PRENATAL DIAGNOSIS
Prenat Diagn 2007; **27**: 104–110.
Published online 7 December 2006 in Wiley InterScience
(www.interscience.wiley.com) **DOI:** 10.1002/pd.1607

Free fetal DNA in maternal circulation: a potential prognostic marker for chromosomal abnormalities?

Ageliki Gerovassili[1]*, Chad Garner[2], Kypros H. Nicolaides[3], Swee Lay Thein[1,4] and David C. Rees[1,4]

[1]*Division of Gene and Cell Based Therapy, King's College London School of Medicine, London, UK*
[2]*Epidemiology Division, University of California, California, USA*
[3]*Harris Birthright Research Centre for Fetal Medicine, King's College Hospital, London, UK*
[4]*Department of Haematological Medicine, King's College Hospital, London, UK*

Objectives Previous studies on the association of fetal cell-free (cf)DNA levels in maternal circulation have produced conflicting results but the sample sizes were small and based on archived material. We aimed to quantify the levels of fetal and total cfDNA on prospectively collected samples, to understand their correlation with other variables and to clarify their diagnostic value.

Methods DNA from pre-CVS maternal plasma was extracted from 264 controls, 72 trisomy 21, 24 trisomy 18, 12 trisomy 13, 16 Turner's syndrome and 8 triploidy first-trimester pregnancies and quantified using real-time PCR. β-globin was used to determine total cfDNA levels and *DYS14* and *SRY* assays to determine fetal cfDNA levels.

Results Fetal cfDNA levels (*DYS14*) showed correlation with crown rump length (CRL) ($p = 0.004$), BMI ($p = 0.01$) and storage time ($p = 0.007$) while there was an inverse correlation of total cfDNA levels with nuchal translucency (NT) ($p = 0.001$). No significant difference was observed between the levels of fetal cfDNA in controls and aneuploidy cases.

Conclusion Quantification of fetal and total cfDNA in maternal circulation showed inverse correlation between NT and total cfDNA levels. Our results also suggest that fetal cfDNA is not an ideal prognostic marker for chromosomal abnormalities in first-trimester pregnancies. Copyright © 2006 John Wiley & Sons, Ltd.

KEY WORDS: circulating nucleic acids; cell-free DNA; non-invasive prenatal diagnosis; aneuploidy

INTRODUCTION

There is conflicting evidence concerning the elevation of fetal cell-free DNA (cfDNA) in maternal plasma or serum in pregnancies with fetal chromosomal defects (Table 1). In trisomy 21, three studies reported increased (Lo *et al*., 1999; Zhong *et al*., 2000; Lee *et al*., 2002) and three no significantly different maternal concentration of fetal cfDNA (Ohashi *et al*., 2001; Hromadnikova *et al*., 2002; Spencer *et al*., 2003). In the case of trisomy 13, the levels were lower and in trisomy 18 they were not significantly different from normal (Zhong *et al*., 2000; Ohashi *et al*., 2001; Wataganara *et al*., 2003). Two studies examined total DNA in trisomy 21 pregnancies and the maternal levels were increased in one (Spencer *et al*., 2003) and not significantly different from normal in the other (Hromadnikova *et al*., 2002). Possible explanations for the conflicting results are the small number of samples examined in each study, the broad ranges of cfDNA levels at each particular gestation age and the degree of apoptosis in the placenta (Wataganara *et al*., 2005). This study concentrates on the issue of sample size.

For the detection of fetal cfDNA in male fetuses, we employed two different markers; the single copy *SRY* gene and the multicopy marker *DYS14*, both situated on Y chromosome as validation of the results.

We aimed to examine in a prospective study, possible quantitative differences in maternal plasma concentration of fetal and total cfDNA in a large number of chromosomally abnormal pregnancies.

MATERIALS AND METHODS

Subjects

Maternal plasma was collected from 1268 singleton pregnancies undergoing CVS for fetal karyotyping following first-trimester screening for trisomy 21 by maternal age (MA) and fetal nuchal translucency (NT) thickness. All patients gave informed written consent to participate in the study, which was approved by King's College Hospital Research Ethics Committee.

Maternal blood (5 mL) was collected into EDTA tubes, prior to CVS and centrifuged within 4 h at 4000 *g* for 10 min. Plasma was transferred into polypropylene tubes and centrifuged at 20.000 *g* for 10 min. For 1 year from September 2004 to September 2005, we collected samples from 72 pregnancies with trisomy 21 fetuses (38 male), 24 with trisomy 18 (9 male), 12 with trisomy 13

*Correspondence to: Ageliki Gerovassili, Division of Gene and Cell Based Therapy, King's College London School of Medicine, London, SE5 9PJ, UK. E-mail: ageliki.gerovassili@kcl.ac.uk

Received: 9 August 2006
Revised: 9 October 2006
Accepted: 11 October 2006
Published online: 7 December 2006

Table 1—Summary of all publication on quantification of fetal and total cfDNA in maternal circulation in trisomic and control pregnancies

Author	Trisomy	Sample type	Gestation weeks	Values expressed in:	Normal N	Normal DNA (GE/mL) Fetal	Normal DNA (GE/mL) Total	Trisomy N	Trisomy DNA (GE/mL) Fetal	Trisomy DNA (GE/mL) Total
Lo *et al*., 1999	21	Plasma	17–18	Median	37	19.8[a]		13	47.1	
Zhong *et al*., 2000	21	Plasma	14 + 4	Mean	29	83.1[a]		15	185.7	
	18	Plasma	14 + 4	Mean	29	83.1		6	95.5	
	13	Plasma	14 + 4	Mean	29	83.1[a]		3	213.2	
Ohashi *et al*., 2001	21	Serum	15–17	Mean	55	31.5		5	23.5	
	18	Serum	15–17	Mean	55	31.5		3	21.5	
Hromadnikova *et al*., 2002	21	Plasma	20	Median	13	24.5	7330	11	23.3	10 165
Lee *et al*., 2002	21	Serum	15–19	Mean	55	24.2[a]		11	41.2	
Spencer *et al*., 2003	21	Serum	15	Median	10	34.1	5833[a]	10	32	36 153
Wataganara *et al*., 2003	18	Serum	15–20	Median	24	40.3		5	31.5	
	13	Serum	15–20	Median	23	40.3[a]		5	97.5	

[a] Indicates significance.

(4 male), 16 with Turner syndrome, and 8 with triploidy (five male). We then selected from the 1026 samples of the chromosomally normal group, a total of 264 controls (including 238 with male fetuses) on the basis of gestational age (GA) and MA so that there were two cases for each of the chromosomally abnormal group.

DNA extraction and analysis

DNA was extracted from plasma using a QIAmp Mini Blood Kit (Qiagen, Crawley UK) according to the 'blood and body fluid' protocol. Starting volume and experimental conditions vary within the literature. Our protocol was as follows. Four-hundred microlitres of plasma sample were used, per column, for a final volume of 50 μL-extracted DNA. Real-time PCR was employed to quantify the levels of fetal and total cfDNA using an ABI Prism 7900HT Sequence Detector. The *SRY* gene sequence was used for the detection of fetal cfDNA. The primers and probe were obtained from ABI's 'assay on demand' service (Ref: Hs00243216_s1). The *DYS14* multicopy gene was also used to quantify fetal cfDNA according to Zimmermann *et al*. (2005). The primers and probe used were: *DYS14* forward-5′-GGG CCA ATG TTG TAT CCT TCT C-3′, *DYS14* reverse-5′-GCC CAT CGG TCA CTT ACA CTT C-3′, *DYS14* probe-5′-TCT AGT GGA GAG GTG CTC-3′. The β-globin gene sequence was used to quantify total cfDNA as described by Lo *et al*. (1998). The primers and probe used were: β-globin forward 5′-GTG CAC CTG ACT CCT GAG GAG A-3′; β-globin reverse-5′-CCT TGA TAC CAA CCT GCC CAG-3′; and β-globin probe-5′-AAG GTG AAC GTG GAT GAA GTT GGT GG-3′. All reagents were from ABI (Warwick, UK) and the probes labelled with 6-carboxyfluorescein (FAM).

PCRs were performed in a total volume of 25 μL containing 1 × TaqMan Universal master mix, 5 μM of each primer, 5 μM of TaqMan probe and 9 μL of plasma DNA extract. DNA samples were tested in duplicate (ABI). Cycling conditions were 2 min at 50 °C and 10 min at 95 °C for initial denaturation of the DNA and polymerase activation followed by 50 cycles of 1 min at 60 °C and 15 s at 95 °C. A calibration curve was run in parallel with each analysis using serial dilutions of a reference genomic DNA. The analysis was performed with the ABI Prism 7700HT SDS Software with automatic baseline setting. Levels of fetal DNA were calculated according to Lo *et al*. (1998) and expressed in genome equivalents per millilitre (GE/mL). Fetal gender was confirmed by karyotype analysis of CVS samples.

Statistical analysis

Additional data collected, including GA, MA, fetal, were sex, storage time prior to quantification and measurements of NT, crown rump length (CRL) before CVS, maternal weight and body mass index (BMI) before pregnancy, as well as smoking status. It was assumed that maternal weight and BMI would not be significantly different between pre-pregnancies and first-trimester pregnancies. All statistical analysis was performed using SAS (SAS Institute, Inc, Cary, NC) and SPSS (SPSS 12.0.1, Chicago: SPSS Inc) software.

Logarithmic transformation was used to normalize storage time, NT, maternal weight and BMI, *SRY, DYS14* and β-globin variables prior to any statistical analysis. Correlation between all the variables was determined and unpaired *t*-test was used to determine the significance of differences in the mean values between the chromosomally normal and each of the abnormal groups. Generalized linear models (GLMs) were tested to measure the effects of the DNA level measures on each chromosomal abnormality. Each model included terms for the effects of each of the possible confounding variables that were measured.

RESULTS

Descriptive statistics were generated including medians and minimum and maximum values (Table 2). There

DOI: 10.1002/pd

was no significant correlation between fetal cfDNA levels analysed with *SRY* marker and any of the variables. However, there was a significant correlation in fetal cfDNA levels as measured by the *SRY* and *DYS14* markers ($r = 0.56$, $p = <0.0001$), which was expected. Fetal cfDNA levels analysed with *DYS14* marker showed an inverse correlation with storage time ($r = -0.16$, $p = 0.007$) (Figure 1(A)) and BMI ($r = -0.17$, $p = 0.01$) (Figure 1(B)). A positive correlation was found between *DYS14* fetal cfDNA levels and CRL ($r = 0.17$, $p = 0.004$) (Figure 1(C)). Lastly, borderline significant correlation was found between total cfDNA levels analysed with β-globin marker and storage time ($r = 0.09$, $p = 0.055$), maternal weight ($r = 0.11$, $p = 0.035$) and BMI ($r = 0.10$, $p = 0.052$). An inverse correlation was also reported between total DNA levels and NT ($r = -0.15$, $p = 0.008$), (Figure 1(D)) (Results summarized in Table 3).

Comparison of mean fetal and total cfDNA levels using *t*-test analysis revealed no differences between chromosomally normal and trisomy 21 fetuses. Borderline significance was found between fetal cfDNA levels analysed with *SRY* and the 9 trisomy 18 male fetuses ($p = 0.04$, 95% CI: 0.033, 1.497) but not with *DYS14* fetal cfDNA and total cfDNA. No difference was also found between chromosomally normal and trisomy 13 or triploidy fetuses in fetal and total cfDNA levels. Additionally, analysis of total cfDNA revealed no differences between chromosomally normal and Turner's syndrome fetuses (results in Table 4). Box plots of fetal and total cfDNA in normal and chromosomally abnormal cases are presented in Figure 2. In pregnancies with female fetuses there was no detectable fetal cfDNA using either the *SRY* marker or the *DYS14* marker (having applied a cut off detection point of 35 amplification cycles), these cases were excluded from further statistical analysis of fetal cfDNA. However, female fetuses were included in the statistical analysis of total cfDNA.

No significant difference was found between the total cfDNA levels and the fetal sex ($p = 0.239$, 95%

Table 2—Descriptive statistics of the subjects taking part in the study for all the different variables

	Normal			Trisomy 21			Trisomy 18		
	N	Mean	Median (min–max)	N	Mean	Median (min–max)	N	Mean	Median (min–max)
Storage time (days)	264			72			24		
For fetal DNA		75.3	56 (5, 480)		37.5	35 (7, 79)		49	41 (15, 129)
Total DNA		88.1	63 (5, 480)		50.7	37.5 (7, 165)		62.5	52.5 (21, 157)
Gestational age (GA) (weeks)	264	12.7	12.6 (11.1, 13.9)	72	12.7	12.6 (11.7, 13.9)	24	12.6	12.6 (12, 13.9)
Maternal age (MA) (years)	264	35.6	36 (21, 45)	72	36	37 (16, 44)	24	37.4	37.5 (28, 45)
Nuchal translucency (NT) (mm)	264	2.7	2.4 (1.2, 8.7)	72	4.9	4.4 (1.3, 15)	24	6	6.6 (1.3, 11)
Crown rump length (CRL) (mm)	264	65.9	66 (48, 84)	72	66.9	68 (50, 83)	24	59.4	59.3 (48, 81)
Maternal weight (kg)	212	64.6	63 (40, 111)	64	64.3	62 (44, 104)	20	65	59.5 (30, 120)
Body mass index (BMI) (kg/m^2)	212	24.5	23.1 (14.8, 58.7)	64	24	22.8 (17.4, 41)	20	25	21.6 (19.2, 43)
Fetal cfDNA levels (GE/mL)									
Assays: *SRY*	238	17	15 (1, 66)	38	19.8	15.5 (1, 61)	9	11.2	7 (1, 46)
DYS14	238	117.8	99 (1, 653.1)	38	146.6	120 (1, 413)	9	85	34 (1, 343)
Total cfDNA levels (GE/mL)	264	454.5	341 (56.6, 1460)	72	437.5	345 (34.8, 1540)	24	439	317 (49.4, 1401)
Smoking status	259		26 smokers	72		7 smokers	23		2 smokers

	Trisomy 13			Turner's			Triploidy		
	N	Mean	Median (min-max)	N	Mean	Median (min-max)	N	Mean	Median (min-max)
Storage time (days)	12			16			8		
For fetal DNA		48.3	51.5 (13, 73)		33.1	26.5 (7, 83)		37.3	35.5 (10, 85)
Total DNA		70.6	56 (22, 161)		44.1	26.5 (7, 171)		48.6	47 (17, 85)
Gestational age (GA) (weeks)	12	12.6	12.8 (11.6, 13.4)	16	12.6	12.7 (11.9, 13.4)	8	12.5	12.5 (11.1, 13.9)
Maternal age (MA) (years)	12	36.8	37 (30, 44)	16	29.8	28.5 (22, 43)	8	34.2	34.5 (27, 45)
Nuchal translucency (NT) (mm)	12	3.6	3.2 (1.6, 7.3)	16	9.8	9.1 (4.9, 14)	8	3.01	2.3 (1.2, 5.4)
Crown rump length (CRL) (mm)	12	61.25	60 (51, 77)	16	62.5	61.5 (51, 83)	8	58.7	57 (42, 79)
Maternal weight (kg)	11	67.4	69 (50, 94)	16	65.8	66.5 (50, 78)	7	60.8	61 (50, 68)
Body mass index (BMI) (kg/m^2)	11	24.6	23.6 (19.7, 33.8)	16	24.2	23.75 (20, 30.8)	7	22.5	22.7 (17.5, 26.6)
Fetal cfDNA levels (GE/mL)									
Assays: *SRY*	4	10.7	7 (3, 26)	16	0	0 (0, 0)	5	33.8	42 (2, 56)
DYS14	4	43.5	46 (11, 71)	16	0	0 (0, 0)	5	162	171 (21, 285)
Total cfDNA levels (GE/mL)	12	282.6	266 (132, 438.5)	16	326.6	234 (103.3, 777.6)	8	506	361 (149.3, 1540)
Smoking status	12		1 smoker	16		1 smoker	8		2 smokers

DOI: 10.1002/pd

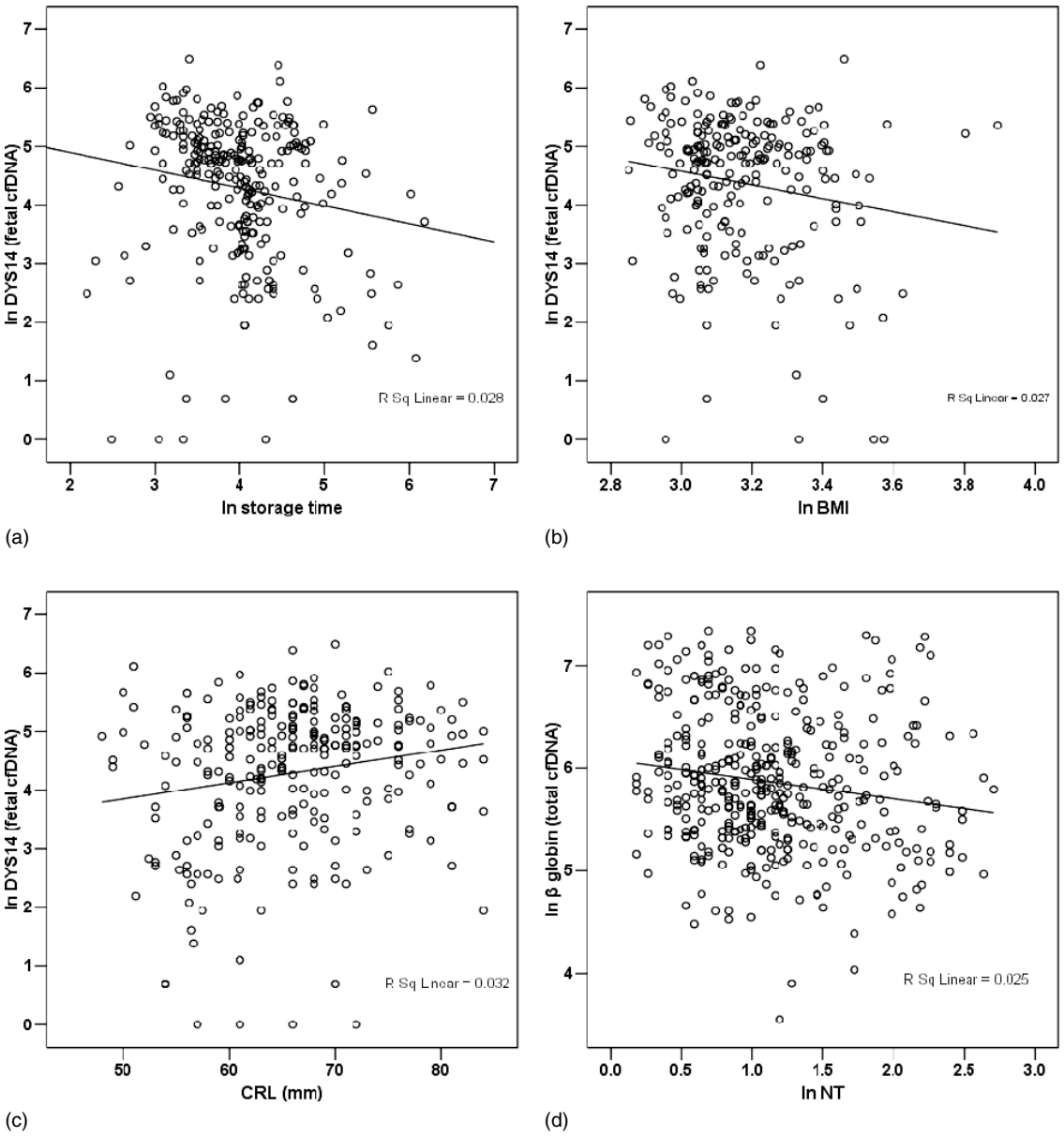

Figure 1—Scatter plots showing correlation between natural logarithmic value (ln) of fetal cfDNA (*DYS14*) and ln of storage time (A), ln of maternal BMI (B), and CRL in mm (C). Scatter plot showing correlation of total cfDNA (β-globin) and ln of NT (D)

Table 3—Association of all the different variables with fetal (*SRY* and *DYS14*) and total (β- globin) cfDNA levels

		Storage	GA	MA	NT	CRL	M. weight	M. BMI
SRY	*p* value	0.159	0.941	0.375	0.977	0.387	0.515	0.224
	r value	−0.082	0.004	0.052	0.002	0.051	−0.042	−0.077
	Cases	294	294	294	294	294	248	248
DYS14	*p* value	0.007[a]	0.106	0.459	0.983	0.004[a]	0.022[a]	0.011[a]
	r value	−0.164	0.099	0.045	0.0013	0.175	−0.153	−0.170
	Cases	262	262	262	262	262	220	220
β-globin	*p* value	0.055	0.203	0.298	0.001[a]	0.540	0.035[a]	0.052
	r value	0.0964	−0.064	0.052	−0.156	0.030	0.1156	0.107
	Cases	396	396	396	396	396	330	329

[a] Indicates significance.
GA, gestation age; MA, maternal age; NT, nuchal translucency; CRL, crown rump length; M. weight, maternal weight; M. BMI, maternal body mass index.

DOI: 10.1002/pd

Table 4—Comparison of mean cfDNA levels between chromosomally normal and abnormal fetuses

cfDNA levels (GE/mL)		Normal	Trisomy 21	Trisomy 18	Trisomy 13	Turner's	Triploidy
SRY	Cases	238	38	9	4	0	5
	Median	15	15.5	7	7	—	42
	Minimum	1	1	1	3		2
	Maximum	66	61	46	26	—	56
t-test	***p*** value	—	0.508	0.04[a]	0.434	—	0.206
	95% CI	—	−0.498 0.247	0.033 1.497	−0.646, 1.500		−1.592 0.344
DYS14	Cases	209	36	9	4	0	4
	Median	99	120	34	46	—	171
	Minimum	1	1	1	11	—	21
	Maximum	653	413	343	71	—	285
t-test	***p*** value	—	0.1851	0.614	0.1592	—	0.4652
	95% CI	—	−0.683 0.1326	−0.036 1.5385	−0.323 1.9572		−1.567 0.7187
β-globin	Cases	264	72	24	12	16	8
	Median	341	345	317	266	234	361
	Minimum	56.5	34.8	49.4	131.9	103.3	149.3
	Maximum	1460	1540	1400.7	438.5	777.6	1540
t-test	***p*** value	—	0.6389	0.2707	0.0876	0.0936	0.8782
	95% CI	—	−0.132 0.214	−0.125 0.4431	−0.049 0.3288	−0.048 0.2837	−0.504 0.431

[a] Indicates significance.
95% CI shows the logarithmic values of the cfDNA markers.

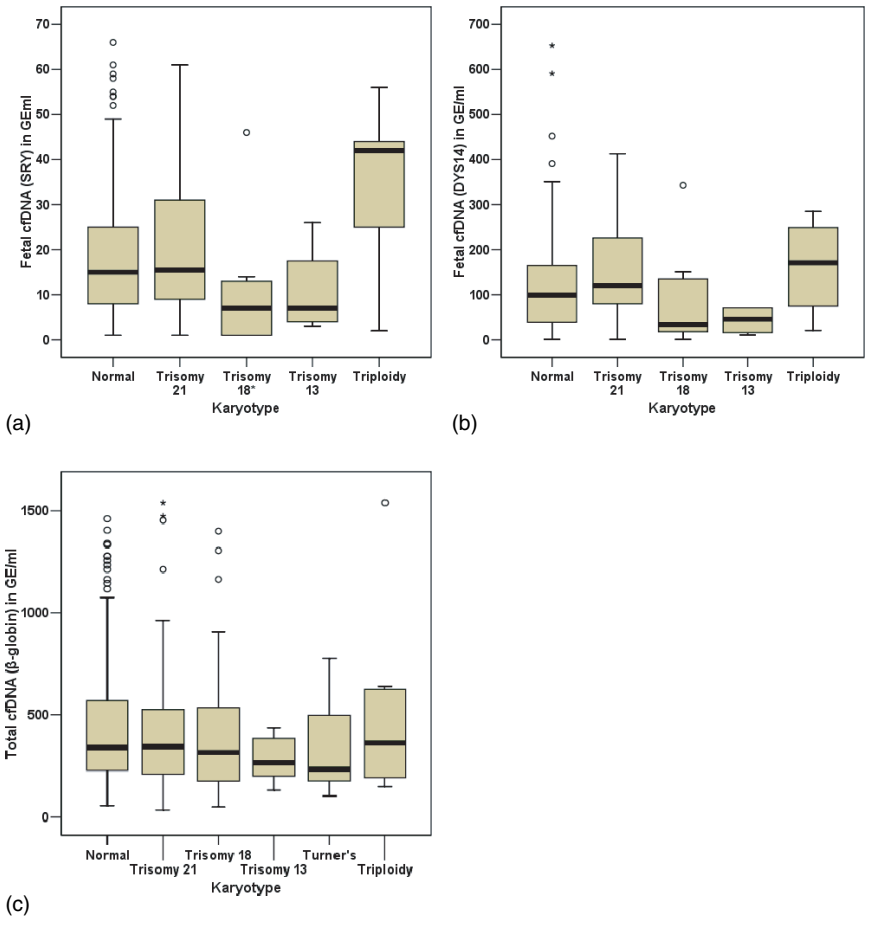

Figure 2—Box plots of concentrations in GE/mL of fetal a) SRY, b) DYS14 and total c) ?-globin cfDNA in karyotypically normal and abnormal fetuses. *Indicates borderline significance

DOI: 10.1002/pd

CI: −0.242–0.0606). Finally, the levels of fetal and total cfDNA were not related with the maternal smoking status (*SRY*: $p = 0.231$ 95% CI: −0.156–0.645; *DYS14*: $p = 0.176$, 95% CI: −0.144–0.777; β-globin: $p = 0.286$ 95% CI: −0.1–0.336).

Consistent with the results of the *t* tests, analysis of the GLMs showed no statistical evidence for an association between any of the measurements of circulating DNA and any of the chromosomal abnormalities.

DISCUSSION

Since 1997, when fetal cfDNA in maternal circulation was discovered, a new field of research has emerged, driven by its potential clinical applications for non-invasive prenatal diagnosis. Quantitative aberrations of fetal cfDNA in maternal plasma were noticed in chromosomal aneuploidies, but conflicting results were reported among different studies. This is thought to be because of variables that might affect the level of circulating fetal cfDNA and the relatively small numbers of subjects studied.

One of the variables could be related to the timing of the plasma sampling. Previous studies have concentrated on second-trimester pregnancies by which time the invasive procedure (CVS) may have been carried out. This study concentrated on first-trimester pregnancies and the advantage is that the samples were obtained concurrently, just prior to the CVS, therefore giving the test prognostic value that can be employed prior to, or in place of any invasive procedure.

We found inverse correlation between total cfDNA circulating maternal plasma and NT measurement of the fetus ($r = -0.15$, $p = 0.001$), which, however, explains only 2.5% of the variability in cfDNA levels ($R^2 = 0.025$). To our knowledge, this is the first study that correlated NT with cfDNA levels.

Our results suggest that there are no differences in total cfDNA levels between 264 controls and 72 Down syndrome pregnancies (median of 341.3 and 345.3 GE/mL, respectively) and contradict previously reported results (median of 36 153 and 5832 GE/mL, respectively) (Spencer *et al*., 2003). Previous studies used serum and archived samples while in this study plasma was separated within 4 h of venepuncture and DNA was extracted from plasma within 2 months. Total cfDNA levels increase after 6 h of venepuncture when samples are left standing (Angert *et al*., 2003). This could explain the difference in the results.

Fetal cfDNA levels did not differ significantly between controls and trisomy 21 samples in this study, in agreement with previous results (Ohashi *et al*., 2001; Hromadnikova *et al*., 2002; Spencer *et al*., 2003). Other studies however, (Lo *et al*., 1999; Zhong *et al*., 2000; Lee *et al*., 2002) show a two-fold increase in trisomy 21 samples compared to controls. Again, the difference may be related to the age of the samples; we utilized fresh plasma samples as opposed to archived ones. Also, the number of cases analysed in this study is larger and the average gestation age is lower compared to the other studies. In first-trimester pregnancies, fetal cfDNA levels in the maternal circulation are very low.

Similarly, fetal cfDNA levels did not differ significantly between controls and trisomy 18 in agreement with all previously reported studies (Zhong *et al*., 2000; Ohashi *et al*., 2001; Wataganara *et al*., 2003). We did not find any significant differences in the levels of fetal cfDNA between controls and trisomy 13 fetuses, which differ from other publications (Zhong *et al*., 2000; Wataganara *et al*., 2003). However, we believe that the number of samples studied in total (four in our study, three in Zhong's, and five in Wataganara's) is too small to draw any conclusions. No relation was found in the levels of total cfDNA between normal and Turner's syndrome bearing pregnancies and any of the triploidy cases. Furthermore, we found a considerable degree of overlap between the values observed in trisomic and control fetuses.

In this study, we did find a small inverse correlation between fetal cfDNA (*DYS14*) and maternal weight ($r = -0.15$, $p = 0.02$) and more importantly with the BMI of the subjects ($r = -0.17$, $p = 0.01$) agreeing partly with previous findings. It was previously discussed that maternal weight is inversely correlated with the levels of free fetal DNA in the second-trimester pregnancies ($r = -0.26$, $p = 0.07$) but not in the first trimester (Wataganara *et al*., 2004). It is thought that since the levels of fetal cfDNA increase with GA, the effects are more pronounced in the second trimester, which could explain a lesser correlation in the first-trimester samples in this study.

A trend towards inverse correlation was also found between the levels of fetal cfDNA (*DYS14*) and storage time. However, our results did not reveal important changes due to storage time in general (small *r* value), similar to the results of Lee *et al*. (2002). Sozzi *et al*. (2005) showed that prolonged storage of both isolated DNA and whole plasma samples led to substantial DNA degradation. We did not notice any storage effect on our samples as on average, the analysis was done within 2 months of their collection.

It has been reported that smoking during pregnancy is associated with increased chromosomal instability in amniocytes (De la Chica *et al*., 2005). However, in our study, no significant difference was found between smoking and non-smoking groups and the fetal and total cfDNA.

In conclusion, we present here the largest study performed to date on prospectively collected samples on the quantification of fetal and total cfDNA in maternal circulation. Our results suggest that quantification of fetal cfDNA in first-trimester pregnancies is not an ideal prognostic marker for chromosomal abnormalities with the methods currently used. While the results may not be applicable to the broader population, this diagnostic test however, would normally be applied to pregnancies at higher risk of chromosomal abnormalities.

ACKNOWLEDGEMENTS

This study was supported by the Fetal Medicine Foundation.

REFERENCES

Angert RM, LeShane ES, Lo YM, Chan LY, Delli-Bovi LC, Bianchi DW. 2003. Fetal cell-free plasma DNA concentrations in maternal blood are stable 24 hours after collection: analysis of first- and third-trimester samples. *Clin Chem* **49**: 195–198.

De la Chica RA, Ribas I, Giraldo J, Egozcue J, Fuster C. 2005. Chromosomal instability in amniocytes from fetuses of mothers who smoke. *JAMA* **293**: 1212–1222.

Hromadnikova I, Houbova B, Hridelova D, *et al*. 2002. Quantitative analysis of DNA levels in maternal plasma in normal and Down syndrome pregnancies. *BMC Pregnancy Childbirth* **2**: 4.

Lee T, LeShane ES, Messerlian GM, *et al*. 2002. Down syndrome and cell-free fetal DNA in archived maternal serum. *Am J Obstet Gynecol* **187**: 1217–1221.

Lo YM, Tein MS, Lau TK, *et al*. 1998. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet* **62**: 768–775.

Lo YM, Lau TK, Zhang J, *et al*. 1999. Increased fetal DNA concentrations in the plasma of pregnant women carrying fetuses with trisomy 21. *Clin Chem* **45**: 1747–1751.

Ohashi Y, Miharu N, Honda H, Samura O, Ohama K. 2001. Quantitation of fetal DNA in maternal serum in normal and aneuploid pregnancies. *Hum Genet* **108**: 123–127.

Sozzi G, Roz L, Conte D, *et al*. 2005. Effects of prolonged storage of whole plasma or isolated plasma DNA on the results of circulating DNA quantification assays. *J Natl Cancer Inst* **97**: 1848–1850.

Spencer K, de Kok JB, Swinkels DW. 2003. Increased total cell-free DNA in the serum of pregnant women carrying a fetus affected by trisomy 21. *Prenat Diagn* **23**: 580–583.

Wataganara T, Peter I, Messerlian GM, Borgatta L, Bianchi DW. 2004. Inverse correlation between maternal weight and second trimester circulating cell-free fetal DNA levels. *Obstet Gynecol* **104**: 545–550.

Wataganara T, Metzenbauer M, Peter I, Johnson KL, Bianchi DW. 2005. Placental volume, as measured by 3-dimensional sonography and levels of maternal plasma cell-free fetal DNA. *Am J Obstet Gynecol* **193**: 496–500.

Wataganara T, LeShane ES, Farina A, *et al*. 2003. Maternal serum cell-free fetal DNA levels are increased in cases of trisomy 13 but not trisomy 18. *Hum Genet* **112**: 204–208.

Zhong XY, Burk MR, Troeger C, Jackson LR, Holzgreve W, Hahn S. 2000. Fetal DNA in maternal plasma is elevated in pregnancies with aneuploid fetuses. *Prenat Diagn* **20**: 795–798.

Zimmermann B, El-Sheikhah A, Nicolaides K, Holzgreve W, Hahn S. 2005. Optimized real-time quantitative PCR measurement of male fetal DNA in maternal plasma. *Clin Chem* **51**: 1598–1604.