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ORIGINAL INVESTIGATION

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Fetal DNA detection in maternal plasma throughout gestation

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Abstract The presence of fetal DNA in maternal plasma may represent a source of genetic material which can be obtained noninvasively. We wanted to assess whether fetal DNA is detectable in all pregnant women, to define the range and distribution of fetal DNA concentration at different gestational ages, to identify the optimal period to obtain a maternal blood sample yielding an adequate amount of fetal DNA for prenatal diagnosis, and to evaluate accuracy and predictive values of this approach. This information is crucial to develop safe and reliable non-invasive genetic testing in early pregnancy and monitoring of pregnancy complications in late gestation. Fetal DNA quantification in maternal plasma was carried out by real-time PCR on the *SRY* gene in male-bearing pregnancies to distinguish between maternal and fetal DNA. A cohort of 1,837 pregnant women was investigated. Fetal DNA could be detected from the sixth week and could be retrieved at any gestational week. No false-positive results were obtained in 163 women with previous embryo loss or previous male babies. Fetal DNA analysis performed blindly on a subset of 464 women displayed 99.4, 97.8 and 100% accuracy in fetal gender determination during the first, second, and third trimester of pregnancy, respectively. No *SRY* amplification was obtained in seven out of the 246 (2.8%) male-bearing pregnancies. Fetal DNA from maternal plasma seems to be an adequate and reliable source of genetic material for a noninvasive prenatal diagnostic approach.

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Introduction

In the last two decades, a huge effort has been spent in developing prenatal diagnostic procedures that do not constitute a risk for the fetus, based on the analysis of fetal genetic material obtained from the rare fetal cells ($1/10^6$–$1/10^7$ maternal nucleated cells) circulating in maternal blood (Bianchi 1998). A great advancement in this field was achieved thanks to the more recent identification of cell-free fetal DNA in the plasma of pregnant women (Lo et al. 1997), this representing a more abundant source of fetal genetic material, accounting for approximately 3–6% of total DNA (Lo et al. 1998). The identification of both fetal cells and, especially, fetal DNA can be considered strongly innovative in the field, since this pushed up the development of alternative approaches for noninvasive prenatal testing of genetic diseases and monitoring of pregnancy complications. Since then, isolated prenatal diagnoses of selected genetic disorders on maternal plasma fetal DNA have been performed (Ding et al. 2004; Fucharoen et al. 2003; Gonzalez-Gonzalez et al. 2002, 2003; Nasis et al. 2004; Saito et al. 2000). Nevertheless, before a large-scale

clinical application of this approach, it is crucial to investigate the presence and distribution of fetal DNA in maternal plasma throughout pregnancy in order to assess whether fetal DNA is present in sufficient quantities in all pregnant women for a reliable prenatal diagnosis and monitoring. Existing data on variation of fetal plasma DNA concentrations show progressive increase throughout pregnancy (Finning et al. 2002; Lo et al. 1998). This is in contrast with our previous findings on DNA extracted from fetal cells in maternal whole blood, which showed a drop from the first to the second trimester (Smid et al. 1997). Another study on fetal DNA extracted from fetal cells circulating in maternal blood during the first trimester reported significantly higher fetal DNA concentrations before 10 weeks of gestation (Falcinelli et al. 1999). Hence, it is crucial to evaluate on a large population sample whether variations of fetal DNA concentration occur also in maternal plasma. This information will be an essential prerequisite in choosing the optimal time for maternal blood sampling in order to establish a new method of early prenatal diagnosis. Some previous papers on smaller population samples reported a sensitivity of approximately 95–100% and specificity close to 100% (Birch et al. 2005; Costa et al. 2001, 2002; Finning et al. 2002; Rijnders et al. 2004; Rouillac-Le Sciellour et al. 2004; Sekizawa et al. 2001), while a multicentric study showed a high interlaboratory sensitivity variation ranging from 31 to 97% (Johnson et al. 2004). However, all these studies were focused mainly on the first or second trimesters of pregnancy.

Indeed, fetal DNA concentration in maternal plasma also needs to be determined in order to allow the identification of the specific factors affecting these values. It has been shown that fetal DNA passage in maternal circulation increases in pathological pregnancies associated with placental abnormalities, such as preeclampsia and preterm labor (Leung et al. 1998; Lo 1999; Lo et al. 1999a; Zhong et al. 2001). Also, in pregnant women carrying karyotypically abnormal fetuses, an increase of free fetal DNA in maternal plasma has been observed (Lo et al. 1999a).

The aim of our work was to quantify fetal DNA levels in maternal plasma at all gestational ages in a very large population of pregnant women in order to determine range and distribution at different gestational ages, and to evaluate accuracy and predictive values of this approach. This would provide reference parameters throughout the whole gestation to be compared with any pathological condition, also allowing the identification of the optimal gestational age for maternal blood sampling.

Materials and methods

Patients

Blood samples were collected at a single time-point, from an unselected population of pregnant women attending both the hospital ambulatory care facilities and the prenatal diagnosis unit. Women who underwent amniocentesis or chorionic villus sampling were sampled before the invasive prenatal procedure, since this could affect fetal DNA passage into maternal circulation.

A total of 1,837 women were sampled at different gestational ages (range 6–40 weeks). Detailed written informed consent, as approved by the local ethical committee, was obtained for each woman.

In order to evaluate accuracy and predictive values of fetal gender determination, two different subsets of samples were analyzed blindly at the beginning (400 samples) and before the end of the work (464 samples); for these samples, fetal gender was unknown at the time of blood sampling and was confirmed at delivery (28 cases) or by karyotyping (436 cases).

Pregnant women were evaluated according to gestational age, maternal age, blood group, and Rh factor, number of previous pregnancies, number of previous abortions or miscarriages, previous pregnancies with male babies, indication for invasive prenatal diagnosis, and smoking habit.

Sample preparation and DNA extraction from plasma

Ten milliliters of maternal peripheral blood were collected in EDTA vacutainer tubes, immediately iced and processed within a few hours. After centrifuging at 1,600 *g*, plasma was carefully removed and re-centrifuged at 13,000 rpm (Chiu et al. 2001; Lo et al. 1998). The supernatant was collected and stored at −20°C until further processing. Blood samples collected in other centers were processed as described above, plasma samples were sent in dry ice to the H. San Raffaele Hospital for any further analysis within a maximum of 15 days from venesection. DNA was extracted from plasma by the use of QIAamp DNA Blood Mini Kit (Qiagen).

In order to identify optimized conditions resulting in the higher plasma DNA recovery, increasing aliquots of plasma samples were extracted by proportionally increasing reagents and DNA was quantified. We then used 400 μl of plasma per column for DNA extraction of all samples analyzed in this study (Lo et al. 1998). Strict precautions against PCR contamination were taken, female operators only performed all the procedures. Moreover, the TaqMan system includes a further anticontamination measure in the form of preamplification treatment by use of uracil *N*-glycosylase, which destroys uracil-containing previous PCR products. Multiple blanks were included in every analysis.

Fetal DNA quantification by real-time quantitative PCR

As a model system, fetal DNA quantification was carried out by amplifying Y-specific sequences in male-bearing pregnancies, since this is the simplest way to

Table 1 Fetal DNA (*SRY*) levels in maternal plasma at different gestational ages. Numbers *in parentheses* indicate male-bearing pregnancies analyzed; *ge/ml* genome equivalents/ml of maternal plasma

Statistical parameter	First trimester (221) ge/ml	Second trimester (677) ge/ml	Third trimester (121) ge/ml	Whole pregnancy (1,019) ge/ml
Mean	23.1	32.4	77.7	35.8
Median	9.1	16.6	51.8	16.6
Range	0–264.7	0–346.1	0–390.9	0–390.9

distinguish between maternal and fetal DNA according to the previously published method (Lo et al. 1998). In plasma samples displaying no *SRY* amplification, the *β-globin* gene was amplified to determine the concentration of the total amount of extracted DNA, both maternal and fetal (Lo et al. 1998).

Statistical analysis

To regularize the data distribution, a log transformation of the DNA level was performed. Log DNA was regressed on week of gestation, mother age, blood group, gravidity, previous number of male children, previous abortion, Rh factor, and smoke habits. Variables to enter into the final model where selected by means of the AIC (Akaike's Information Criterion) (Atkinson 1980), which enables the comparison of the fitting of different models. The effect of the gestational week on DNA levels was accounted for by inserting the week into the model.

The best fitting model accounted for the effect of week, age and blood group, while all other variables did not contribute significantly. All the analysis were performed using the R statistical package version 2.0.1 (R Development Core Team 2004).

Results

Optimization of the extraction protocol

Evaluation of DNA extraction and purification performed on increasing plasma amounts (200–800 μl) confirmed that 400 μl of plasma allows a proportionally higher fetal DNA recovery with respect to the 200 μl suggested by the manufacturers; higher plasma amounts (600 and 800 μl) enable the recovery of only a slightly higher fetal DNA amount, in non-linear proportion with what expected on the basis of the increased aliquot of plasma extracted. This could be ascribed to column overloading and interference of an excess of plasma components with DNA binding and elution to and from the column. This could lead to an underestimation in the calculations of fetal DNA concentration in maternal plasma. Moreover, since the original protocol has been designed for loading 200 μl of plasma onto the column, extraction of more than 400 μl of plasma would demand a corresponding increase in the number of loading and washing steps which could also increase the risk of contamination.

Patient analysis

According to this protocol, we analyzed a cohort of 1,837 pregnant women, including 1,681 pregnancies with a single fetus, 83 with multiple fetuses, and 73 with chromosomally abnormal fetuses. Of the 1,681 singleton pregnancies sampled throughout pregnancy, 1,019 delivered male and 662 female babies. Among male bearing pregnancies, 221 were sampled during the first, 677 during the second, and 121 during the third trimester of gestation.

Mean, median, and range of fetal DNA levels in maternal plasma throughout pregnancy in 1,019 male-bearing pregnancies are reported in Table 1. Statistical analysis showed a direct relationship between DNA and

Table 2 Statistical parameters at different gestational ages. Numbers *in parentheses* indicate male bearing pregnancies analyzed

Statistical parameters[a]	First trimester (78)	Second trimester (138)	Third trimester (30)	Whole pregnancy (246)
Sensitivity	98.7	95.6	100	97.1
Specificity	100	100	100	100
Positive predictive value	100	100	100	100
Negative predictive value	98.8	95.7	100	96.7
Accuracy	99.4	97.8	100	98.5

[a] *Sensitivity* number of male-bearing pregnancies correctly identified/total number of male-bearing pregnancies tested; *specificity* number of female-bearing pregnancies correctly identified/total number of female-bearing pregnancies; *positive predictive value* number of male-bearing pregnancies correctly identified/total number of positive results at real-time quantitative PCR; *negative predictive value* number of female-bearing pregnancies correctly identified/total number of negative results at real-time quantitative PCR; *accuracy* total number of male- and female-bearing pregnancies correctly identified/total number of pregnancies tested

gestational week, with a raise in DNA level at increasing week resulting in a significant increase both in the second trimester (median DNA level 1.86 higher relative to first trimester, $P < 0.001$) and third trimester (median DNA level more than nine times higher relative to first trimester, $P < 0.001$).

All female fetuses were correctly identified. No contamination of blank samples was detected in any of the assays performed. No false-positive result was obtained in 163 pregnant women bearing a female fetus among whom 73 had previous sons and 90 had a previous embryo loss.

Overall 8.6% (88/1019) of the values obtained from male-bearing pregnancies were zero. During the first part of the study performed blindly on the first consecutive 400 samples, no *SRY* amplification had been obtained in 10% (40/400) of male-bearing pregnancies. Thus, in order to further optimize the extraction protocol, we found that washing the column after plasma application with larger volumes of buffers than those indicated by the original protocol of the kit facilitated DNA recovery and contributed to reduce the rate of false-negative results. In the optimized final protocol, extensive washing after plasma application onto the column was performed with 1 ml of buffers AW1 and AW2 to facilitate DNA recovery. This quantity is doubled with respect to that suggested by the manufacturers. Hence, a second subset of 464 pregnancies was analyzed blindly by applying this modification. Among these, 7/246 male bearing pregnancies gave no *SRY* amplification signal, corresponding to an overall 2.8% false-negative results. Of the 464 pregnancies, 160 were analyzed during the first trimester (78 male-bearing pregnancies), 272 during the second trimester (138 male-bearing pregnancies), and 32 during the third trimester (30 male-bearing pregnancies). Statistical parameters concerning reliability of fetal gender identification are reported in Table 2.

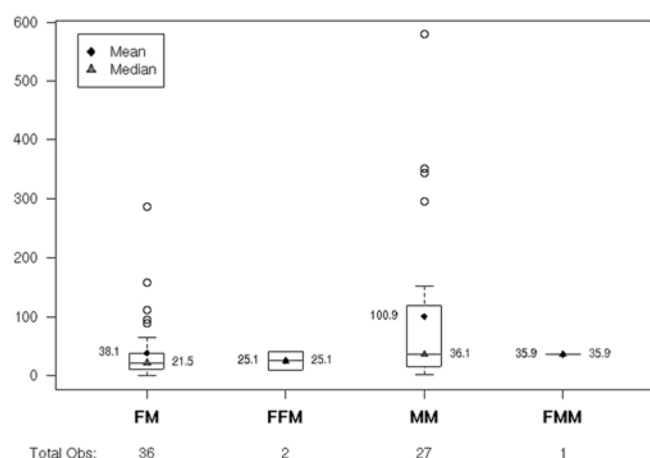

Fig. 1 Plasma *SRY* concentrations vs twin type . *M* male fetus, *F* female fetus, *Total obs.* total observations. All the female-bearing multiple pregnancies (FF and FFF) gave no *SRY* amplification signal. Values on the *left side of boxes* indicate the mean fetal DNA concentration, those on the *right side* indicate median fetal DNA concentration

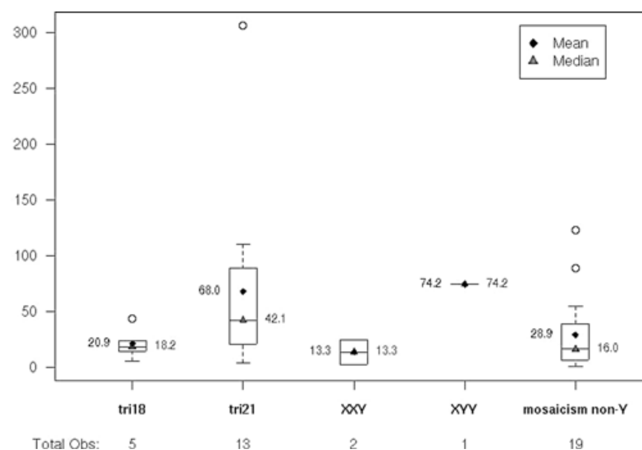

Fig. 2 Fetal DNA concentration in different subsets of pregnancies bearing fetuses with chromosomal abnormalities. Pregnancies bearing male fetuses affected by trisomy 21 (*tri21*) were sampled from 10 to 19 weeks of gestation; those with male fetuses affected by trisomy 18 (*tri18*) were sampled from 11 to 23 weeks of gestation; one pregnancy with an XYY fetus at 12 weeks of gestation; two with an XXY fetus at 11 and 13 weeks of gestation, respectively; 19 with a male fetus carrying different kinds of mosaicism not involving the Y chromosome were sampled from 10 to 17 weeks of gestation. One pregnancy with a fetus affected by trisomy 13 was sampled at 11 weeks and gave no *SRY* amplification. Values on the *left side of boxes* indicate the mean fetal DNA concentration, those on the *right side* indicate median fetal DNA concentration

Concerning anamnestic, demographic and clinical parameters evaluated in this study, fetal DNA levels were found to correlate only with maternal age and maternal blood group ($n = 812$ pregnancies with complete record). In particular, an average increase of about 4% of DNA median level per 1-year increase in maternal age was estimated ($P = 0.004$). Also restricting the analysis to women aged 25–45 (87.8% of the entire sample) the relationships were consistently significant.

Regarding blood group, DNA median levels did not differ among the 0 (17.3 ge/ml), A (15.1 ge/ml), and AB (19.9 ge/ml) blood groups, while pregnancies with B group ($n = 110$; median 9.7 ge/ml) showed a significantly lower DNA level (59% of the 0 group median, $P = 0.006$, corrected for week of gestation and maternal age).

Additionally, 83 pregnancies were found to bear multiple fetuses, among whom 66 male-bearing ones. Figure 1 displays *SRY* concentration in different subsets of twins. These findings are an extension of our previously published results (Smid et al. 2003a), and further confirm that in multiple pregnancies *SRY* median concentration relates to the number of male fetuses.

Moreover, in the course of the present study 73 pregnancies among which 41 male-bearing ones with chromosomal abnormalities were identified; among these, one male bearing-pregnancy gave no *SRY* amplification signal. Fetal DNA analysis showed that trisomy 21 is associated with a highly increased median fetal DNA concentration relative to physiological pregnancies (fold change = 3.02, $P < 0.01$, corrected for week of gestation, maternal age and blood group) (Fig. 2).

Discussion

Since the first report concerning the identification of fetal DNA in maternal plasma (Lo et al. 1997) an increasing interest has been raised toward the development of safe and reliable non-invasive prenatal approaches for both monitoring of pregnancy complications and diagnosis of genetic diseases. However, before any clinical application, extensive knowledge about the physiology of fetal DNA release into maternal plasma is needed. The data presented here are the first findings concerning the evaluation of fetal DNA concentration in a large population of women, comprehensive of all gestational ages, which might serve as reference values.

Our study confirmed that fetal DNA release into maternal plasma seems to be a very early phenomenon since it could be detected from the sixth week of gestation, which was the earliest gestational period available to us in our sample cohort. Interestingly, fetal DNA could be retrieved at any week and a statistically significant correlation was found with increasing gestational age. Our data showed that fetal DNA concentration detected throughout the whole pregnancy and, in particular, in the first and second trimester is adequate for prenatal genetic testing by the use of highly sensitive techniques (Cremonesi et al. 2004; Ding et al. 2004).

In our study, all female fetuses were correctly identified and no false-positive result was registered. No false-positive result was obtained even in pregnant women with a female fetus who had a male baby or an embryo loss in previous pregnancies. These data further support previous observations that fetal DNA is cleared from maternal circulation within a short time (Lo et al. 1999b; Smid et al. 2003b) and no persistence is detectable during the following pregnancies. Thus, the absence of false-positive results due to the combination of both the anticontamination procedures employed and the clearance of fetal DNA from previous pregnancies further enhances the potential of maternal plasma analysis in view of non-invasive prenatal diagnosis and monitoring.

However, for the reliability of noninvasive testing, the presence of false-negative results might be a problem. Similarly to the previous multicentric study (Johnson et al. 2004), our data confirm that a methodological approach is crucial to reduce false-negative rates. Both from the multicentric study and from our findings it appears that the critical step is DNA extraction from plasma. We demonstrated that a simple modification of the original protocol allowed the reduction of false-negative rate from 10 to 2.8%.

Notably, we obtained on a large sample cohort a high sensitivity both referred to the whole pregnancy (97.1%) and to the first and second trimesters, which combined with a 100% specificity results in a very high accuracy.

No previous report had evaluated the possible influence of demographic, clinical, and anamnestic data on fetal DNA release into maternal plasma. Our study shows that fetal DNA concentration does not seem to be affected by the majority of these parameters. This further implies that fetal DNA can be considered as a quite independent marker and indicates that reference values can be applied to different typologies of pregnancy, such as nulliparous and multiparous women, smokers and non-smokers, etc. Furthermore, out of all these factors, only maternal age and blood group showed a significant correlation with fetal DNA levels. With no evident explanation, a significant decrease in fetal DNA levels was observed only in the B blood group, which is the second rarest in the general population (0.10) and involves only a small subset of pregnant women. Concerning fetal DNA increase with increasing maternal age, this phenomenon might be related to a higher risk of developing placental complications with the progression of maternal age.

It is important to notice that absolute fetal DNA quantification becomes particularly relevant in view of a possible prenatal non-invasive screening for trisomy 21 during the first and second trimesters. Concerning chromosomal abnormalities, our findings support the previously reported indication (Lo et al. 1999a) that trisomy 21 is associated with a highly increased median fetal DNA concentration relative to physiological pregnancies, while fetal DNA levels are not increased in trisomy 18 (Wataganara et al. 2003). Moreover, data obtained in the third trimester may be useful as reference values to be compared with those obtained in placental pathological conditions, such as preeclampsia and preterm labor, where increased fetal DNA concentrations were reported. In all these cases, reference values should be corrected for maternal age and eventually for maternal blood group.

All our findings indicate that fetal DNA in maternal plasma may represent a valid alternative source of fetal genetic material for a clinical application of non-invasive prenatal testing and pregnancy monitoring.

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