Reproductive BioMedicine Online (2012) **25**, 508–520

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ARTICLE

Circulating trophoblastic cells provide genetic diagnosis in 63 fetuses at risk for cystic fibrosis or spinal muscular atrophy

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Abstract This study sought to determine whether a reliable non-invasive prenatal diagnosis (NI-PND) of cystic fibrosis (CF) or spinal muscular atrophy (SMA) can be achieved through analysis of circulating fetal trophoblastic cells (CFTC). The kinetics of CFTC circulation were also studied. CFTC were isolated by isolation by size of epithelial tumour/trophoblastic cells at 9–11 weeks of gestation, before chorionic villus sampling (CVS), from the blood of 63 pregnant women at 25% risk for having a child affected by either CF ($n = 32$) or SMA ($n = 31$). Collected cells were laser-microdissected, short tandem repeat-genotyped to determine fetal origin and blindly assessed for mutation analysis. CFTC were independently analysed weekly (4–12 weeks of gestation) in 14 women who achieved pregnancy following IVF. Diagnostic results were compared with those obtained by CVS. All seven CF and seven SMA pregnancies carrying an affected fetus were correctly identified as well as non-affected pregnancies. CFTC provided 100% diagnostic sensitivity (95% CI 76.8–100%) and specificity (95% CI 92.7–100%) in these 63 consecutive pregnancies at risk for CF or SMA. CFTC were found to circulate from 5 weeks of gestation and can be used to develop an early and reliable approach for NI-PND.

KEYWORDS: circulating fetal cells, cystic fibrosis, diagnostic accuracy, ISET, non-invasive prenatal diagnosis, spinal muscular atrophy

1472-6483/$ - see front matter
http://dx.doi.org/10.1016/j.rbmo.2012.08.002

Introduction

Definitive non-invasive prenatal genetic diagnosis (NI-PND) is a long-standing goal to avoid miscarriage related to invasive prenatal diagnostic procedures (Mujezinovic and Alfirevic, 2007). Initial advances involved recovery of intact fetal cells, usually erythroblasts, from maternal blood (Bianchi et al., 1990; Herzenberg et al., 1979; Holzgreve et al., 1992; Simpson and Elias, 1993). In the National Institute of Child Health and Development collaboration reported by Bianchi et al. (2002), the fluorescence-activated cell sorting and magnetic-activated cell sorting approaches used to isolate circulating fetal erythroid cells resulted in 74% sensitivity in detecting trisomy 21. However, the approach only targeted pregnant women carrying a male fetus and consistently obtaining results has not been possible (Bianchi et al., 2002), thus precluding clinical introduction. Other approaches have since been pursued and current emphasis has shifted to cell-free fetal DNA (Wright and Chitty, 2009). However, the amount of free fetal DNA in plasma is highly variable, with usually a low proportion of fetal free DNA (3.4–6.2%) (Lo et al., 1998). Furthermore, admixture of fetal DNA with the large majority of maternal DNA makes detection of fetal abnormalities technically complex. Thus, the recovery of intact fetal cells still has a unique potential to develop a reliable NI-PND method.

Previous studies has showed proofs of principle for the ability to isolate circulating fetal trophoblastic cells (CFTC) and use them for NI-PND of cystic fibrosis (CF) (Saker et al., 2006) and spinal muscular atrophy (SMA), in mothers with both male and female fetuses (Beroud et al., 2003). CFTC are consistently present in pregnant mothers and do not persist in maternal blood after pregnancy termination (Bianchi et al., 1996; P. Paterlini Brechot, unpublished results), making their use for NI-PND especially attractive.

The present work explores further the potential of CFTC for NI-PND. A prospective, blind validation study was conducted involving a non-invasive test versus chorionic villus sampling (CVS), targeting five or 10 CFTC per mother in consecutive couples at risk of CF or SMA. The kinetics of CFTC circulation were also studied in pregnant women after IVF using precise information of the date of conception. The goal was to assess the possibility of CFTC recovery providing a consistent early NI-PND test. The results show that the CFTC-based test, when compared with the CVS-based test, obtained 100% diagnostic sensitivity and specificity. Furthermore, it was demonstrated that CFTC circulate in maternal blood beginning at 5 weeks of gestation. Having verified the diagnostic accuracy of this method for early and reliable NI-PND of CF and SMA, by analogy the approach can be extended to many other genetic disorders.

Materials and methods

Pregnant women

Blood from two groups of pregnant women were studied. First, a validation study was performed involving 63 pregnant women (mean age 37.9 years) at risk for either CF (mean age 35.3 years), or SMA (mean age 40.5 years). Maternal blood was obtained at 9–11 weeks of gestation prior to CVS and any invasive prenatal genetic procedure. Some couples had a previously affected child. Except for pregnancy terminations, for reasons to be described, pregnancy complications were unusual. Maternal blood samples (20 ml) were collected in EDTA buffer. In each couple, 1 ml of paternal and 1 ml of maternal blood were reserved for parental genotyping. The at-risk groups of 32 CF and 31 SMA women represented a consecutive series recruited in the Necker Enfants Malades Hospital (Paris-France), thus mimicking circumstances under which a definitive NI-PND test would be offered clinically. Data concerning gestational ages are shown in **Tables 1** and **2**. During this study period, no other couples at risk for CF or SMA presented to this clinic.

In 26 of the 32 couples at risk for having a child affected by CF, both parents were F508del carriers; in five couples (nos. 11, 26–28, 31; **Table 1**) one parent was a F508del carrier whereas the other was not a carrier for F508del; in one couple (no. 12; **Table 1**) neither parent had F508del. It is known that other CF mutations existed and could be detected, but testing CFTC for other CF mutations was not necessary to obtain a diagnostic result in this series. In all 31 couples at risk for SMA, each parent carried the heterozygous deletion for SMN1. Thus, the affected fetus in each at-risk pregnancy should be homozygous for the SMA deletion.

Second, a blood sample was obtained weekly from 4 to 12 weeks of gestation from 14 women (nos. 1–14 **Figure 3;** mean age 38.4 years) who conceived by IVF, the goal being to determine the number of CFTC per ml and per gestational age. The 14 women undergoing IVF were recruited in the Antoine Béclère Hospital (Clamart-France). None were at increased risk for having a fetus affected by CF or SMA, or for any other genetic disease.

This study received the approval of both the Ethical Committee of Ile de France XI (approval reference number CCP02001, approval date 11 April 2002), and the local ethics committee. All women participating in this study gave written informed consent.

None of these subjects were among those previously reported by this study group during proof of principle studies (Beroud et al., 2003; Saker et al., 2006).

ISET and identification of CFTC

In ISET (isolation by size of epithelial tumour/trophoblastic cells; Beroud et al., 2003; Saker et al., 2006; Vona et al., 2002), circulating epithelial cells (including trophoblasts) are efficiently recovered on the basis of size, being larger than leukocytes (**Figure 1**). Within 4 h of collection in EDTA, each 10 ml of whole maternal blood is diluted 1:10 using a proprietary buffer that lyses erythrocytes and fixes any nucleated cells (Rarecells, Paris, France). Precisely 10 min thereafter, the solution is passed through a cartridge-containing filter under negative pressure (Rarecells). Cells smaller than 8 μm pass through the filter's calibrated pores, which are 8 μm in diameter. Larger cells remain on the filter, which can be stored at −20°C. Epithelial cells were microdis-

Table 1 Non-invasive-prenatal diagnosis for cystic fibrosis.

Couple no. (weeks of gestation)	*STR markers*	*Microdissected cells (n)*	*CFTC (n)*	*CFTC result for F508del (n)*			*Test results*	
				Homozygous	*Heterozygous*	*Without F508del*	*NI-PND*	*Invasive PND*
1 (11)[a]	D7S486, D16S539, D21S1435	14	7	0	5	0	C	C
2 (9)[a]	D7S486, D16S3018, D21S1437	12	6	0	5	0	C	C
3 (11)	D16S539, D21S1435, D21S1437	16	7	0	0	5	N	N
4 (10)	D16S3018, D21S1437, D21S1435	15	8	0	5	0	C	C
5 (10)	D16S539, D16S3018, D21S1435	14	6	0	0	5	N	N
6 (9)[a]	D7S480, D16S539, D16S3018	13	6	0	0	5	N	N
7 (11)	D16S539, D21S1437, D21S1435	19	9	0	5	0	C	C
8 (11)	D16S539, D16S3018, D21S1435	15	7	0	5	0	C	C
9 (10)[a]	D7S480, D16S3018, D21S1435	12	6	5	0	0	A	A
10 (9)[a]	D7S486, D7S490, D7S523	11	5	0	5	0	C	C
11 (10)[b]	D7S486, D21S1435, D21S1437	13	6	0	5	0	C	C
12 (10)[c]	D7S480, D7S486, D7S523	17	8	0	0	5	N	N
13 (11)[a]	D7S486, D21S1435, D16S3018	13	7	0	5	0	C	C
14 (11)[a]	D7S480, D21S1435, D21S1435	13	6	5	0	0	A	A
15 (11)	D7S486, D16S539, D21S1435	11	5	0	5	0	C	C
16 (10)[a]	D7S486, D16S539, D16S3018	15	7	5	0	0	A	A
17 (10)[a]	D16S539, D21S1437, D7S486	23	11	0	0	10	N	N
18 (11)	D16S3018, D21S1435, D1S1171	27	12	0	10	0	C	C
19 (10)[a]	D16S3018, D7S486, D5S615	24	11	0	10	0	C	C
20 (9)[a]	D16S3018, D5S816, D7S480	26	12	0	0	10	N	N
21 (11)	D16S539, D16S3018, D21S1435	21	10	0	10	0	C	C
22 (11)	D16S539, D21S1437, D5S637	24	11	0	10	0	C	C
23 (11)	D16S539, D21S1435, C272	21	10	0	10	0	C	C
24 (11)[a]	D7S480, D16S3018, D16S539	22	10	0	0	10	N	N
25 (11)	D16S539, D5S816, D21S1437	21	11	0	0	10	N	N
26 (10)[b]	D7S486, D16S539, D5S816	30	13	10	0	0	A	A
27 (9)[b]	D7S486, D7S490, D21S1437	23	11	0	0	10	N	N
28 (10)[b]	D7S523, D21S1435, D16S539	22	11	0	0	10	N	N
29 (11)[a]	D7S486, D16S539, D5S816	25	12	10	0	0	A	A
30 (11)	D21S1435, D7S490, D5S681	21	10	0	10	0	C	C
31 (11)[b]	D7S523, D21S1435, D7S490	26	12	10	0	0	A	A
32 (10)	D7S523, D21S1435, D7S490	27	12	10	0	0	A	A
Total CF		606	285	55	105	80		

A = affected; C = carrier; CFTC = circulating fetal trophoblastic cells; CVS = chorionic-villous sampling; N = normal; NI-PND = non-invasive prenatal diagnosis; STR = short tandem repeat.
[a]Cases redundantly tested by indirect diagnosis.
[b]One parent with non-F508del mutation.
[c]Both parents with non-F508del mutation.

Table 2 Non invasive-prenatal diagnosis for spinal muscular atrophy.

Couple no. (weeks of gestation)	*STR markers*	*Microdissected cells (n)*	*CFTC (n)*	*CFTC result (n)*		*Test results*	
				With gene deletion	*Without gene deletion*	*NI-PND*	*Invasive PND*
1 (11)	D16S539, D16S3018, D21S1435			0	5	NA	NA
2 (11)[a]	D7S480, D16S3018, D5S465	13	6	5	0	A	A
3 (10)	D7S486, D7S490, D7S523	11	5	0	5	NA	NA
4 (10)	D7S486, D21S1435, D21S1437	15	7	0	5	NA	NA
5 (9)[a]	D16S539, C272, D21S1435, D17S800	17	8	5	0	A	A
6 (9)	D16S539, D5S816, D1S1171	15	8	0	5	NA	NA
7 (9)	D21S1437, D17S800, D5S816	13	6	0	5	NA	NA
8 (11)	D16S539, D5S816, D5S1360	14	6	0	5	NA	NA
9 (11)	D16S539, D5S615, D21S1435	12	7	0	5	NA	NA
10 (10)	D7S523, D16S539, D5S681	11	5	0	5	NA	NA
11 (9)	D7S486, D16S539, D21S1435	19	9	5	0	A	A
12 (11)	D7S486, D16S3018, D21S1437	16	7	0	5	NA	NA
13 (10)	D5S637, D16S539, D5S816	14	6	5	0	A	A
14 (11)[a]	D16S539, D5S1360, C272	11	5	0	5	NA	NA
15 (11)	D16S539, D21S1437, D5S465	12	7	0	5	NA	NA
16 (9)[a]	D16S539, D5S615, D5S816	25	13	0	10	NA	NA
17 (11)[a]	D16S539, D5S346, D21S1435	24	11	0	10	NA	NA
18 (10)[a]	D7S523, D16S539, D5S681, D17S800	21	10	0	10	NA	NA
19 (10)[a]	D16S3018, D5S615, D17S800	25	12	0	10	NA	NA
20 (11)[a]	D16S539, D5S1360, D7S523	21	10	10	0	A	A
21 (11)	D16S539, D21S1435, D1S1171	27	12	0	10	NA	NA
22 (10)[a]	D16S539, D21S1437, D5S465	22	10	0	10	NA	NA
23 (10)[a]	D16S539, D5S681, D21S1435, D17S800	27	13	10	0	A	A
24 (10)[a]	D16S539, D5S816, D5S1360	27	12	10	0	A	A
25 (11)[a]	D16S539, D5S681, D5S637	23	12	0	10	NA	NA
26 (9)[a]	D21S1437, D17S800, C272	23	11	0	10	NA	NA
27 (11)	D16S539, D21S1437, D21S1435	22	10	0	10	NA	NA
28 (10)	D16S539, D21S1435, D1S1171, D7S486	21	10	0	10	NA	NA
29 (11)	D21S1435, D7S486, D17S800	24	10	0	10	NA	NA
30 (11)	D16S539, D21S1435, D16S3018	22	10	0	10	NA	NA
31 (11)	D21S1435, D1S1171, D7S486	21	10	0	10	NA	NA
Total SMA		586	276	50	185		

A = affected; C = carrier; CFTC = circulating fetal trophoblastic cells; CVS = chorionic-villous sampling; N = normal; NA = not affected; NI-PND = non-invasive prenatal diagnosis; STR = short tandem repeat.

[a]Cases redundantly tested by indirect diagnosis.

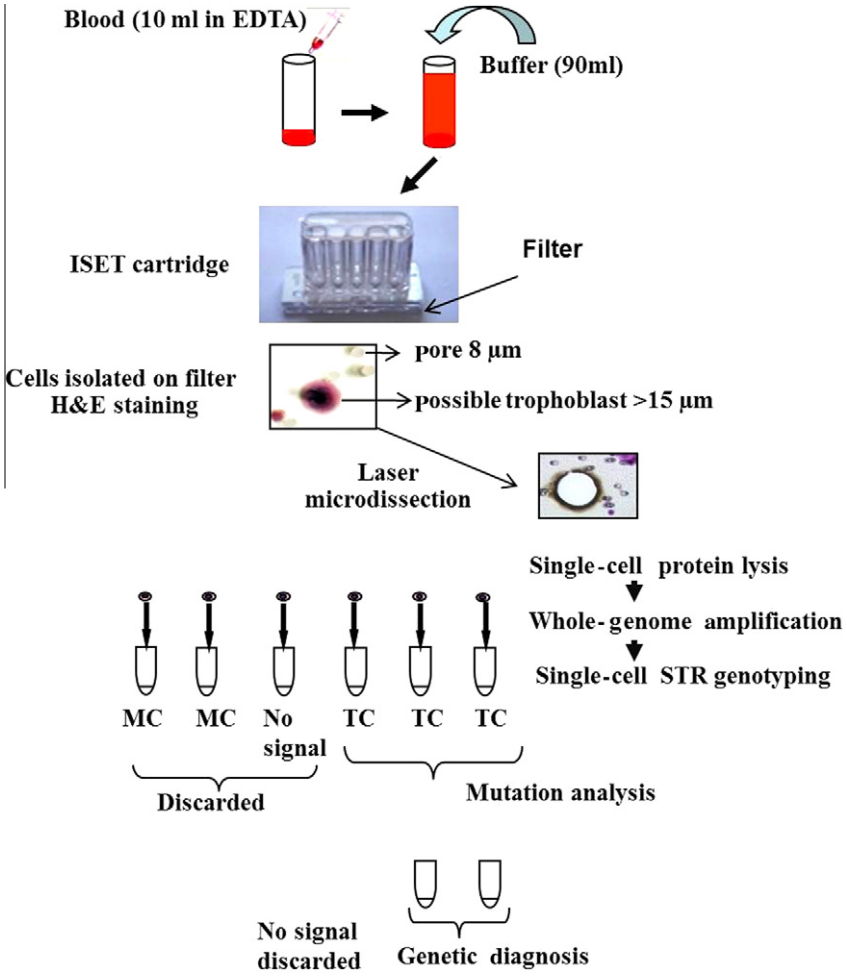

Figure 1 Protocol for non-invasive prenatal diagnosis through isolation by size of epithelial tumour/trophoblastic cells (ISET). Single-cell STR genotyping was performed using short tandem repeat (STR) markers, defined as informative by analysing paternal and maternal DNA extracted from blood. Thus fetal cells were identified through a paternity test. H and E = haematoxylin and eosin; MC, maternal cell; TC, trophoblastic cell. See the main text for details.

sected using a Nikon microscope (Zurich, Switzerland) with equipment and software from Molecular Machines and Industries (MMI, Glattbrugg, Switzerland). The filter was then placed in the microscope with cells facing downward and the laser directly cut the filter around the cell of interest to be microdissected. The back of the filter then adhered to the centre of the underside of the lid of the MMI tube, making it possible to expose the lysis buffer to the cell.

To target epithelial cells for laser microdissection, this study assessed cell size using CellCut software (Molecular Machines and Industries, MMI) and filter-calibrated pore size as a reference. A preliminary analysis to this study performed a retrospective study of 663 KL1-immunostained, microdissected and genotyped cells that were obtained from blood of 16 different pregnant mothers to investigate fetal status according to cell size and positivity or negativity of KL1 labelling. Among the 663 cells, 403 were KL1 positive; all the KL1-positive cells were >13 µm. Furthermore, results showed that one out of two cells ≥ 15 µm (all KL1 positive) had a fetal genotype (see Supplementary Material and Supplementary Figure 1, available online only). Thus, this study microdissected only cells ≥ 15 µm, avoiding KL1 labelling, as used in previous studies (Beroud et al., 2003; Saker et al., 2006; Vona et al., 2002), and assumed that cells ≥ 15 µm are all epithelial.

Genotyping

After ISET treatment and haematoxylin and eosin staining of filters, cells ≥ 15 µm were laser microdissected. This study then carried out protein lysis, whole-genome amplification by primer extension preamplification (PEP) (Zhang et al., 1992), short tandem repeat (STR) genotyping and genetic analysis, as previously described (Beroud et al., 2003; Saker et al., 2006; Vona et al., 2002) (**Figure 1**). For each couple, informative STR markers were identified by analysing paternal and maternal DNA extracted from blood. The informative STR markers were then used to genotype laser-microdissected cells and identify CFTC through a NI-PND paternity test. The informative STR used in this study are shown in **Tables 1** and **2**. STR genotyping was also used to count the number of CFTC per ml to determine the kinetics of appearance of CFTC in maternal blood in the 14 women of known gestation who became pregnant by IVF.

NI-PND sensitivity, specificity and reliability

The operating NI-PND protocol under which this prospective, consecutive series was conducted was set in 2004 and unchanged thereafter. The goal was assessing diagnostic accuracy in comparison to the gold standard reference invasive test (CVS). Invasive PND was obtained in all cases by CVS with results confirmed on post-natal infants or post-abortion fetuses. The protocol was implemented from 2004 to 2007 for NI-PND of SMA and from 2007 to 2009 for NI-PND of CF.

Comparison of NI-PND with invasive PND

In accordance with requirements of a diagnostic test (Sackett and Haynes, 2002), this study performed a blind comparison of the NI-PND results, expressing results as affected or non-affected fetus. In couples at risk for CF, this study also non-invasively determined the fetal carrier status. All results were compared with the reference invasive PND (CVS) approach. NI-PND results were compiled in the laboratory of INSERM Unit 807, Necker Faculty of Medicine, whereas invasive CVS results were made by completely different operators in the Laboratoire de Génétique Médicale in the Hopital Necker-Enfants Malades.

The non-invasive team of the principal investigator had no knowledge of results concurrently obtained by the invasive team, and vice-versa. The non-invasive team was aware only of the specific genetic disease to be diagnosed (CF or SMA) and the gestational age. Maintaining this firewall between the two teams kept the clinical trial blind. Unaware of results of invasive tests, the non-invasive team (H.M., A.S., P.P.B.) communicated its results at a single meeting to both the obstetrician (A.B.) and the geneticist (J.P.B.).

Non-invasive analysis of trophoblasts for fetal genetic diagnosis

This study used two genetic diagnostic approaches, depending on parental genotypes. In the direct approach, the exact mutation in an obligate heterozygote is known, in this series either F508del or SMN1 deletion. There is thus no requirement for the DNA from an index case (an affected individual born to the same couple). In the indirect approach, the gene mutation carried by one or both parents is, by contrast, unknown in this study, i.e. not F508del or SMN1 deletion. In this circumstance, DNA is required from an index case in order to establish phase for linkage analysis. Provided that the DNA from an index case is available, the indirect approach allows one to perform PND for both characterized as well as non-characterized mutations. Irrespective, it is not necessary to screen for other CF or SMN1 mutations if the proband is available.

Cystic fibrosis

For CF, direct diagnosis as defined above was performed in 26 cases in which both parents were F508del carriers (nos. 1—10, 13—25, 29, 30, 32; **Table 1**). Single-cell genomes shown to be fetal by STR genotyping were tested for the presence of F508del mutation (deletion of three nucleotides) as described (Saker et al., 2006).

Indirect diagnosis was necessary in six cases in which either one (nos. 11, 26—28, 31; **Table 1**) or both (no. 12; **Table 1**) parents were not heterozygous for F508del. A protocol for Fdel508 versus non-Fdel508 is also more generally applicable and amenable to inclusion of other mutations if needed. CFTC DNA and DNA of the index case were amplified using informative STR markers (D7S480, D7S486, D7S490, D7S523) on chromosome 7 (which carries the CFTR gene). This identified STR alleles segregating with the mutated CFTR gene. To validate the indirect approach, the same method was independently applied in selected cases in which both parents were F508del carriers and DNA of the index case was available (nos. 1, 2, 6, 9, 10, 13, 14, 16, 17, 19, 20, 24, 29; **Table 1**).

Spinal muscular atrophy

For SMA, the direct approach was applicable for all 31 cases because in each pregnancy both parents had a heterozygous deletion of SMN1. CFTC genomes were amplified with primers encompassing the *SMN1* exon 7 sequence, as described (Beroud et al., 2003). Because *SMN1* exon 7 differs from *SMN2* exon 7 by one nucleotide (codon 280: G in *SMN1* and A in *SMN2*), the loss of G/A heterozygosity in exon 7 in a CFTC confirms homozygous absence of *SMN1*, thus characterizing an affected fetus. Sequence analysis, in the context of a duplicated gene, can thus identify affected cases. However, this method does not distinguish heterozygous (carrier) from genetically normal fetuses.

Although unnecessary here for diagnostic purposes because in all pregnancies both parents genotyped heterozygous for the SMN1 deletion, this study validated the indirect approach that would be needed if this were not so. That is, the indirect approach would be required to achieve a diagnosis in couples in which one or both carry a mutation other than the typical deletion. DNA from an index case was available (nos. 2, 5, 14, 16—20, 22—26; **Table 2**). In these cases, CFTC DNA and DNA from an index case were amplified using informative STR markers (D5S346, D5S465, D5S615, D5S681, D5S816, D5S1360, C272) on chromosome 5 (which encodes the SMN gene) in order to identify the STR alleles segregating with the deleted or mutated SMN1 gene.

Controls for PCR specificity

Extensive controls for specificity are implemented in the study laboratory (Saker et al., 2006). In particular, a negative control (buffer without sample) is inserted for each tested sample at the lysis step and maintained for the duration of the assay. When performing laser microdissection, at least one microdissection from a portion of the filter not containing cells is always included; these blanks are run in parallel with samples as an additional control.

Statistical methods

Determining sensitivity and specificity

The diagnostic accuracy of NI-PND performed on CFTC was evaluated by computing the sensitivity (Se) and specificity

(Sp) to that achieved using invasive diagnosis as the gold standard. Confidence intervals at a 95% confidence level (CI) were estimated by the exact method implemented in the binom.test function of R (Brown et al., 2001) considering either the CFTC or the fetus as the statistical unit. Sensitivities and specificities for the two disease conditions were combined because both tests share the same molecular approach; only amplification primers at the diagnostic step change.

Determining cell-to-cell reliability

Reliability was determined on the basis of cell-to-cell replicability. In each pregnancy, 'blind' and independent analysis was performed on each of a cohort of either five or 10 CFTC from a given pregnancy. The likelihood of making the same mistake simultaneously on all five or all 10 CFTC from the same mother was computed by varying the error rate of the test and the correlation between CFTC evaluations from the same mother. Probabilities were estimated with the CorrBinom library of R, assuming a qpower distribution for correlated binary responses (Kuk, 2004).

Results

NI-PND of cystic fibrosis

A total of 32 pregnant women at risk for having a fetus with CF were tested before CVS at 9–11 weeks of gestation (**Table 1**, **Figure 2**). Results achieved blindly by the non-invasive approach were identical to those from villi obtained by CVS and analysed in the Laboratoire de Génétique Médicale in the Hopital Necker-Enfants Malades. In every subject, a NI-PND result was obtained. Clinical sensitivity and specificity were both 100% (Se 95% CI 59–100%, Sp 95% CI 86.3–100%).

CFTC were identified by STR genotyping using informative STR markers as defined based on preliminary STR genotyping of paternal and maternal DNA (**Figure 2**B). At the cellular level, in the first 16 pregnancies, five CFTC giving a result at the genetic diagnosis step were studied per mother; in the next 16, 10 CFTC giving a result at the genetic diagnosis step were studied per mother. As shown in **Table 1**, 606 cells were laser microdissected and 285 CFTC were identified by STR genotyping. Among the 285 CFTC, 240 (55 + 105 + 80) permitted genetic diagnosis of CF; the others were discarded because of PCR failure or other technical problems. As noted, either five or 10 of these CFTC were analysed per pregnancy. Results thus obtained through the analysis of 55 CFTC – 15 CFTC from three mothers having 5-cell analysis (nos. 9, 14, 16) and 40 CFTC from four mothers having 10-cell analysis (nos. 26, 29, 31, 32) – showed that seven women were carrying an affected fetus (homozygous F508del mutated alleles; **Figure 2**C, lower line). Results obtained through the analysis of 105 CFTC – 45 total CFTC from nine mothers (nos. 1, 2, 4, 7, 8, 10, 11, 13, 15) and 60 total from six mothers (nos. 18, 19, 21, 22, 23, 30) – showed that, among the other 25 women, 15 were carrying a non-affected carrier fetus (heterozygous F508del mutated allele; **Figure 2**C, upper line). Finally, results obtained through the analysis of 80 CFTC – 20 total CFTC from four mothers (nos. 3, 5, 6, 12) and 60 total from six mothers (nos. 17, 20, 24, 25, 27, 28) – showed that the other 10 mothers carried a completely normal fetus (homozygous normal alleles, without F508del mutation; **Figure 2**C, middle line).

Direct diagnosis was applied to all women except nos. 11, 12, 26, 27, 28 and 31; in these six pregnancies, at least one parent was a carrier of a mutation other than F508del. In these cases, genetic diagnosis could be obtained only by the indirect linkage analysis method (**Figure 2**C′). In addition to the obligatory indirect diagnosis for these six couples, DNA from an index case was available in women 1, 2, 6, 9, 10, 13, 14, 16, 17, 19, 20, 24 and 29 and used to verify replicability of the indirect approach.

A total of 42 CFTC – from nos. 17 (four CFTC), 18 (five CFTC), 20 (four CFTC), 24 (six CFTC), 25 (four CFTC), 26 (three CFTC), 29 (six CFTC), 31 (five CFTC) and 32 (five CFTC) – were tested twice for genetic diagnosis of CF, always giving identical results.

NI-PND of spinal muscular atrophy

A total of 31 pregnant women at risk for having a fetus affected by SMA were tested before CVS at 9–11 weeks of gestation (**Table 2**, **Figure 2**). Results achieved blindly by the non-invasive approach were identical to those obtained in the Laboratoire de Génétique Médicale in the Hopital Necker-Enfants Malades for CVS samples. In every subject, a NI-PND result was provided. At the clinical level, sensitivity and specificity were both 100% (Se 95% CI 59–100%, Sp 95% CI 85.8–100%).

CFTC were identified by STR genotyping using informative STR markers as defined based on preliminary STR genotyping of paternal and maternal DNA (**Figure 2**B). At the cellular level, in the first 15 pregnancies, five CFTC giving result at the genetic diagnosis step were studied per mother; in the next 16 women 10 CFTC giving result at the genetic diagnosis step were studied per mother. As shown in **Table 2**, 586 cells were laser microdissected and 276 CFTC were identified by STR genotyping. Among the 276 CFTC, 235 (50 + 185) allowed genetic diagnosis of SMA. Results obtained through the analysis of 50 CFTC – 20 total CFTC from four women having 5-cell analysis (nos. 2, 5, 11, 13) and 30 total from three women having 10-cell analysis (nos. 20, 23, 24) – showed that seven women were carrying an affected fetus. Results obtained through analysis of 185 CFTC – 55 CFTC from 11 mothers (nos. 1, 3, 4, 6–10, 12, 14, 15) and 130 from 13 mothers (nos. 16–19, 21, 22, 25–31) – showed that 24 women were carrying a non-affected fetus. Twelve of the 24 had a heterozygous fetus, established by the invasive approach and confirmed post pregnancy. Although direct diagnosis could be applied to all couples because both parents were carriers of heterozygous deletion of SMN1, indirect diagnosis was additionally validated in nos. 2, 5, 14, 16–20 and 22–26 given DNA from an index case being available (**Figure 2**D′).

Clinical outcome and accuracy: overall results

Women at risk for having a child with either SMA or CF were recruited, from 2004 to 2007 and from 2007 to 2009,

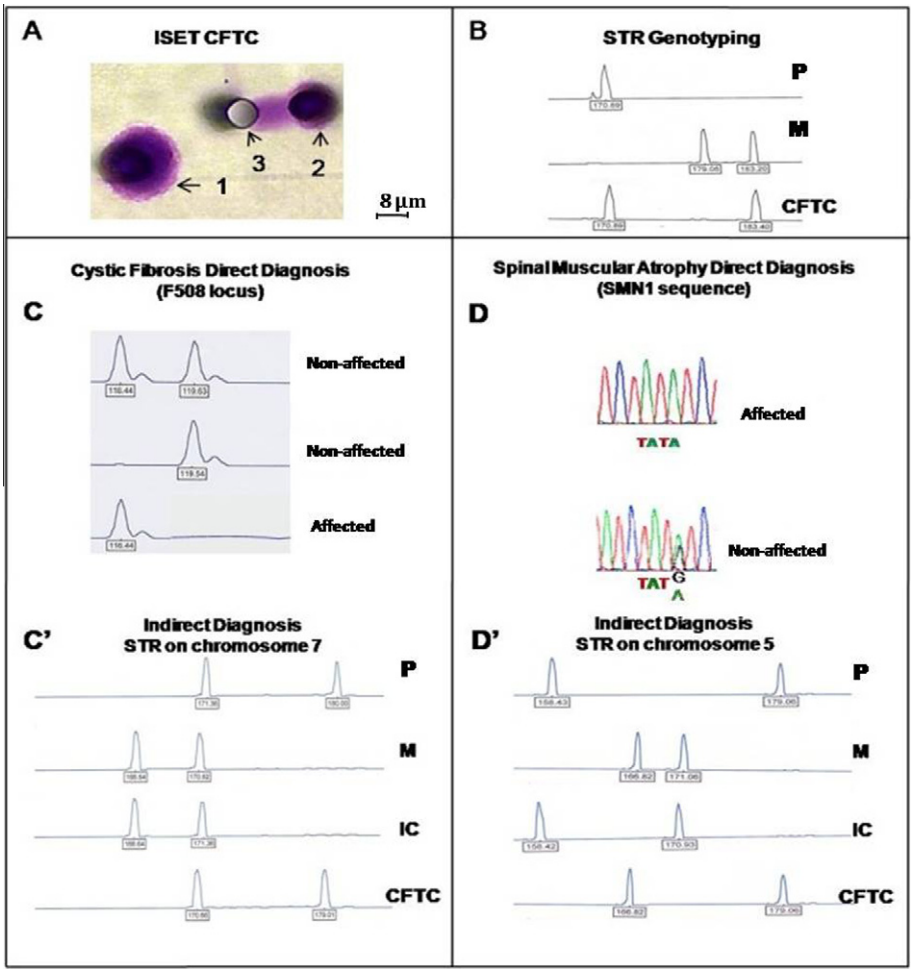

Figure 2 Non-invasive prenatal diagnosis of cystic fibrosis and spinal muscular atrophy: representative results. (A) Circulating fetal trophoblastic cell (CFTC) >15 µm obtained by isolation by size of epithelial tumour/trophoblastic cells (ISET) (arrow 1); by comparison, the filter pore size is 8 µm (arrow 3), similar to that of a lymphocyte (arrow 2); haematoxylin and eosin staining, original magnification 40×. (B) Short tandem repeat (STR) single-cell genotyping compared with the paternal (P) and maternal (M) allelic profiles, showing that the genotyped cell is fetal (CFTC) because it carries both paternal and maternal alleles. (C) Cystic fibrosis direct diagnosis through the amplification and fragment analysis of the F508 locus, showing the carrier (heterozygous) non-affected profile (mutated allele on the left and normal allele on the right), the normal profile (homozygous normal allele) and the affected profile (homozygous mutated allele). (C′) Cystic fibrosis indirect diagnosis through STR allele typing of chromosome 7; the index case (IC) genotyping shows the two STR alleles segregating with the CFTR mutated alleles, whereas the CFTC shows both a maternal normal allele and a paternal normal allele; the CFTC is thus derived from a normal fetus (see Methods). (D) Spinal muscular atrophy direct diagnosis through sequence analysis of the SMN gene; the affected profile shows a TATA sequence only, indicating homozygous loss of G and thus homozygous deletion of the SMN1 gene, whereas the non-affected profile shows a TATA/G sequence. (D′) Spinal muscular atrophy indirect diagnosis through STR allelotyping of chromosome 5; the IC shows the two STR alleles segregating with the SMN mutated alleles, whereas the CFTC shows both a maternal normal allele and a paternal normal allele. The CFTC is thus derived from a normal fetus.

respectively. All participants satisfied the inclusion criteria and underwent both non-invasive and invasive PND. The mean time-interval between the blood sample collection for NI-PND and the CVS was 3 h. On average, NI-PND diagnosis was completed in 3 days, while the invasive prenatal diagnosis was completed in 2 days. The NI-PND approach applied to the 63 pregnant women at risk of having a fetus affected by CF or SMA correctly identified in a blind protocol all 14 mothers carrying an affected fetus (**Tables 1** and **2**, **Figure 2**). Results achieved blindly by the non-invasive approach were identical to those obtained in the Laboratory of Medical Genetics after CVS. Clinical sensitivity and specificity were 100% (Se 95% CI 76.8—100%, Sp 95% CI 92.7—100%). This accuracy was achieved by laser-microdissecting 1192 cells, identifying by STR genotyping 561 CFTC.

Among 561 CFTC, 475 gave informative results for SMA or CF (**Tables 1** and **2**). The other 86 cells showed PCR failure, i.e. allele drop out (ADO) (Piyamongkol et al., 2003) or other technical vicissitudes like insufficient PEP product, and were discarded (**Figure 1**). A total of 105 CFTC was collectively used to show that 14 pregnant women were carry-

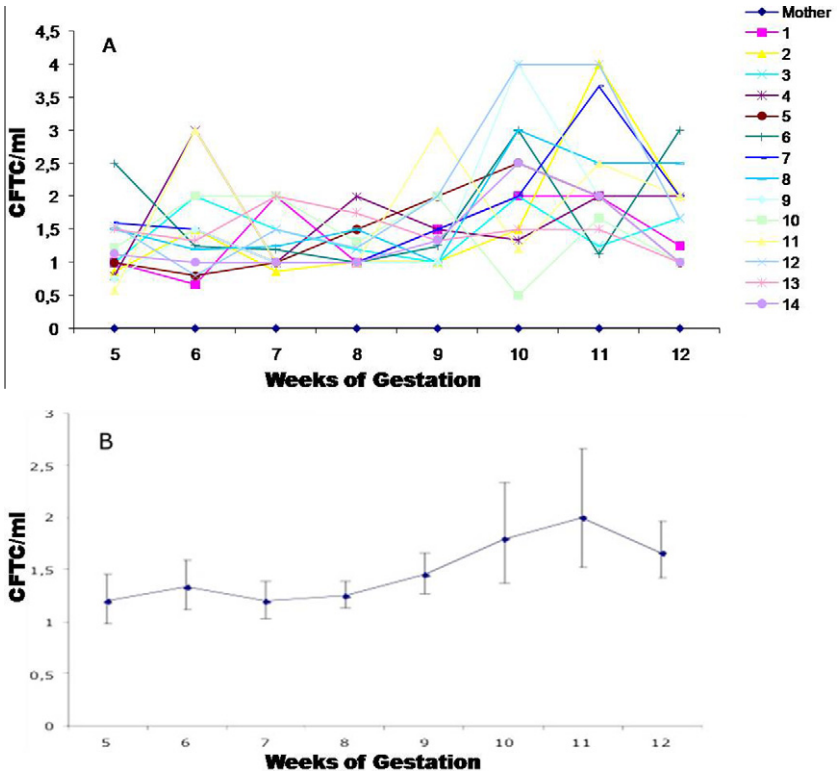

Figure 3 Kinetics of circulating fetal trophoblastic cells (CFTC) during the first trimester of pregnancy in 14 pregnant women who achieved pregnancy through IVF (nos. 1—14) from 5 to 12 weeks of gestation. (A) Number of CFTC per ml of blood. (B) Mean and 95% CI of CFTC number per ml of blood estimated by the Poisson (generalized estimating equations) model (Zeger and Liang, 1986).

ing an affected fetus (CF or SMA). Results obtained through the analysis of 370 CFTC showed that 49 other women were carrying a non-affected fetus. Direct diagnosis was performed in 57 couples; in six, indirect diagnosis was required. Indirect diagnosis was performed redundantly to the direct diagnosis in 26 other couples, giving identical results.

No variability in diagnostic accuracy was observed between the two groups of women, namely those at risk for CF and SMA. Test reproducibility was estimated to be 100%.

Effect of CFTC replications on overall reliability of the test

To assess the reliability of the test, this study computed the likelihood of making an error simultaneously on all replicated CFTC, considering that discordant results would lead to failure in the diagnostic process and to a non-conclusive call. This probability was computed both for five and 10 CFTC replicates, error rates from 4 to 20% and correlation between measures from 0 (independence) to 1 (perfect dependence). Dependence (correlation = 1) means that a possible error in a single test (one CFTC) would be accompanied by a similar error in the other CFTC in the same mother. When the correlation is total (i.e. equal to 1), the probability of simultaneous error when testing replicate CFTC approximates the individual CFTC testing error rate. Testing replicates is by definition useless in this case. Independence (correlation = 0) means that an error in a single test (one CFTC) does not modify the risk of error for the other CFTC from the same mother. In order to maximize 'independent' testing at the diagnostic step, replicate CFTC were tested for NI-PND and results were interpreted without knowledge of the CFTC origin (i.e. from a given couple). With the protocol of independence (correlation = 0) performed here, the test virtually will never arise to a global (diagnostic) mistake if five CFTC are tested and the error rate is limited to 1% ($P = 10^{-10}$). Performance is even better with 10 replicates (10 CFTC tested for NI-PND), with the test virtually never arising to a diagnostic mistake even given an error rate of 10% ($P = 10^{-10}$). In fact, probability of simultaneous error markedly increases with the correlation; for instance, with 10 replicates, a correlation of 0.5 and an error rate of 1%, the probability of simultaneous error is less than 1/1000.

Appearance of CFTC and kinetics in maternal blood

As described, the study utilized blood samples obtained weekly (4—12 weeks of gestation) from 14 women pregnant after IVF. DNA of collected cells was genotyped using three informative STR markers to identify and count the number of CFTC per ml at different gestational weeks. Mean number of CFTC collected by ISET was 1.61 per ml. Eight mothers (nos. 1—8) were tested at 4 weeks of gestation: a total of three CFTC per blood sample were found in four of these mothers (nos. 1, 3, 6, 7). CFTC were found in all 14 mothers followed from 5 to 12 weeks of gestation; CFTC counts per ml of blood increased from 1.19 (95% CI 0.98—1.45) at

5 weeks to 2.00 (95% CI 1.51—2.65) at 11 weeks of gestation. The observed decrease to 1.66 (95% CI 1.41—1.96) at 12 weeks of gestation was not significant compared with the CFTC number found at 11 weeks of gestation. Trend analysis using a coding of time by orthogonal polynomials showed a significant positive linear trend ($P = 1.7 \times 10^{-5}$), all other components being not significant except for the polynome of order 4 ($P = 0.038$). Overall, CFTC counts were significantly lower during the second month of gestation (1.24, 95% CI 1.15—1.34) in comparison with the third month (1.72, 95% CI 1.55—1.90) ($P = 2.8 \times 10^{-5}$).

The genotype of the live born child was available in eight of the 14 cases (nos. 5, 7—13; **Figure 3**). Each was identical to the corresponding CFTC. All samples were obtained prior to any prenatal diagnostic analysis.

Discussion

As far as is known, this study is the first to provide validation, by accepted criteria (Sackett and Haynes, 2002), of definitive NI-PND for cystic fibrosis and spinal muscular atrophy. In 63 consecutively studied at-risk women, all representative of couples traditionally presenting for prenatal genetic diagnosis, trophoblasts were recovered in all cases from maternal blood obtained prior to CVS. Results obtained from NI-PND were identical to that from chorionic villi (100% sensitivity and specificity). Clinical subjects were studied between 9—11 weeks of gestation. In a separate experiment involving 14 women, trophoblasts were recovered from 5 weeks of gestation onward, demonstrating that an early and non-invasive prenatal diagnosis of genetic diseases is feasible.

The ISET approach reported previously (Beroud et al., 2003; Saker et al., 2006) has been improved. Initially, KL1 immunolabelling of the enriched cells was required to identify epithelial cells that could be of fetal origin (Vona et al., 2002). The current study reports success in distinguishing circulating cells (potentially trophoblasts) after ISET solely on the basis of their size. In the retrospective study targeting 663 single cells microdissected and genotyped after KL1 labelling, all cells >13 μm were KL1 positive and one out of two cells ≥ 15 μm (all KL1 positive) were fetal in nature. Thus, the analysis was restricted to cells ≥ 15 μm in size, avoiding expensive and time-consuming immunolabelling with the KL1 antibody. This procedure also facilitates automated cell-size-based laser microdissection. Cells ≥ 15 μm, thus epithelial cells likely to be trophoblasts, were individually laser microdissected, STR genotyped to confirm fetal status and genotyped at the mutant locus if confirmed. Thus, trophoblastic cells were identified through a NI-PND paternity test. However, paternal DNA analysis may be avoided in the future as identification of fetal cells can be performed solely by comparing the STR profile of the microdissected single cell to the STR profile of maternal DNA extracted from blood using an established number of highly polymorphic STR markers, as used in forensic medicine.

Any genetic analysis relying on single cells can be adversely affected by loss of PCR signal from one of the two alleles (Allele Drop Out, ADO) (Piyamongkol et al., 2003). Presumably ADO is related to the lack of availability of one DNA strand to primers and/or to breakage of one or both DNA strands. In the current method, the integrity of single-cell DNA is tested at the genotyping step for fetal cell identification, using at least three informative markers. If, at this step, both maternal and paternal alleles are not identified, the cell is not analysed further because its DNA is considered not accessible to primers. When the single-cell DNA does prove fetal by genotyping, diagnostic analysis can be pursued. At this step, 86 of 561 CFTC were discarded because of PCR failure, insufficient PEP product or other technical problems. A successful strategy was reliably performed on 475 of 561 CFTC; 42 CFTC were tested twice and gave identical results. These data demonstrate that NI-PND is feasible for CF and SMA, even when the DNA from one or both parents is characterized by an unknown (here non-F508del) mutation requiring (indirect) linkage analysis. In other words, NI-PND should be feasible whenever invasive PND is feasible, including detection of uniparental isodisomy, intragenic perturbation and de-novo mutations. Protocols already in use for single-cell preimplantation genetic diagnosis can be readily adapted to cells isolated by ISET.

This study's protocol used molecular techniques that proved error free compared with results obtained by the gold standard, invasive procedure-based, diagnosis. Of special note is that a (correct) result was obtained in 63 consecutive cases without any exclusion or selection whatsoever. Several key issues are pivotal to the non-invasive approach: (i) it was systematically ascertained that the diagnostic analysis targeted pure fetal DNA, in cells with an intact nucleus, not fetal DNA mixed with maternal DNA, as is currently applied for cell-free fetal DNA analysis in maternal blood; (ii) the genetic analysis was performed on a single cell rather than pooled cells or tissues; and (iii) the protocol, based on independent replicative diagnoses using different CFTC obtained from the same blood sample, established guards against diagnostic errors due to ADO or human foibles (labelling, technical errors) or persistence of CFTC from a preceding or ignored pregnancy. The protocol provided replicate diagnostic analysis of multiple cells in a given sample, each showing the same genotyping (STR) profile for parentally transmitted STR and mutation analysis.

This NI-PND method appears at least as reliable as traditional invasive PND, in which genetic analysis is determined on the basis of DNA pooled from many cells. **Figure 4** shows that, when using 10 CFTC replicates, the protocol of independent diagnostic analysis (correlation = 0), virtually never gave rise to a global (clinical) mistake at an error rate of 10% ($P = 10^{-10}$, i.e. the probability of global diagnostic error rate is 1 in 10 billion). With five CFTC replicates, this extraordinary performance can be reached if the error rate is 1% or less.

The presence of trophoblasts in maternal blood was first reported in 1893 (Schmorl, 1893). Thereafter came the fundamental discovery that several different fetal cell types not only exist in the maternal circulation but may remain for decades (Bianchi et al., 1996). Researchers faced three technical challenges: (i) only erythroid and trophoblastic cells, among the four types of circulating fetal cells (Bianchi, 1998) (also including lymphoid and myeloid cells (Walknowska et al., 1969)), appear cleared from blood rapidly after delivery, thus being potential targets for

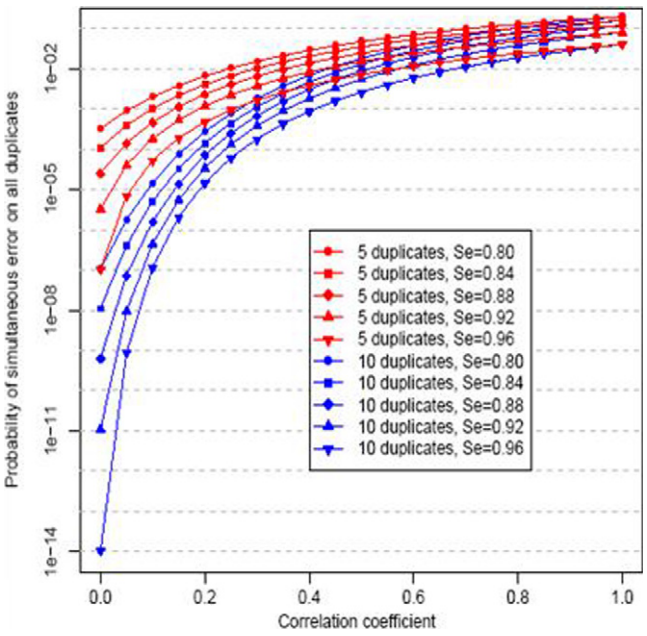

Figure 4 Effect of circulating fetal trophoblastic cell replicated tests on the overall reliability of the diagnostic procedure. Probability of simultaneous error on all replicates according to the number of replicates (5 or 10), the individual error rate (1%, 5%, 10%, 20%, 40%) and the correlation between measurements. A null correlation corresponds to the independence case (i.e. the error rate does not depend on the subject). When the correlation is equal to 1, the overall error rate is equal to the one individual as the replicates do not carry supplemental information.

NI-PND; (ii) circulating fetal cells are very rare, up to approximately one fetal cell per ml of blood (thus one cell admixed with an average of 10 million leukocytes and 5 billion erythrocytes); and (iii) genetic diagnosis of a fetal genome must avoid contamination by maternal genome in order to avoid false positive and false negative results. Culturing circulating fetal cells has also failed to bring a substantial advance in this field, as it has proved impossible to target cell proliferation stimuli specifically to fetal cells and in particular to target selectively circulating erythroid and trophoblastic fetal cells (Guetta et al., 2005). The current study reports 63 consecutive cases with results identical to those obtained by traditional PND (CVS).

Previous studies have addressed the issue of kinetics of fetal cell circulation and in particular the week of gestation when these cells consistently appear in maternal blood (Ariga et al., 2001; Bischoff et al., 2002; Lim et al., 2001; Oudejans et al., 2003; Shulman et al., 1998); however, the immunological approaches previously used to enrich and/or identify circulating trophoblastic cells suffer from specificity bias (Tjoa et al., 2007). The current study of 14 pregnant women of known gestation demonstrated by CFTC genotyping that CFTC circulate very early during pregnancy, consistently (14/14) from 5 weeks of gestation onwards. The results showed 1—2 CFTC per ml during the first 12 weeks of gestation and noticed that the mean numbers slightly decreased at 12 weeks of gestation, compared with the mean CFTC numbers found at 11 weeks of gestation, but the difference was not significant (**Figure 3**). However, mean CFTC counts were significantly lower during the second month of gestation as compared with the mean CFTC numbers found at 11 weeks of gestation. If confirmed by studying a higher number of pregnant women, this observation could be related to the fact that trophoblastic invasion is known to occur by two waves, the first completed by the late first trimester and the second completed by the early second trimester. Thus, the lower CFTC number in the second month of gestation could represent the lower level before the first wave.

Of considerable recent interest are data on NI-PND aneuploidy detection using cell-free fetal DNA in maternal blood. Lo et al. (1997, 1998) found that a variable amount (3.4—6.2%) of free DNA in maternal plasma was of fetal origin and thus potentially suitable for NI-PND. Circulating free fetal DNA (ffDNA) is already used clinically for prenatal diagnosis of fetal gender and Rh(D) status. However, until recently, difficulty in verifying ffDNA in pregnant women carrying a female fetus generated difficulty, hampering exclusion of a false-negative diagnosis. Use of ffDNA for aneuploidy testing is now more promising and commercially available. Several industrial and academic teams are engaged in extensive analyses. Despite impressive data accuracy, inability to obtain results in consecutive cases and without any type of sample selection (filtering data according to DNA quality control criteria, targeting undefined 'subset' groups or other exclusion criteria) still falls short of the essentially 100% informative and accurate results expected with an invasive procedure (CVS or amniocentesis) (Chiu et al., 2008, 2011; Ding et al., 2004; Fan et al., 2008; Lo et al., 2007; Ehrich et al., 2011; Papageorgiou et al., 2011; Chen et al., 2011; Tsui et al., 2011; Palomaki et al., 2012; Bianchi et al., 2012; Sparks et al., 2012). Perhaps 5—8% of cases result in non-informative results. Of course, this may improve and the assay could be repeated a week or more later.

Detecting fetal aneuploidy using a mixed maternal and ffDNA sample at present requires considerable technological effort, namely several steps of complexity and high costs. As an example of the principle employed, suppose the total number of chromosome 21 sequences in plasma cell-free DNA is 100 and the fetal DNA proportion is 5%. Ability to make a diagnosis of trisomic fetus depends on the technical ability to distinguish 100 (euploid) from 102.5 (trisomic) sequences of chromosome 21, a challenging goal. By contrast, with pure fetal DNA (from trophoblastic cells), one would merely need to distinguish 100 (euploid) sequences from 150 sequences (trisomies) of chromosome 21 in order to make a reliable diagnosis of a trisomic fetus. The task becomes even easier by pooling trophoblastic cells, generating more rapid results with lower likelihood of error. Using ffDNA alone, simultaneous diagnosis of aneuploidies and single-gene disorders (Lun et al., 2008) also seems unfeasible at present. Yet this could be achieved readily using pure fetal DNA from circulating trophoblasts. Finally, the optimal cytogenomic test is surely array comparative genomic hybridization, essentially precluded at present using free DNA. While studies on circulating trophoblastic cells have lately received less attention than studies targeting ffDNA, intact fetal cells thus remain valuable, if not preferable, for NI-PND. The ffDNA strategy is, however, expected to

be easier, cheaper and more rapid for detection of fetal genetic targets which are absent in the maternal DNA, such as male fetal gender (Y-specific) and RHD-positive genetic targets in RHD-negative mothers.

In conclusion, as far as is known, this study is the first to report a consecutive series in which NI-PND using intact trophoblastic cells was successfully applied to consecutive pregnancies at risk for monogenic disorders, here by illustration CF and SMA. Trophoblasts recovered from maternal blood prior to CVS yielded 100% clinical sensitivity and specificity. Every case generated a correct diagnosis. In 63 cases, this study correctly identified the 14 affected fetuses. Furthermore, this study has demonstrated that CFTC circulate in the blood from 5 weeks of gestation. Thus, early and reliable definitive NI-PND of genetic disorders using CFTC in maternal blood appears to be a short-term reachable goal.

Acknowledgements

The authors thank Colette Denolf for formatting the manuscript and the couples who participated in this study.
Funding: This work was supported by grants from INSERM (Institut National de Santé et Recherche Médicale), AFM (Association Française contre les Myopathies) and Anjou Mucoviscidose, ABCF, Mucoviscidose, Vaincre la Mucoviscidose and Université Paris Descartes.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.rbmo.2012.08.002.

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Declaration: P.P.B. is the inventor of the ISET method and co-inventor of the method for NI-PND with J.P.B. and A.S. The relevant patents belong to INSERM (Institut National de Santé et Recherche Médicale), Université Paris Descartes and AP-HP (Assistance Publique-Hopitaux de Paris). P.P.B. is scientific advisor of Rarecells and Rarecells US and holds stock in each company. J.L.S. is on the Scientific Advisory for Rare Cells and Novartis, for which he receives honoraria. A.S. has received research grants from INSERM and Vaincre la Mucoviscidose, H.M. has received research grants from INSERM, AFM (Association Française contre les Myopathies) and Anjou Mucoviscidose.

Received 14 March 2012; refereed 16 August 2012; accepted 16 August 2012.