Cell-free fetal DNA is not present in plasma of nonpregnant mothers

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As noted in our report *(1)*, in 25 of 55 female samples testosterone was <2.06 nmol/L by ID/GC-MS. These women could be similar to the female patients of Torjesen and Sandnes. The testosterone concentrations measured by the AutoDelfia system were clearly overestimated, as shown in Fig. 3 of our report *(1)*. In addition, in 42 of 55 females, testosterone was lower than the upper limit of the ID/GC-MS range (0.50–2.55 nmol/L) as measured in normally menstruating women 19–35 years of age with no evidence of hirsutism, acne, or alopecia and taking no oral contraceptives for at least 6 months before being tested. Hyperandrogenism has been diagnosed in 24 of these 42 females based on an increase in at least one serum androgen concentration at baseline: dehydroepiandrosterone sulfate >9.50 μmol/L; 17-hydroxypregnenolone >11.50 nmol/L; dehydroepiandrosterone >38.00 nmol/L; or androstenedione >7.85 nmol/L. Eighteen of 42 were "normoandrogenic" females. These 42 females had 17-hydroxyprogesterone concentrations within the reference interval. Mean (SD) testosterone measured by ID/GC-MS was 1.37 (0.56) nmol/L in normoandrogenic females and 1.93 (0.33) nmol/L in females with hyperandrogenism, whereas it was 2.94 (1.77) and 5.30 (1.74) nmol/L, respectively, for these two groups when measured by AutoDelfia. One would expect that if some particular interfering substances were present in the female samples there would be an intriguing individual serum response in some, if not all, of the direct assays tested, but we did not observe this.

AutoDelfia with Immulite 2000 gives the highest mean testosterone values, widely dispersed results, and the highest overestimation, as shown in Fig. 3 of our report *(1)*. This observation confirmed a previous report *(3)* in which serum pools were tested. The use of pools is more favorable to assay performance because interferences in individual samples are diluted. In that study, the testosterone concentrations in the two female pools tested by ID/GC-MS were 0.74 and 2.67 nmol/L, whereas they were 1.99 and 4.99 nmol/L, respectively, when measured by AutoDelfia (differences of 169% and 87%). In a dilution test, AutoDelfia results were high by 13–176% *(3)*.

We accept the possibility of a change in the assay's reagents between our two studies. Many relevant reports have highlighted interferences in immunoassays *(4–7)*. Interfering substances and factors relating to blood collection *(8)* have been identified by the manufacturers and considered limitations of the assay procedure. We think that most of the sources of errors involved in direct steroid assays are directly related to the assay format, such as the matrix *(9)*, the preparation and the purity of the labeled molecule, the specificity of the antibody, the flexibility of the labeled analyte–antibody complex, and assay optimization.

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Cell-Free Fetal DNA Is Not Present in Plasma of Nonpregnant Mothers

To the Editor:

Fetal DNA sequences are present in the plasma of pregnant women *(1)* and can be studied to determine fetal sex *(1)* and RhD type *(2)*. Unlike fetal cells, fetal cell-free DNA is cleared rapidly from plasma after delivery *(3)*, with a half-life of 16–28 min. Even when clearance is slowed, as in preeclampsia *(4)*, the mean half-life for clearance of fetal DNA is 114 min *(5)*.

Recently, Invernizzi et al. *(6)* reported positive PCR results for a Y-chromosome-specific sequence in 36 of 160 (22%) women who had given birth to a son up to several years previously. By contrast, other published studies on pregnant women have described no false-positive results *(1, 2, 7–14)*. It is conceivable that fetal cells remain and proliferate in the maternal circulation or are engrafted in maternal organs after delivery and that this proliferation is suppressed again in a subsequent pregnancy. Lambert et al. *(15)* also recently reported the presence of fetal DNA in plasma of nonpregnant women. They also found male DNA sequences in 8 of 22 (36%) healthy nonpregnant women who had previously given birth to sons, but filtration studies showed that this DNA was not cell free.

We recruited 120 nonpregnant women who, with their consent, do-

nated 10 mL of blood. We recorded the numbers and sexes of their children and numbers of abortions and extra-uterine pregnancies. Blood was collected in an EDTA-containing Vacutainer Tube and sent to the Central Laboratory of the Dutch Red Cross in Amsterdam, where samples were processed within 24 h. Each sample was centrifuged at 1289*g* for 10 min. The supernatant was collected and centrifuged again at 2773*g* for 20 min and stored at −30 °C or processed immediately. DNA was extracted from 1.0 mL of plasma by use of the Magna Pure LC (Roche) with the "Total Nucleic Acid Large Volume" reagent set (Roche Biochemicals) according to the manufacturer's instructions. As a control in the fetal DNA isolation, we included a *SRY*- or *RHD*-positive plasma from a pregnant woman in each run. The isolated DNA was eluted in 50 μL.

Within 8 h after isolation the DNA was automatically dispensed into a 96-well plate by the Magna Pure LC. Real-time quantitative PCR (ABI Prism 7700; Applied Biosystems) was used for amplification of *SRY* and *albumin* gene sequences. PCR for *SRY* used the primers SRY-109F (5′-TGG CGA TTA AGT CAA ATT CGC-3′) and SRY-245R (5′-CCC CCT AGT ACC CTG ACA ATG TAT T-3′) and the probe SRY-142T (5′-FAM-AGC AGT AGA GCA GTC AGG GAG GCA GA-TAMRA-3′), where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine. As a control, we amplified part of the *albumin* gene with primers Alb-F (5′-TGA AAC ATA CGT TCC CAA AGA GTT T-3′) and Alb-R (5′-CTC TCC TTC TCA GAA AGT GTG CAT AT-3′) and the probe Alb-T72 (5′-FAM-TGC TGA AAC ATT CAC CTT CCA TGC AGA-TAMRA-3′). The *SRY* PCR was performed in triplicate and the *albumin* PCR in duplicate.

Reaction mixtures of 50 μL contained 300 nM each of the primers, 100 nM probe, 25 μL of TaqMan Universal Master Mix (Applied Biosystems), and 10 and 5 μL of the eluted DNA samples for the *SRY* and *albumin* PCRs, respectively. Thermocycling was 2 min at 50 °C, 10 min at 95 °C, and then 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Our real-time *SRY* PCR was identical to the one used by Invernizzi et al. *(6)* as first described by Lo et al. *(16)*. The number of copies used for conversion to genome (cell)-equivalents was 6.6 pg/cell. This number represents a minimum concentration because it does not allow for losses of DNA during extraction. Amplification data were analyzed with ABI Prism 7000 SDS software (Applied Biosystems).

In 192 RhD-negative pregnant women at 16–18 weeks of gestation, all *RHD* genotyping results agreed with cord blood serology and/or amniotic fluid genotyping. Sixty-nine of these women carried a RhD-negative child. Serial dilution studies showed that both the *RHD* and *SRY* PCRs could detect 1 genome-equivalent. At least 16 of the women carrying a RhD-negative child previously had given birth to a RhD-positive child, and 24 had previously had miscarriages. The absence of false-positive results in this group argues against the persistence of cell-free fetal DNA from previous pregnancies.

Of the 120 women, 77 had given birth to children and 43 had not. Of the 43 childless women, 5 had previously had a spontaneous abortion, extra-uterine pregnancy, or first-trimester termination of pregnancy. Sixty-four women had previously delivered one or more boys. The mean age of the youngest son was 13.8 years (range, 1–41 years), and the mean age of all participating women was 43 years (range, 22–73 years). The mean (SD) age of women who previously delivered one or more sons was comparable [44 (9) years] to the mean age of women of the control group without children or with only daughters [41 (10) years]. No *SRY* sequences were amplified in 192 PCRs on DNA isolated from plasma of 64 nonpregnant mothers of sons, nor from the plasma of the other 56 nonpregnant women.

Our results contrast with those of Invernizzi et al. *(6)* and Lambert et al. *(15)* but are in full concordance with those of Smid et al. *(17)* and Benachi et al. *(18)*, who detected no *SRY* sequences in 70 and 30 nonpregnant mothers of sons, respectively. In pregnant women with previous sons and currently carrying a girl, no *SRY* was detected in 47 and 67 cases, respectively. These authors hypothesized that the centrifugation protocol of Invernizzi et al. *(6)* led to fetal *SRY* amplification originating from fetal cells remaining in the supernatant after centrifugation. Lo et al. *(16)* centrifuged the samples at 3000*g* and then centrifuged the resulting supernatants at 3000*g*. Smid et al. *(17)* centrifuged the sample at 1600*g* and then centrifuged the resulting supernatants at maximum speed: 16 000*g*. Benachi et al. *(18)* used serum that was obtained after centrifugation of the clotted blood sample once at 3000*g*. Invernizzi et al. *(6)* centrifuged the blood samples only once at 3000*g* and omitted the second centrifugation of the plasma. Lambert et al. *(15)* also gently centrifuged the plasma only once at 400*g*.

We tested the hypothesis that gentle centrifugation influences fetal DNA detection in nonpregnant women. Blood samples from 18 women who had previously delivered at least one boy were centrifuged at 1606*g* for 5 min instead of 10 min, and the second centrifugation was omitted. The remainder of the procedure was as described above. In none of these 18 cases was *SRY* sequences amplified. Lambert et al. *(15)* in their study clearly demonstrated that the fetal DNA found in plasma samples from nonpregnant women who had previously delivered one or more sons derived from cells of fetal origin. All plasma samples that were positive for fetal DNA became negative after a filtration process that removes cells or cell-derived particles but keeps cell-free fetal DNA. These cells or cell fragments are probably already removed in our first centrifugation step.

Invernizzi et al. *(6)* reported extremely high fetal DNA concentrations in plasma (mean, 983 ng/L). Given the scarcity of circulating fetal cells *(19)* or apoptotic cells *(15)* in the maternal circulation, this is unexpected. Lambert et al. *(15)* found a maximum fetal DNA concentration

in plasma of only 66 ng/L in healthy nonpregnant mothers of sons. We found a mean *SRY* concentration of 237 ng/L in early pregnancy (8–14 weeks) and 520 ng/L in week 30 *(11)*. Lo et al. *(16)* reported concentrations of 167 ng/L in early pregnancy and 1928 ng/L in late pregnancy. We have no explanation for the high concentrations of fetal DNA in nonpregnant women as described by Invernizzi et al. *(6)*. However, in view of the possible detection in plasma of fetal DNA from apoptotic cells, we strongly advise centrifugation or filtration of maternal plasma before testing for genetic diagnosis to reduce the possible risk of false-positive results.

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To the Editor:

Following conflicting reports on the presence of fetal DNA in plasma samples of nonpregnant healthy women *(1–4)*, Rijnders et al. report an additional 64 nonpregnant women with previous sons who lacked fetal DNA in plasma. The earlier finding of Lambert et al. *(2)* that fetal DNA in such plasma samples derived from apoptotic cells of fetal origin suggests that the male DNA we found *(1)* had a similar origin.

For the risk of false-positive results in prenatal diagnosis, the origin and concentration of fetal DNA in maternal plasma may be less important than its presence. We thus advise caution in the use of plasma fetal DNA for genetic diagnosis until the issue of the presence of fetal DNA in maternal plasma long after pregnancy is better understood.

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