



The Potential Usefulness of Free Fetal DNA in Maternal Blood for Prenatal Fetal Gender Determination in Multiple Pregnancies

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We applied a noninvasive prenatal test for the determination of fetal gender in multiple pregnancies by using free fetal DNA circulating in maternal blood in order to evaluate whether the quantification of male DNA could distinguish the fetal gender and the number of male and female fetuses in multiple pregnancies. We enrolled consecutively 44 women with twin pregnancies between 11–14 weeks of gestation. Peripheral maternal blood was collected, and genomic DNA was extracted from maternal plasma and analyzed for the multicopy DYS14 sequence by using real-time PCR to quantify male DNA. Results showed that male DNA concentration was significantly higher in twin pregnancies with at least one male fetus, compared to twin pregnancies with only female fetuses. Comparing male DNA concentration in pregnancies with two male fetuses versus pregnancies with one female fetus and one male fetus, we did not obtain a significant difference between the two groups due to a slight overlapping of the range values. Therefore, our test correctly predicted fetal gender, distinguishing twin pregnancies with at least one male fetus from twin pregnancies with only female fetuses, with a diagnostic accuracy of 100%. For distinguishing pregnancies with two male fetuses from pregnancies with both female and male fetuses, a diagnostic accuracy of 76.1% was achieved.

■ **Keywords:** Noninvasive prenatal diagnosis, fetal gender, multiple pregnancies, ffDNA, DYS14.

Noninvasive prenatal determination of fetal gender using free fetal DNA (ffDNA) circulating in maternal blood during pregnancy (Lo et al., 1997) offers a valid alternative to invasive diagnosis of X-linked diseases and fetal disorders of ambiguous genitalia (Bustamante-Aragones et al., 2008; Costa et al., 2002; Rijnders et al., 2001).

In our previous studies (Centra et al., 2011; Picchiassi et al., 2008), we developed an accurate diagnostic tool for an early noninvasive fetal gender determination by using ffDNA extracted from maternal plasma and a real-time quantitative PCR (qPCR) detection system with the DYS14 sequence as marker of the Y chromosome. With this approach, we reached an overall diagnostic accuracy of 99.7% in a large-scale study (Centra et al., 2011). The significant result, obtained by analyzing 513 singleton pregnancies between 10–15 weeks of gestation, has encouraged us to apply the DYS14–ffDNA test on multiple pregnancies because, in recent years, we have observed a high number of these pregnancies, principally

due to the increased use of assisted reproduction techniques (D'Angelo, et al., 2011).

Very few studies (Attilakos et al., 2011; Orendi et al., 2011; Smid et al., 2003) have assessed the content of ffDNA in multiple pregnancies, but report significantly higher ffDNA concentration in twin pregnancies with two male fetuses compared to singleton male pregnancies; no difference was evident between mixed-gender twin pregnancies and singleton male pregnancies. All these data were principally obtained from multiple pregnancies during the second and third trimesters of pregnancy.

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Because little is known about the correlation of the ffDNA concentration and the gender of fetuses in multiple pregnancies at first trimester of gestation, we evaluated ffDNA concentration in twin pregnancies at this gestational age. The aim of the present study was to evaluate whether the quantification of ffDNA concentration in maternal plasma could distinguish male and female fetuses and the number of male and female fetuses in twin pregnancies between 11–14 weeks of gestation. We did not verify whether DYS14–ffDNA concentration could be useful to distinguish between twin and singleton pregnancies, because this information is usually determined in a noninvasive way by ultrasound at the beginning of pregnancy.

Moreover, because in previous studies no difference was found in ffDNA levels of mono-chorionic and di-chorionic male twin pregnancies (Orendi et al., 2011; Smid et al., 2003), we did not consider this parameter in our study, assuming that it should not interfere with qPCR results.

Finally, we verified the diagnostic accuracy of our test in twin pregnancies in order to establish the feasibility of its use in clinical practice.

Methods

Patients

All pregnant women recruited in the study attended the Department of Obstetrics and Gynecology of Perugia University Hospital for prenatal routine checkup or counseling for prenatal invasive procedures. All pregnant women provided informed consent, after they were made aware of the purpose and experimental nature of the study. Gestational age was based on menstrual date and was confirmed by ultrasound, which was used also to distinguish multiple from singleton pregnancies.

We enrolled consecutively 44 women with twin pregnancies between 11–14 weeks of gestation. The characteristics of pregnant women enrolled in the study are reported in Table 1.

Maternal blood samples were drawn before any invasive procedures. Fetal gender, obtained in a blind manner

from the quantification of DYS14–ffDNA concentration by qPCR, was verified by the analysis of karyotypes from invasive procedures, or confirmed by phenotype at birth.

Blood Sampling

Peripheral maternal blood (5 ml) was collected into tubes containing EDTA as the anticoagulant and treated within 4 hours of sampling. Blood samples were stored at 4 °C until treatment. Plasma was obtained by centrifugation, divided into 1 ml aliquots and stored at –20 °C until use, as previously described (Picchiassi et al., 2008).

DNA Extraction

Genomic DNA was extracted from 1000 µl of maternal plasma using a QIAmp DSP Virus kit (Qiagen, Hilden, Germany, www.qiagen.com). The manufacturer's protocol was modified as required (Picchiassi et al., 2008). Moreover, all reagent volumes were adjusted for the increased volume of plasma used in each extraction. Extracted DNA was stored at 4 °C until qPCR analysis.

qPCR

For the analysis of plasma, we employed a real-time PCR 7300 detection system (Applied Biosystems, Foster City, USA, www.appliedbiosystems.com). Extracted DNA was analyzed for the multicopy DYS14 sequence in three replicates for each qPCR experiment to measure the quantity of male ffDNA and for the TERT gene (telomerase reverse transcriptase) to confirm the presence and quality of total (fetal and maternal) DNA in each sample.

Single-plex qPCR reaction setup, thermal cycling conditions, and the quantification method were the same as those previously described (Picchiassi et al., 2008). The ffDNA concentration was expressed as genome equivalent per milliliter of maternal plasma (GE/ml) (Lo et al., 1997).

Blood sampling, plasma preparation, DNA extraction and qPCR analysis were carried out with possible measures to minimize the risk of contamination (Picchiassi et al., 2008). The results of each experiment were considered

TABLE 1

Descriptive Data of Pregnant Women Enrolled in the Study

	Median (range)
Maternal age at the time of blood sampling	32 (24–43) years
Gestational age at blood sampling	12 (11–14) weeks
Gestational age at delivery	36 (20–40) weeks
	Number of pregnancies
Normal physiological pregnancies	35
Complicated pregnancies	9
Total	44
Spontaneously conceived pregnancies	33
Assisted pregnancies	11
Total	44

acceptable only when extraction and amplification protocols gave an optimal performance.

Statistical Analysis

The Shapiro-Wilk test was used to check Gaussian distribution of continuous data and, due to skewness of the data distribution, nonparametric tests were applied. The Mann-Whitney test was used to assess whether there were differences in DYS14-ffDNA concentration between twin pregnancies with at least one male fetus and female-only twin pregnancies.

The Kruskal-Wallis test followed by the Dwass-Steel-Critchlow-Fligner test for post hoc multiple comparisons was used to assess whether there were differences in DYS14-ffDNA concentration among the three types of pregnancies: twin pregnancies bearing two male fetuses (MM), two female fetuses (FF) and one male and one female fetus (FM).

Results are expressed as median values with range for descriptive statistics, which are represented as box-and-whisker plots. The box represents the interquartile range, which contains 50% of the values. The lower and upper margins of the box indicate the 25th and the 75th percentile, respectively. The median is indicated as a line across the box. Whiskers (error bars) above and below the box indicate the 90th and 10th percentiles, excluding outliers.

ROC curve analysis was applied to set a cutoff of DYS14-ffDNA concentration in order to evaluate the diagnostic accuracy of plasma DYS14-ffDNA quantification in noninvasive prenatal determination of fetal gender in multiple pregnancies. The level of statistic significance was set at $p < .05$. All calculations were carried out with Predictive Analytic Software (PASW, release 17.0.2, SPSS Inc., Chicago, USA, 2009).

Results

The DNA extraction of each sample was excellent as TERT amplification products were revealed in each analyzed sample. The performance of the DNA amplification was optimal in all experiments, as demonstrated by the high values of amplification efficiency (always over 90%) and by the high analytical sensitivity in DYS14-ffDNA detection

(the lowest concentration point of the standard curve was always detected).

Fetal gender information, obtained from karyotype or phenotype at birth, showed that 30 multiple pregnancies had at least one male fetus (M) and 14 multiple pregnancies had only female fetuses (F). We classified the twin pregnancies into three different groups on the basis of the gender of the fetuses. Table 2 shows the number of twin pregnancies in each group and their relative DYS14-ffDNA concentrations.

As shown in Table 2, the ranges of DYS14-ffDNA concentration were significantly higher ($p < .0001$) in twin pregnancies with at least one male fetus (MM+FM; range, 8.53–246.94 GE/ml) than in twin pregnancies with only female fetuses (range: .00–3.30 GE/ml). An analogous difference in DYS14-ffDNA concentration ranges was found between MM and FM groups ($p = .049$). The differences of DYS14-ffDNA concentration among the three groups are shown in Figure 1.

Figure 1 shows that the range of values of DYS14-ffDNA concentration for the FF and FM groups did not overlap, but partially overlapped for the MM and FM groups. Moreover, the DYS14-ffDNA concentration values showed an increasing trend related to the number of male fetuses in twin pregnancy.

In order to evaluate the applicability of the test in clinical practice, we calculated its diagnostic accuracy in predicting fetal gender in twin pregnancies. Figure 2 shows that, when adopting a cutoff of > 3.3 GE/ml for DYS14-ffDNA concentration, the test achieved 100% diagnostic accuracy (area under the curve = 1.000, 95% [0.919, 1.000]; $p < .0001$) in predicting fetal gender between pregnancies with at least one male fetus and twin pregnancies with only female fetuses (100% sensitivity, 95% CI [88.3, 100]; 100% specificity, 95% CI [76.7, 100]).

By constructing another ROC curve, we tried to individuate a DYS14-ffDNA concentration value useful as the best diagnostic cutoff to distinguish between MM and FM pregnancies. From Figure 3, it can be seen that, with a cutoff of > 44.01 GE/ml, the test has a diagnostic accuracy of 76.1% (area under the curve = .761, 95% CI [.571, .896]; 81.8% sensitivity, 95% CI [48.2, 97.2]; 68.4% specificity, 95% CI [43.5, 87.3]).

TABLE 2

Multiple Pregnancy Groups and Relative Concentrations of DYS14-ffDNA

Fetal gender group	N	DYS14-ffDNA (GE/ml)		
		Median	Minimum	Maximum
FF	14	0.17	0.00	3.30
FM	19	35.78	11.07	224.45
MM	11	81.82	8.53	246.94
MM+FM	30	48.16	8.53	246.94

Note: FF = twin pregnancy with two female fetuses, FM = twin pregnancy with one female and one male fetus, MM = twin pregnancy with two male fetuses.

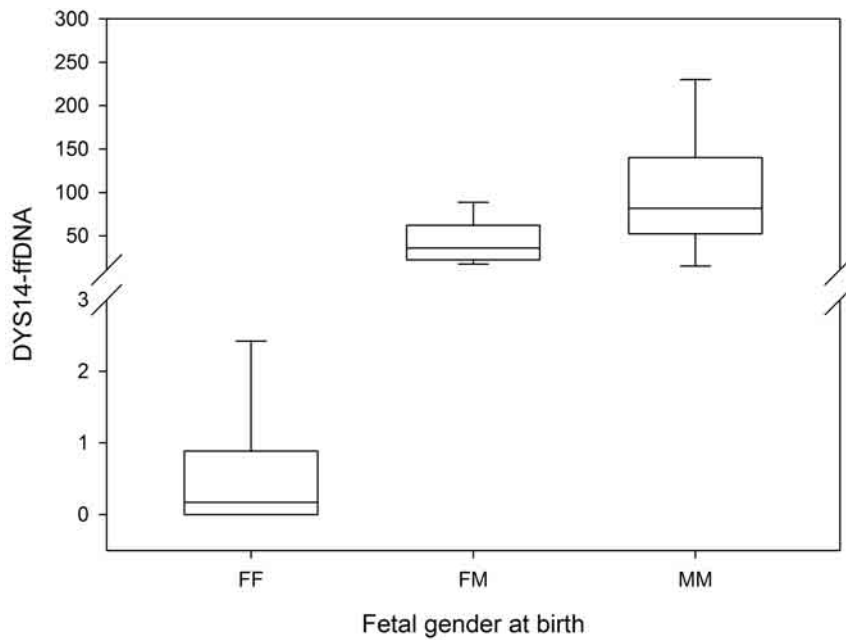


FIGURE 1

Box-and-whisker plot showing the median and range of DYS14-ffDNA concentrations among all groups of the enrolled women.

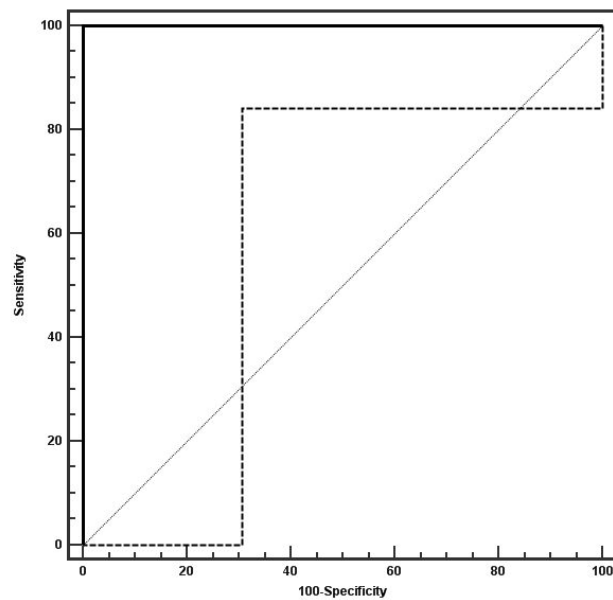
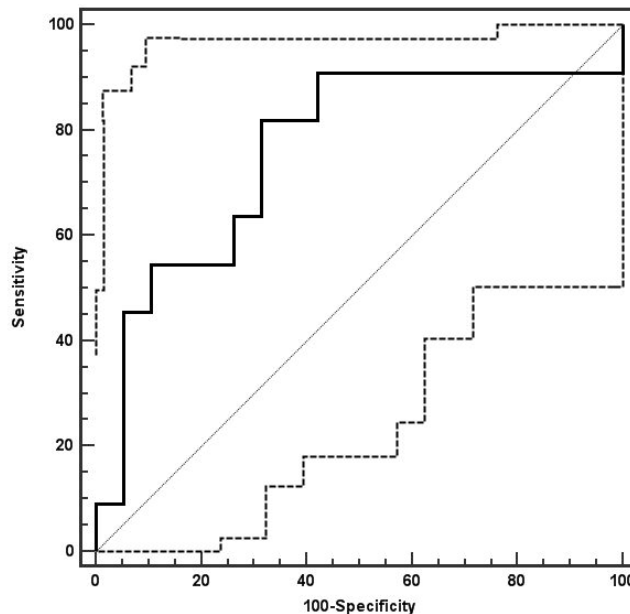


FIGURE 2

ROC curve analysis performed on the results of samples from twin pregnancies with at least one male fetus and twin pregnancies with only female fetuses to set a cutoff value for achieving the best diagnostic accuracy in predicting fetal gender (cutoff value = 3.30 GE/ml, area under the curve = 1.000, 95% CI [.919, 1.000], $p < .0001$).

**FIGURE 3**

ROC curve analysis performed on the results of samples from MM and FM pregnancies to set a cutoff value for achieving the best diagnostic accuracy in predicting fetal gender (cutoff value = 44.01 GE/ml, area under the curve = .761, 95% CI [.571, .896]).

Discussion

In the present study we assessed the *ffDNA* concentration in maternal plasma of twin pregnancies in the first trimester of gestation by using qPCR and primers and probes specific for the *DYS14* Y chromosome sequence, in order to evaluate whether this quantification could be a useful tool for assessing the gender of fetuses in twin pregnancies.

To our knowledge, this is the first study on fetal gender prediction in twin pregnancies performed at an early gestational age (11–14 weeks of gestation). Other similar studies reported in the literature have enrolled pregnant women mainly in the second and third trimesters of gestation (Attilakos et al., 2011; Orendi et al., 2011; Smid et al., 2003).

First, we verified the technical performance of the *DYS14* qPCR methodological protocol for detecting low concentrations of *ffDNA*. The results showed a very high analytical sensitivity and reproducibility, confirming our previous results (Centra et al., 2011; Picchiassi et al., 2008).

Subsequently, we demonstrated a higher *DYS14-ffDNA* concentration in twin pregnancies with at least one male fetus than in multiple pregnancies with only female fetuses. These results suggest that the test could be a useful diagnostic tool to distinguish between these types of pregnancies, because the diagnostic accuracy was 100%. This observation confirms the results obtained in our previous studies performed on singleton pregnancies carrying male and female fetuses — when our test was applied to 145 samples (Picchiassi et al., 2008) and 398 samples (Centra et al., 2011), we reached high diagnostic accuracies of 97.9% and 99.7%, respectively.

However, in this study we were not able to perfectly discriminate between MM and FM pregnancies, because the *DYS14-ffDNA* concentration ranges of the two groups partially overlapped and, therefore, the diagnostic accuracy of the test was 76.1%.

Our results confirm that our noninvasive test could be safely translated into clinical practice to discriminate between female-only twin pregnancies and those with at least one male fetus, with a diagnostic accuracy of 100%. Our test cannot accurately assess the number of male fetus in twin pregnancies; nevertheless, the *DYS14-ffDNA* concentration trend suggests a direct correlation with the number of male fetuses. Our hypothesis is not supported by statistical significance, probably due to the restricted number of twin pregnancies analyzed. We believe that a large-scale study could allow us to individuate a criterion to significantly distinguish the number of male fetuses in twin pregnancies and increase the diagnostic power of our test.

In conclusion, although our test cannot determine the number of male fetuses in twin pregnancies, it can accurately establish whether the mother is carrying at least one male fetus. This information could have importance in the management of multiple pregnancies when one or both parents are known carriers of, or sufferers from, X-linked diseases. A big additional advantage of this test is that it avoids invasive procedures such as amniocentesis or sampling of chorionic villi in women without a real risk of bearing fetuses affected by X-linked diseases. Moreover, we can conclude that this prenatal testing is reliable for the unrestricted assessment of all twin pregnancies, because

these encouraging results were obtained from pregnant women with both spontaneous and assisted pregnancies, and also from women with both physiologically normal and complicated pregnancies.

These new findings expand the horizons of noninvasive prenatal testing of fetal gender, based on the quantification of ffDNA (Kooij et al., 2009), beyond the population of singleton pregnancies to include twin pregnancies.

Confirmation of these preliminary results could lead to the development of an innovative tool that could be combined with ultrasound screening at early gestation to better discriminate male singleton or twin pregnancies, with the advantage of reducing invasive methods of prenatal diagnosis.

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