thyroxine levels after controlled ovarian hyperstimulation. J Clin Endocrinol Metab 2000;85:545–8.
5. Oppenheimer JH. Role of plasma proteins in binding distribution, and metabolism of the thyroid hormones. N Engl J Med 1968;278:1153–62.
6. Ain KB, Mori Y, Refetoff S. Reduced clearance rate of thyroxine-binding globulin (TBG) with increased sialylation: a mechanism for estrogen-induced elevation of serum TBG concentration. J Clin Endocrinol Metab 1987;65: 689–96.
7. Zidan J, Rubenstein W. Effect of adjuvant tamoxifen therapy on thyroid function in postmenopausal women with breast cancer. Oncology 1999;56: 43–5.
8. Kostoglou-Athanassiou I, Ntalles K, Markopoulos C, Athanassiou P, Gogas J, Proukakis C. Thyroid function in postmenopausal women with breast cancer on tamoxifen. Eur J Gynecol Oncol 1998;19:150–4.
9. Delmas PD, Bjarnason NH, Mitlak BH, Ravoux AC, Shah AS, Huster WJ, et al. Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. N Engl J Med 1997;33:1641–7.
10. Prestwood KM, Gunness M, Muchmore DB, Lu Y, Wong M, Raisz LG. A comparison of the effects of raloxifene and estrogen on bone in postmenopausal women. J Clin Endocrinol Metab 2000;85:2197–202.
11. Johnston CC, Bjarnason NH, Cohen FJ, Shah A, Lindsay R, Mitlak BH, et al. Long-term effects of raloxifene on bone mineral density, bone turnover, and serum lipid levels in early postmenopausal women: three-year data from 2 double-blind, randomized, placebo-controlled trials. Arch Intern Med 2000; 160:3444–50.
12. Walsh BW, Kuller LH, Wild RA, Paul S, Farmer M, Lawrence JB, Shab AS, et al. Effects of raloxifene on serum lipids and coagulation factors in healthy postmenopausal women. JAMA l998;278:1445–51.
13. Cummings M, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, et al. The effect of raloxifene on the risk of breast cancer in postmenopausal women: results from the MORE randomized trial. JAMA 1999;281:2189–97.
14. Goldstein SR, Scheele WH, Rajagopalan SK, Wilkie JL, Walsh BW, Parsons AK. A 12-month comparative study of raloxifene, estrogen, and placebo on the postmenopausal endometrium. Obstet Gynecol 2000;95:95–103.
15. Tsai KS, Cheng WC, Chen CK, Sanches TV, Su CT, Chieng PU, et al. Effect of bone area on spine density in Chinese men and women in Taiwan. Bone 1997;21:547–51.
16. Walsh BW, Paul S, Wild RA, Dean RA, Tracy RP, Cox DA, et al. The effects of hormone replacement therapy and raloxifene on C-reactive protein and homocysteine in healthy postmenopausal women: a randomized, controlled trail. J Clin Endocrinol Metab 2000;85:214–8.

Large Amounts of Cell-free Fetal DNA Are Present in Amniotic Fluid, *Diana W. Bianchi,* Erik S. LeShane, and Janet M. Cowan* (Division of Genetics, Departments of Pediatrics and Obstetrics and Gynecology, New England Medical Center and Tufts University School of Medicine, Boston, MA 02111; * author for correspondence: fax 617-636-1469, e-mail Dbianchi@Lifespan.org)

Until recently, the focus of our research in the noninvasive diagnosis of Down syndrome was the genetic analysis of intact fetal nucleated erythrocytes from the circulation of pregnant women *(1)*. The initial step in the physical isolation of these target cells involves the layering of diluted maternal blood on a density gradient *(2)*. Before 1997, we routinely discarded the plasma layer of the density gradient, unaware of the fact that it could contain nucleic acids. In 1997, prompted by reports of large quantities of tumor-specific DNA sequences in the plasma and serum of cancer patients *(3–5)*, Lo et al. *(6)* demonstrated the presence of male fetal DNA sequences in the serum and plasma of pregnant women. Subsequently, this same group extended their observations by quantifying the fetal DNA in maternal plasma *(7)* and studying its kinetics and physiology *(8)*.

The detection and/or quantification of fetal DNA sequences in maternal plasma have been used for a variety of clinical applications, including diagnosis of gender, Rhesus D genotype, single gene disorders, aneuploidy, and preeclampsia [reviewed in Refs. *(9, 10)*]. More recently, it has also been suggested that the genetic material in the plasma consists of a continuum of intact and apoptotic cells as well as cell-free DNA *(11, 12)*. Fetal DNA, however, appears to exist predominantly in a cell-free form in the maternal plasma *(13)*. Despite advances in the clinical applications of this technology, to date not much is known about the tissue of origin of the cell-free fetal DNA and how it is metabolized in the pregnant woman.

Botezatu et al. *(14)* demonstrated that male DNA sequences could be found in the urine of women who received a blood transfusion from a male donor, as well as women who were pregnant with a male fetus. The mechanism of DNA transfer across the kidney was unknown. Other groups have not yet replicated these findings in the literature.

To understand the metabolism of fetal DNA in the mother, we decided to examine amniotic fluid because at 16–20 weeks of gestation it is in transition from being identical to fetal plasma to being composed of fetal urine *(15)*. We hypothesized that demonstration of the existence of cell-free fetal DNA in amniotic fluid might provide a clue to the tissue source of DNA in the maternal plasma, as well as lead to novel clinical applications.

This study was performed with approval from the hospital's Institutional Review Board. Thirty-eight coded frozen discarded amniotic fluid specimens were obtained from the Cytogenetics Laboratory. All samples were collected for routine indications, such as advanced maternal age, abnormal maternal serum screening results, or detection of a fetal sonographic abnormality. The standard protocol in the Cytogenetics Laboratory is, upon receipt, to centrifuge the amniotic fluid sample at 350*g* for 10 min in a bench-top centrifuge, place the cell pellet in tissue culture, assay an aliquot of the fluid for α-fetoprotein and acetyl cholinesterase, and store the remainder at −20 °C as a back-up in case of assay failure. After 6 months, the frozen amniotic fluid supernatant samples usually are discarded. Results of the fetal karyotype were revealed only after the study was completed.

The frozen amniotic fluid samples were initially thawed at 37 °C and then vortex-mixed for 15 s. An aliquot of 500 μL of fluid was centrifuged at 13 500*g* in a microcentrifuge to remove any remaining cells. A final volume of 400 μL of the supernatant was used for extraction of DNA. Extraction was performed with the QIAamp Blood Kit (Qiagen) using the "Blood and Body Fluid" protocol as described by the manufacturer. The DNA was eluted into a final volume of 100 μL. Aerosol-resistant tips were used throughout the protocol to prevent contamination with exogenous DNA.

Real-time quantitative PCR analysis was performed using a Perkin-Elmer Applied Biosystems (PE-ABI) 7700 Sequence Detector. Analysis was based on the 5′-to-3′ exonuclease

activity of the Taq DNA polymerase, using the *FCY* locus as a basis for detecting male DNA if the fetus was male. The *FCY* primers were derived from the Y-chromosome-specific sequence, Y49a (DYS1) *(16)*. The *FCY* amplification system consisted of the amplification primers *FCY*-F (5′-TCCTGCTTATCCAAATTCACCAT-3′), *FCY*-R (5′-ACTTCCCTCTGACATTACCTGATAATTG-3′), and a dual-labeled fluorescent TaqMan probe *FCY*-T (5′-FAM-AAGTCGCCACTGGATATCAGTTCCCTTGT-TAMRA-3′). The β-globin gene was used to confirm the presence of DNA and estimate its overall concentration.

Amplification reactions were set up as described previously by Lo et al. *(7)*, except that each primer was used at 100 nmol/L and the probe was used at 50 nmol/L. Amplification data were collected by the 7700 Sequence Detector and analyzed using the Sequence Detection System software, Ver. 1.6.3 (PE-ABI). Each sample was run in quadruplicate with the mean results of the four reactions used for further calculations. An amplification calibration curve was created using titrated purified male DNA. The extractions and subsequent quantitative assays were performed twice for each sample, with the mean of the two results used for final analysis.

The mean gestational age of the pregnancies in which the fluids were obtained was 16 weeks, 6 days (range, 14 weeks, 3 days to 20 weeks). In one sample, the gestational age was not recorded. In 21 samples, the fetal karyotype was 46,XX; in 15 samples, the karyotype was 46,XY; and in 2 samples, the karyotype was 47,XY,+21. The actual amounts of total and fetal DNA detected in the 38 samples are shown in Table 1. The mean amount of β-globin DNA detected was 3427 genome-equivalents (GE)/mL (range, 293–15 786 GE/mL). There was no correlation between gestational age and the total amount of DNA detected. In the female fetuses, 0 GE/mL of Y DNA was detected in the amniotic fluid. The mean amount of male DNA detected in male fetuses was 2668 GE/mL (range, 228–12 663 GE/mL). Linear regression analysis showed a correlation between fetal DNA and gestational age ($r = 0.6225$; $P = 0.0231$). In all 38 cases, the predicted fetal gender was correct. These results are statistically significant ($P < 0.0001$, Fisher's exact test). In the two cases of fetal Down syndrome, there was no increase in the amount of fetal DNA compared with the karyotypically normal male fetuses.

Amniotic fluid is obtained via an invasive procedure, but it represents another source of material for the study of cell-free fetal DNA production and clearance. We have demonstrated the presence of large quantities of cell-free fetal DNA in stored amniotic fluid supernatant samples. There is ~100- to 200-fold more fetal DNA per milliliter of amniotic fluid compared with maternal plasma *(7)*. This higher amount may permit new studies and clinical applications to be performed on this typically discarded material.

Although gender prediction was 100% correct, there was no evidence of increased fetal DNA in the two fetuses with trisomy 21. We previously hypothesized that mothers carrying fetuses with Down syndrome have increased amounts of fetal DNA in their plasma because of placental abnormality *(17)*. If the source of fetal DNA in the amniotic fluid were placental, one would expect to observe more fetal DNA in amniotic fluid from Down syndrome pregnancies. This suggests that either our original hypothesis was incorrect or that the source of fetal DNA in the amniotic fluid is not placental.

In some cases, there was a discrepancy between the amounts of fetal and total DNA detected. A possible explanation for the detection of more copies of *FCY* than β-globin sequence per milliliter is that some men may have more than one copy of *FCY* present in their genome.

Table 1. Absolute amounts of fetal DNA amplified from amniotic fluid supernatant samples.

Study ID[a]	Gestational age[b]	Total DNA (β-globin), GE/mL	Fetal DNA (*FCY*), GE/mL	Fetal karyotype
921	15	375	0	46,XX
922	16 5/7	2973	1817	46,XY
923	17	1128	1770	46,XY
928	18 3/7	15786	0	46,XX
929	15	699	600	47,XY,+21
930	16	859	0	46,XX
942	17 5/7	4968	0	46,XX
943	18	1562	2370	46,XY
944	17	4943	0	46,XX
945	20	5068	0	46,XX
946	17 3/7	2787	0	46,XX
951	17	3330	3405	47,XY,+21
953	14 3/7	2637	2338	46,XY
956	16	1782	1833	46,XY
957	16	2746	2528	46,XY
958	16	1943	2588	46,XY
959	15 3/7	293	228	46,XY
974	17	551	861	46,XY
986	17 4/7	3337	0	46,XX
987	16 4/7	599	502	46,XY
989	16	1299	0	46,XX
999	18	11231	0	46,XX
1001	17 1/7	9120	0	46,XX
1002	16 4/7	2703	0	46,XX
1003	15 5/7	373	0	46,XX
1005	18	5553	6121	46,XY
1006	16 5/7	2204	2059	46,XY
1012	15	494	0	46,XX
1017	NA	2124	2749	46,XY
1018	16 6/7	2576	0	46,XX
1021	18 4/7	3411	0	46,XX
1022	17	1642	0	46,XX
1025	16 5/7	415	0	46,XX
1029	19	14741	0	46,XX
1030	16 6/7	440	0	46,XX
1031	18 5/7	11892	12663	46,XY
1032	16 1/7	461	932	46,XY
1034	16 2/7	1214	0	46,XX

[a] ID, identification; NA, not available.
[b] Given in weeks and fractions of weeks.

The fact that, in a few cases, more β-globin than *FCY* sequences were detected was unexpected. At the present time, however, we think it is unlikely that significant amounts of maternal cell-free DNA are present in the amniotic fluid.

The fetal DNA in the amniotic fluid is either excreted by the fetal kidneys, locally degraded, or permeates through the nonkeratinized fetal skin. If excreted, it may be that the relatively immature fetal kidney (at 16–18 weeks) functions differently than the adult pregnant female kidney. If it originates from local degradation, potential sources of apoptosis and DNA liberation include the fetal kidney, blood cells, and direct degradation of placenta or membranes, skin, or other organs undergoing apoptosis with direct contact with the amniotic fluid. One example is the lung, which is actively developing and remodeling during the second trimester, and fetal lung fluid is in direct contact with amniotic fluid.

Although the present study adds another piece to the puzzle, our findings do not solve the mystery of how the pregnant woman clears the fetal DNA from her circulation. In fact, it raises more questions, such as how the fetus eliminates the DNA from its amniotic cavity. Does the free DNA cross the placenta, or is it swallowed and degraded in the fetal intestines? Future studies will attempt to identify the specific tissue of origin of the fetal DNA in amniotic fluid and track its metabolism and/or transfer into the circulation of the pregnant woman.

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References

1. Bianchi DW. Fetal cells in the maternal circulation: feasibility for prenatal diagnosis. Br J Haematol 1999;105:574–83.
2. Zheng YL, DeMaria M, Zhen D, Vadnais TJ, Bianchi DW. Flow-sorting of fetal erythroblasts using intracytoplasmic anti-fetal haemoglobin: preliminary observations on maternal samples. Prenat Diagn 1995;15:897–906.
3. Chen XQ, Stroun M, Magnenat JL, Nicod LP, Kurt AM, Lyautey J, et al. Microsatellite alterations in plasma DNA of small cell lung cancer patients. Nat Med 1996;2:1033–5.
4. Nawroz H, Koch W, Anker P, Stroun M, Sidransky D. Microsatellite alterations in serum DNA of head and neck cancer patients. Nat Med 1996;2:1035–7.
5. Stroun M, Anker P, Maurice P, Gaban PB. Circulating nucleic acids in higher organisms. Int Rev Cytol 1977;51:1–48.
6. Lo YMD, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CWG, Wainscoat JS. Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350:485–7.
7. Lo YMD, Tein MSC, Lau TK, Haines CJ, Leung TN, Poon PMK, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet 1998;62:768–75.
8. Lo YMD, Zhang J, Leung TN, Lau TK, Chang AMZ, Hjelm NM. Rapid clearance of fetal DNA from maternal plasma. Am J Hum Genet 1999;64:218–24.
9. Pertl B, Bianchi DW. Fetal DNA in maternal plasma: emerging clinical applications. Obstet Gynecol 2001;in press.
10. Lo YMD. Fetal DNA in maternal plasma: biology and diagnostic applications. Clin Chem 2000;46:1903–6.
11. Van Wijk IJ, de Hoon AC, Jurhawan R, Tjoa ML, Griffioen S, Mulders MAM, et al. Detection of apoptotic fetal cells in plasma of pregnant women. Clin Chem 2000;46:729–31.
12. Poon LLM, Leung TN, Lau TK, Lo YMD. Prenatal detection of fetal Down's syndrome from maternal plasma. Lancet 2000;356:1819–20.
13. Lo YMD. Studies on bi-directional DNA trafficking. Abstracts of the 12th Fetal Cell Workshop, Prague, May 12–13, 2001.
14. Botezatu I, Serdyuk O, Potapova G, Shelepov V, Alechina R, Molyaka Y, et al. Genetic analysis of DNA excreted in urine: a new approach for detecting specific genomic DNA sequences from cells dying in an organism. Clin Chem 2000;46:1078–84.
15. Fox H. Development and structure of the placenta and fetal membranes; formation of the liquor amnii. In: Philipp E, Setchell M, Ginsberg J, eds. Scientific foundations of obstetrics and gynaecology, 4th ed. Oxford: Butterworth Heinemann, 1991:287–98.
16. Lucotte G, Fabrice D, Mariotti M. Nucleotide sequence of p49a, a genomic Y-specific probe with potential utilization in sex determination. Mol Cell Probes 1991;5:359–63.
17. Lo YMD, Lau TK, Zhang J, Leung TN, Chang AMZ, Hjelm NM, et al. Increased fetal DNA concentrations in the plasma of pregnant women carrying fetuses with trisomy 21. Clin Chem 1999;45:1747–51.