conclusief. Vergelijking van de expressie in moederlijk plasma van C21orf105 in beide groepen vertoonde geen significante verschillen. Ook indien berekend als ratio, hPL/C21orf105, bleven de verschillen tussen trisomie 21 en normale zwangerschappen niet significant. Geconcludeerd werd dat de hoeveelheid C21orf105 mRNA in moederlijk plasma, ondanks het feit dat het in de Down syndrome critical region op chromosome 21 gelokaliseerd is en opgereguleerd in trisomie 21 placenta's, niet hoger was in plasma van vrouwen die een zwangerschap droegen met een foetus met trisomie 21.

In dezelfde tijd werd een andere strategie ontwikkeld en beschreven, de RNA 'single-nucleotide polymorphism' (SNP) allelic ratio strategie. Volgens deze strategie wordt door kwantitatieve vergelijking van de allel expressie ratio van een chromosoom-21 gecodeerd gen (die voldoet aan de eerder genoemde criteria voor een trisomie 21 marker) detectie van het verschil tussen 2 en 3 kopieën van chromosoom 21 mogelijk gemaakt. Vanwege het feit dat polymorfisme de essentie is van het onderscheidend vermogen, is de RNA-SNP allel ratio strategie alleen informatief bij een deel van de populatie met een heterozygotie voor de gebruikte SNP.

Theoretisch kan populatie dekking worden vergroot door de resultaten van de verschillende markers te combineren. Om deze reden werd de beschikbaarheid van potentieel bruikbare SNPs bestudeerd. In **hoofdstuk 8** worden 44 SNPs zoals aanwezig op 7 chromosoom 21- coderende, placentaspecifieke genen getest op hun mogelijke toepasbaarheid voor niet-invasieve prenatale diagnostiek. Bloed monsters werden verzameld in EDTA en PAX gene buizen. In de beoogde transcriptie gebieden werden 44 SNPs geïdentificeerd. Nagestreefd werd om de primers benodigd voor deze SNPs op gelijke thermodynamische eigenschappen uit te kiezen opdat RT-PCR in een enkele run kon worden uitgevoerd. Alle primers zijn intron spanning, behoudens de primers van PLAC4. Gebruikmakend van gevoelige, betrouwbare methode werd de marker set getest in placenta weefsel, plasma van zwangere en van niet-zwangere vrouwen. Van het RNA dat geïsoleerd werd uit volbloed, afgenomen in de PAX gene buizen, voldeed geen enkele SNP aan het criterium afwezig in bloed van niet-zwangere vrouwen. Door middel van identieke analyse van hPL RNA kon fout-positiviteit uitgesloten worden; in RNA verkregen uit volbloed in PAXgene buizen, bleek deze marker duidelijk aanwezig en afwezig in monsters afgenomen bij respectievelijk zwangere en niet-zwangere vrouwen. Gebruik van P AX gene buizen in prenatale diagnostiek lijkt beperkt te zijn tot genen met grote verschillen in relatieve expressie tussen placenta weefsel en moederlijke bloed cellen. Gebruik makend van RNA geïsoleerd uit het plasma afgenomen in EDTA buizen, waren 5 van de 44 SNP markers detecteerbaar in moederlijk plasma en afwezig in niet zwanger plasma. Dit resultaat maakt een specifiekere op bewijs gebaseerde meer evidence-based keuze van kandidaat genen (markers) mogelijk met het doel populatie dekking te verhogen.

In de RNA-SNP allel ratio studie van Lo *et al* is de gebruikte assay gebaseerd op verlenging van de polymorfe plaats om een klein maar zeer specifiek allel afhankelijk verschil in grootte te bewerkstelligen. Voor deze benadering is zeer gespecialiseerde techniek en apparatuur nodig, die implementatie op grote schaal voor de dagelijkse diagnostische routine bemoeilijkt. In **hoofdstuk 9** passen we de Transgenomic WAVE System en de quencher extension (QEXT) techniek toe om heterozygotie en allel ratio van placenta transcripts te bepalen. SNP (rs217247) in exon 2 van het in placenta weefsel tot expressie komende, chromosoom 21-coderende C21orf105 gen werd getest in een trisomie 21 model systeem. Hiertoe werd RNA, selectief verkregen uit syncytiotrophoblast van normaal en trisomie 21 placenta weefsel, bevestigd door middel van karyotypering, verzameld in het late eerste trimester. Een exacte overeenkomst werd gevonden tussen de resultaten van identificatie van heterozygote samples gebaseerd zowel op sequencing als van de WAVE System. Wat betreft de benodigde tijd om de test uit te voeren, was het WAVE systeem

beter. Ten tweede, na optimalisatie en validatie met behulp van ijklijnen bestaande uit cDNA fragmenten (262 bp) van C21orf105, was de real-time QEXT zeer accuraat in bepaling van allel ratios na optimalisatie van de zuivering stappen, hoeveelheid benodigd DNA, concentraties van labels, en computer analyse. Ten derde onderscheid de geoptimaliseerde en gevalideerde QEXT assay correct normale van trisomie 21 placenta's zoals werd getest in klinisch relevante combinaties: diploid homozygoot (CC), diploid heterozygoot (AC), triploid homozygoot (AAA), triploid heterozygoot (ACC of AAC). Concluderend: de QEXT methode, die direct toegepast kan worden op de huidige real-time PCR apparatuur, gecombineerd met snelle identificatie van informatieve monsters door middel van de WAVE system, kan implementatie van de RNA-SNP strategie voor niet-invasieve, aneuploidie diagnostiek in de dagelijkse praktijk vergemakkelijken.

Ten slotte wordt in **hoofdstuk 10** de quencher extension techniek uitgetest in klinische samples om trisomie 21 plasma samples van controle samples te onderscheiden. Ondanks het feit dat het geteste aantal laag is, kon als 'proof of principle' aangetoond worden dat de allel ratio strategie ook uitgevoerd kan worden met een minder bewerkelijke assay.

Agathe Titia Jantine Ida Go is geboren op 16 mei 1964 te Bandung, Indonesië. In 1982 behaalde zij haar eindexamen aan het Stedelijk Gymnasium te Leiden. Aansluitend heeft zij een jaar op het conservatorium te Rotterdam, afdeling Dans, gestudeerd en deed in de avonduren natuurkunde, het zogenaamde 'opheffen van deficiëntie'. In 1983 begon zij de studie geneeskunde aan de RU Leiden. Het doctoraal examen volgde in januari 1989, het arts examen in 1991. Aansluitend kon zij in het Sophia kinderziekenhuis aan de slag als AGNIO op de afdeling kinderchirurgie. Met de opleiding Gynaecologie en Verloskunde begon zij op 1 januari 1993 in het Leyenburg ziekenhuis te Den Haag (opleider dr. J.P. Holm), van 1994-1996 het academische deel in het Leids Universitair Medisch Centrum (opleiders prof. Dr. E.V. van Hall en prof. Dr. H.H.H. Kanhai). De laatste 2 jaar van de opleiding deed zij in het Leyenburg Ziekenhuis (opleider. Dr. P.A. de Jong). Sinds 1999 is zij gynaecoloog en is zij werkzaam in het VUmc, aanvankelijk als fellow, vanaf 2000 als staflid met de subspecialisatie obstetrische perinatologie. Zij is al jaren actief bestuurslid van de werkgroep Prenatale Diagnostiek en Therapie en thans ook afgevaardigd in het bestuur van de koepel Perinatologie. In 2003 werd gestart met het onderzoek naar foetaal DNA/ mRNA in moederlijk bloed, een samenwerking tussen de Klinische Chemie afdeling moleculair biologie onder leiding van dr. C.B.M. Oudejans en de Verloskunde/Prenatale diagnostiek onder leiding van prof. Dr. J.M.G. van Vugt.

Attie is getrouwd met Meindert Jan de Vries en samen zijn ze de gelukkige ouders van David en Yara.

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Tight two birds together
And they won't be able to fly,
Although they have four wings now.
(Rumi)