Clinical Chemistry 49:5
727–731 (2003)

Molecular Diagnostics and Genetics

The Concentration of Circulating Corticotropin-releasing Hormone mRNA in Maternal Plasma Is Increased in Preeclampsia

Enders K.O. Ng,[1] Tse N. Leung,[2] Nancy B.Y. Tsui,[1] Tze K. Lau,[2] Nirmal S. Panesar,[1] Rossa W.K. Chiu,[1] and Y.M. Dennis Lo[1*]

Background: **Increased fetal DNA in maternal plasma/serum has been reported in pregnancies complicated by preeclampsia. We hypothesize that fetal RNA may also be increased in maternal plasma in preeclampsia.**

Methods: **We developed a real-time quantitative reverse transcription-PCR assay to measure the concentration of the mRNA of the *corticotropin-releasing hormone* (*CRH*) locus. Peripheral blood samples were obtained from healthy pregnant women both before and 2 h after delivery. Peripheral blood samples were also obtained from women suffering from preeclampsia and controls matched for gestational age. Plasma was harvested from these samples, and RNA was extracted. Plasma RNA was subjected to analysis by the reverse transcription-PCR assay.**

Results: ***CRH*** **mRNA was detected in the plasma of 10 healthy pregnant women in the third trimester. *CRH* mRNA was found to be cleared very rapidly after cesarean section, with no detectable signal by 2 h postpartum. Plasma *CRH* mRNA concentrations were 1070 and 102 copies/mL, respectively, in 12 preeclamptic women and 10 healthy pregnant women matched for gestational age (Mann–Whitney test, $P < 0.001$).**

Conclusion: **Plasma *CRH* mRNA represents a new molecular marker for preeclampsia. Maternal plasma RNA is gender- and polymorphism-independent and may allow noninvasive gene-expression profiling of an unborn fetus.**

The discovery of fetal DNA in maternal plasma has opened up new possibilities for noninvasive prenatal diagnosis *(1)*. This technology has been used for the noninvasive determination of many disorders, including fetal rhesus D status *(2)*, myotonic dystrophy *(3)*, achondroplasia *(4)*, and β-thalassemia *(5)*. In addition, quantitative abnormalities of fetal DNA in maternal plasma/serum have also been reported in preeclampsia *(6–8)*, fetal trisomy 21 *(9, 10)*, and hyperemesis gravidarum *(11)*. For the latter investigations, most groups have used Y-chromosomal markers, which are present only in male fetuses, as a fetal-specific marker. This approach has limited the application of this technology to the 50% of pregnant women who are carrying male fetuses.

The demonstration of the presence of fetal RNA in maternal plasma provides an approach for detecting fetal nucleic acids in maternal plasma that is independent of the gender and genetic polymorphisms present in a fetus *(12, 13)*. Recently, methods to enhance our ability to use plasma RNA as a potential molecular diagnostic tool have been developed. Our group has developed a protocol for the quantitative analysis of plasma RNA *(14)*, demonstrated the unexpected stability of circulating RNA *(15)*, and shown that the placenta is an important source of fetal RNA in maternal plasma *(16)*.

In this study, we tested the hypothesis that the ability to measure plasma RNA would provide us with a gender- and polymorphism-independent marker for monitoring pregnancy-associated disorders. Because of its importance, we chose preeclampsia as our model system *(17)*. For the mRNA target, we chose the mRNA of the *corticotropin-releasing hormone* (*CRH*)[3] locus, which is known to

Departments of [1] Chemical Pathology and [2] Obstetrics and Gynaecology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong SAR.

*Author for correspondence. Fax 852-2194-6171; e-mail loym@cuhk.edu.hk.

Received December 11, 2002; accepted February 12, 2003.

[3] Nonstandard abbreviations: CRH, corticotropin-releasing hormone; RT-PCR, reverse transcription-PCR; and GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

be expressed in the placenta *(18)* and is released into the maternal circulation *(19, 20)*. Its exact role in human pregnancy is not yet fully understood. A significantly higher CRH peptide content has been reported in placentas of preeclamptic pregnancies *(21, 22)*. Abnormally increased maternal plasma CRH has also been reported by various groups in pregnancies complicated by preeclampsia *(23–25)*. In this study, we investigated whether maternal plasma *CRH* mRNA might also be increased in preeclamptic pregnancies; if this hypothesis is confirmed, then plasma *CRH* mRNA might represent a new noninvasive marker for preeclampsia.

Patients and Methods

PATIENTS

Peripheral blood samples were collected with informed consent and Research Ethics Committee approval from pregnant women who attended the Department of Obstetrics and Gynaecology at the Prince of Wales Hospital, Hong Kong. Women were recruited between April and October 2002.

In the first part of this study, blood samples were obtained from 10 healthy pregnant women during the third trimester of gestation. In the second part of the project, pregnant women with uncomplicated pregnancies were recruited just before elective cesarean section. Peripheral blood samples were taken from these women just before delivery and at 2 h postdelivery. In the third part of the study, two patient groups were studied: (*a*) 12 preeclamptic women, and (*b*) 10 control pregnancies. The median gestational ages of the preeclamptic and control groups were 37 weeks (interquartile range, 36.6–38.9 weeks) and 38 weeks (interquartile range, 37.3–38.3 weeks), respectively. Preeclampsia was defined on the basis of a sustained increase in diastolic blood pressure >110 mmHg on one occasion or >90 mmHg on two or more occasions at least 4 h apart, with the presence of significant proteinuria in women with no history of hypertension. Significant proteinuria was defined as proteinuria >0.3 g/day or ≥2+ on dipstick testing in two clean-catch midstream urine specimens collected at least 4 h apart. The control group included pregnant women with no preexisting medical diseases or antenatal complications.

PROCESSING OF BLOOD SAMPLES

Plasma harvesting was performed immediately on arrival at the laboratory (within 1 h of venesection). Blood samples were processed based on a previously reported protocol *(14)*. In brief, 10-mL blood samples were collected in EDTA-containing tubes and centrifuged at 1600*g* for 10 min at 4 °C. Plasma was then carefully transferred into plain polypropylene tubes. The plasma samples were recentrifuged at 16 000*g* for 10 min at 4 °C, and the supernatants were collected in fresh polypropylene tubes.

RNA EXTRACTION

We mixed 1.6 mL of plasma harvested after the centrifugation steps described above with 2 mL of Trizol LS reagent (Invitrogen) and 0.4 mL of chloroform *(14)*. The mixture was centrifuged at 11 900*g* for 15 min at 4 °C, and the aqueous layer was transferred to new tubes. One volume of 700 mL/L ethanol was added to one volume of the aqueous layer. The mixture was then applied to an RNeasy mini column (Qiagen) and processed according to the manufacturer's recommendations. Total RNA was eluted with 30 μL of RNase-free water and stored at −80 °C. DNase treatment (RNase-Free DNase Set; Qiagen) was carried out to remove any contaminating DNA.

REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTION-PCR

One-step real-time quantitative reverse transcription-PCR (RT-PCR) was used for all mRNA quantifications *(14)*. The *CRH* primer sequences were 5′-GCCTCCCATCTCCCTGGAT-3′ (forward) and 5′-TGTGAGCTTGCTGTGCTAACTG-3′ (reverse), and the dual-labeled fluorescent probe was 5′-(FAM)TCCTCCGGGAAGTCTTGGAAATGGC(TAMRA)-3′, where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine. Calibration curves for *CRH* mRNA quantification were prepared by assaying serial dilutions of HPLC-purified single-stranded synthetic DNA oligonucleotides (Genset Oligos) specifying a 89-bp *CRH* amplicon (GenBank Accession No. NM_000756), with concentrations ranging from 1×10^7 copies to 1×10^1 copies. Absolute concentrations of *CRH* mRNA were expressed as copies/mL of plasma. The sequence of the synthetic DNA oligonucleotides for *CRH* calibrations was 5′-GGAGCCTCCCATCTCCCTGGATCTCACCTTCCACCTCCTCCGGGAAGTCTTGGAAATGGCCAGGGCCGAGCAGTTAGCACAGCAAGCTCACAGCA-3′. A calibration curve for *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) quantification was prepared as described previously, with results expressed in pg/mL of plasma *(14)*.

The RT-PCR reactions were set up according to the manufacturer's instructions (EZ r*Tth* RNA PCR reagent set; Applied Biosystems) in a reaction volume of 25 μL. The fluorescent probes (Genset Oligos) were used at concentrations of 100 nM. The PCR primers (Genset Oligos) were used at a concentration of 200 nM for both the *CRH* and *GAPDH* systems. We used 5 μL of extracted plasma RNA for amplification. Each sample was analyzed in duplicate, and the corresponding calibration curve was run in parallel with each analysis. Samples were also tested to ensure that they were negative for DNA by substituting the r*Tth* polymerase with the Ampli*Taq* Gold enzyme (Applied Biosystems). No amplification was observed for this control analysis, indicating the specificity of the assays for the respective mRNAs. Multiple negative water blanks were also included in every analysis. All analyses were performed by two of the authors (E.K.O.N. and N.B.Y.T.).

The thermal profile used for the *CRH* and *GAPDH* analyses was as follows: the reaction was initiated at 50 °C for 2 min for the included uracil *N*-glycosylase to act, followed by reverse transcription at 60 °C for 30 min. After a 5-min denaturation at 95 °C, 40 cycles of PCR were carried out with denaturation at 94 °C for 20 s and 1 min of annealing/extension at 58 and 62 °C for *CRH* and *GAPDH*, respectively.

Statistical analysis

Statistical analysis was performed with the Sigma Stat 2.03 software (SPSS). The Mann–Whitney test was used for the comparison of maternal plasma *CRH* mRNA concentrations between preeclamptic and control groups. The Wilcoxon test was used for the comparison of maternal plasma *GAPDH* mRNA concentrations before and at 2 h postdelivery.

Results

Establishment of real-time quantitative RT-PCR

To determine the quantitative performance of the *CRH* RT-PCR assay, we used this system to amplify serially diluted calibrators that were synthetic DNA oligonucleotides based on the *CRH* sequence. Previous data have shown that such single-stranded oligonucleotides reliably mimic the products of the reverse transcription step and produce calibration curves that are identical to those obtained using T7-transcribed RNA *(26)*. The calibration curve for the *CRH* amplification systems demonstrated a dynamic range from 2.5×10^1 to 1×10^6 copies and had a correlation coefficient of 0.983. The sensitivities of the amplification steps of these assays were sufficient to detect 25 copies of the *CRH* target. To determine the precision of the whole analytical procedure, including the RNA extraction, reverse transcription, and amplification steps, we performed 10 replicate RNA extractions from a plasma sample obtained from a healthy pregnant woman (gestational age, 38 weeks) and subjected these extracted RNA samples to RT-PCR analysis. The CV for the threshold cycle values of these replicate analyses for *CRH* mRNA was 2.8%. The development and performance of the real-time quantitative *GAPDH* RT-PCR assay was described previously *(14)*.

Detectability of *CRH* mRNA in maternal plasma

To test whether *CRH* mRNA transcripts were detectable in maternal plasma, we analyzed plasma samples from 10 pregnant women in the third trimester of pregnancy (gestational age, 37–41 weeks) by the *CRH* RT-PCR assay. *CRH* mRNA was detected in all tested samples. The median concentration of plasma *CRH* mRNA was 73 copies/mL (interquartile range, 51–177 copies/mL). As a positive control, *GAPDH* mRNA was also detectable in all of these plasma samples.

Clearance of *CRH* mRNA from maternal plasma after delivery

To demonstrate that the maternal plasma *CRH* mRNA was derived from the fetus, we analyzed maternal plasma for *CRH* mRNA both before and at 2 h postdelivery. Four women who delivered by cesarean section (gestational age, 38–40 weeks) were studied. *CRH* mRNA was detected in 100% of predelivery maternal plasma samples, whereas *CRH* mRNA was not detected in any of the postdelivery samples. *GAPDH* mRNA was detectable in all plasma samples, thus demonstrating the quality of the samples. No systematic alternation in maternal plasma *GAPDH* mRNA concentration was observed (Wilcoxon test, $P = 0.25$). The results are shown in Fig. 1.

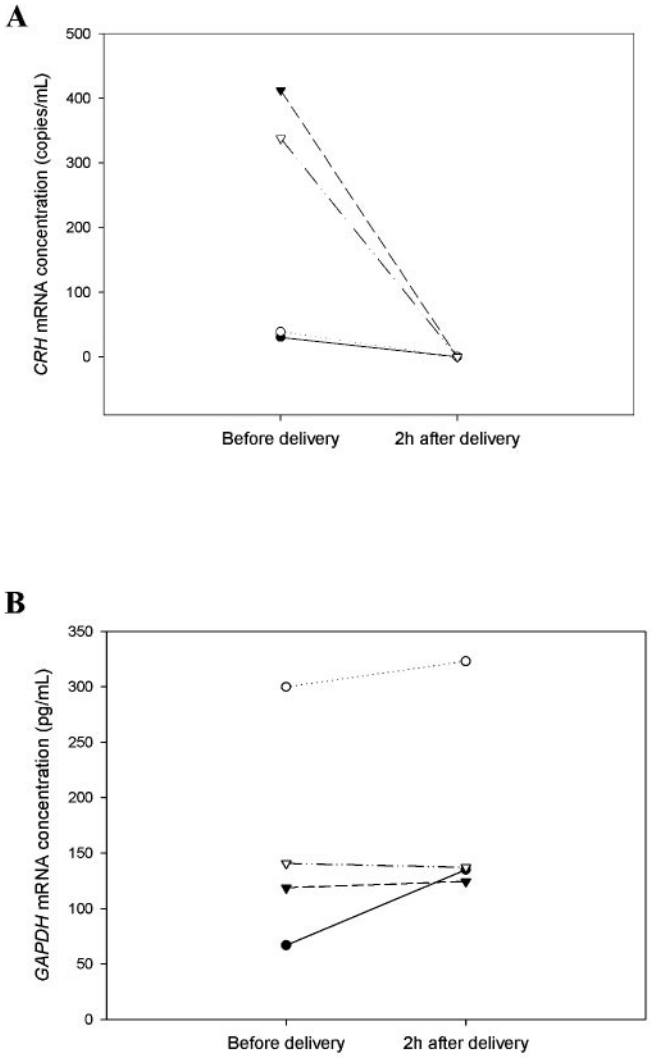

Fig. 1. Clearance of *CRH* mRNA from maternal plasma after delivery.

CRH (*A*) and *GAPDH* (*B*) mRNA concentrations in maternal plasma are shown before delivery and at 2 h after delivery. Each *line* represents one plasma sample obtained from one woman.

QUANTITATIVE ANALYSIS OF *CRH* mRNA IN THE PLASMA OF PREECLAMPTIC PREGNANT WOMEN

To compare the concentration of *CRH* mRNA in maternal plasma of preeclamptic and control pregnant women, we obtained plasma samples from 12 preeclamptic women and 10 control pregnant women with matched gestational age. The median *CRH* mRNA concentration in the plasma of preeclamptic women and control pregnancies were 1070 copies/mL (interquartile range, 535-1468 copies/mL) and 102 copies/mL (interquartile range, 51–158 copies/mL), respectively (Fig. 2). The median plasma *CRH* mRNA concentrations were 10.5 times higher in preeclamptic than control pregnancies (Mann–Whitney test, $P < 0.001$).

Discussion

In this report, we have demonstrated that *CRH* mRNA is easily detectable in the plasma of pregnant women in the third trimester of pregnancy. The fact that such mRNA is cleared within 2 h postpartum provides evidence that such mRNA is derived from the fetus. These results support our previous data demonstrating that the placenta is an important source for releasing fetal mRNA species into maternal plasma *(16)*.

We also considered the possibility that *CRH* mRNA produced by the mother, rather than the placenta, might also be detectable in the plasma, but the postpartum data suggest that this is improbable. Furthermore, because CRH is produced by the hypothalamus, we think that it is unlikely that large amounts of such mRNA will be released into the blood (possibly even requiring the passage of mRNA through the intact blood–brain barrier). Conversely, the relatively large surface area of the placenta would make it a much more plausible source of *CRH* mRNA release.

The data presented here have demonstrated that the concentration of maternal plasma *CRH* mRNA is increased in pregnancies complicated with preeclampsia. The median plasma *CRH* mRNA concentration was increased 10.5 times in preeclampsia, compared with nonpreeclamptic pregnancies matched for gestational age. In comparison, our previously published results showed a fivefold increase in circulating fetal DNA in maternal plasma in preeclamptic pregnancies *(6)*.

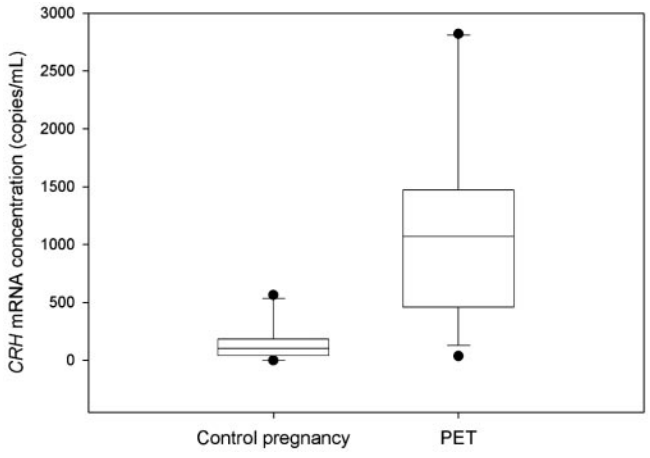

Fig. 2. Box plot of *CRH* mRNA concentration in maternal plasma of preeclamptic (*PET*) and control groups.

CRH mRNA concentrations are expressed in copies/mL. The *lines inside* the *boxes* denote the medians. The *boxes* mark the interval between the 25th and 75th percentiles. The *whiskers* denote the interval between the 10th and 90th percentiles. ● indicate data points outside the 10th and 90th percentiles.

Our results suggest that maternal plasma *CRH* mRNA might be a new molecular marker for preeclampsia. This approach offers an alternative to current studies that involve the measurement of maternal plasma CRH using immunoassays. For immunoassays, the specificity of the method is critically dependent on the specificity of the antibodies used. On the other hand, at least at the present time, the mRNA approach is probably more expensive on a case-by-case basis than a well-established immunoassay system. Future studies should aim at a direct comparison of these potentially complementary approaches in the same patient cohort.

The mechanism producing the increase in such quantitative aberration in plasma RNA requires further investigation. Several theoretical possibilities exist. The first is that increased concentrations of *pro-CRH* mRNA have been detected in placental tissues in preeclamptic pregnancies *(22)*, which may lead to increased liberation of such transcripts into the plasma. The second possibility is that, because plasma nucleic acids have been postulated to be related to cell death *(27–29)*, it is possible that the increase in cell death within the placenta in preeclampsia *(30)* may contribute to the increased release of placenta-expressed mRNA species into maternal plasma. Concerning the third possibility, we have recently demonstrated that impaired clearance of maternal plasma fetal DNA is observed in preeclampsia *(31)*. In theory, a similar phenomenon may also exist for plasma RNA clearance in preeclampsia. This is particularly relevant because the data in the present study demonstrate the rapid clearance of *CRH* mRNA after delivery (Fig. 1), which is similar to the rapid clearance of fetal DNA from maternal plasma after delivery *(32)*.

Compared with fetal DNA measurements in maternal plasma *(6–8)*, quantitative analysis of circulating fetal RNA, such as placenta-expressed mRNA, has the advantage of being applicable to all pregnant women irrespective of fetal gender and polymorphism status. Furthermore, numerous targets can be selected for plasma RNA analysis, including the numerous genes that are known to be expressed in the placenta. It could therefore be worthwhile to systematically investigate the detectability of such transcripts in maternal plasma. In addition, because CRH is a hormone, our data have also opened up the possibility that a similar approach can be used for the investigation of other hormonal systems, with new diagnostic and research opportunities.

This project was supported by the Innovation and Technology Fund (AF/90/99). The Chinese University of Hong Kong has filed patent applications covering aspects of the technology of circulating nucleic acid analysis for prenatal diagnosis and monitoring.

References

1. Lo YMD, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al. Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350:485–7.
2. Lo YMD, Hjelm NM, Fidler C, Sargent IL, Murphy MF, Chamberlain PF, et al. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. N Engl J Med 1998;339:1734–8.
3. Amicucci P, Gennarelli M, Novelli G, Dallapiccola B. Prenatal diagnosis of myotonic dystrophy using fetal DNA obtained from maternal plasma. Clin Chem 2000;46:301–2.
4. Saito H, Sekizawa A, Morimoto T, Suzuki M, Yanaihara T. Prenatal DNA diagnosis of a single-gene disorder from maternal plasma. Lancet 2000;356:1170.
5. Chiu RWK, Lau TK, Leung TN, Chow KCK, Chui DHK, Lo YMD. Prenatal exclusion of β-thalassaemia major by examination of maternal plasma. Lancet 2002;360:998–1000.
6. Lo YMD, Leung TN, Tein MS, Sargent IL, Zhang J, Lau TK, et al. Quantitative abnormalities of fetal DNA in maternal serum in preeclampsia. Clin Chem 1999;45:184–8.
7. Zhong XY, Laivuori H, Livingston JC, Ylikorkala O, Sibai BM, Holzgreve W, et al. Elevation of both maternal and fetal extracellular circulating deoxyribonucleic acid concentrations in the plasma of pregnant women with preeclampsia. Am J Obstet Gynecol 2001;184:414–9.
8. Swinkels DW, de Kok JB, Hendriks JC, Wiegerinck E, Zusterzeel PL, Steegers EA. Hemolysis, elevated liver enzymes, and low platelet count (HELLP) syndrome as a complication of preeclampsia in pregnant women increases the amount of cell-free fetal and maternal DNA in maternal plasma and serum. Clin Chem 2002; 48:650–3.
9. Lo YMD, Lau TK, Zhang J, Leung TN, Chang AM, 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.
10. Zhong XY, Burk MR, Troeger C, Jackson LR, Holzgreve W, Hahn S. Fetal DNA in maternal plasma is elevated in pregnancies with aneuploid fetuses. Prenat Diagn 2000;20:795–8.
11. Sekizawa A, Sugito Y, Iwasaki M, Watanabe A, Jimbo M, Hoshi S, et al. Cell-free fetal DNA is increased in plasma of women with hyperemesis gravidarum. Clin Chem 2001;47:2164–5.
12. Poon LLM, Leung TN, Lau TK, Lo YMD. Presence of fetal RNA in maternal plasma. Clin Chem 2000;46:1832–4.
13. Poon LLM, Lo YMD. Fetal RNA in maternal plasma: the next stage in noninvasive prenatal diagnosis. In: Bruns DE, Lo YMD, Wittwer CT, eds. Molecular testing in laboratory medicine. Washington, DC: AACC Press, 2002:321–2.
14. Ng EKO, Tsui NBY, Lam NY, Chiu RW, Yu SC, Wong SC, et al. Presence of filterable and nonfilterable mRNA in the plasma of cancer patients and healthy individuals. Clin Chem 2002;48: 1212–7.
15. Tsui NBY, Ng EKO, Lo YMD. Stability of endogenous and added RNA in blood specimens, serum, and plasma. Clin Chem 2002; 48:1647–53.
16. Ng EKO, Tsui NBY, Lau TK, Leung TN, Chiu RWK, Panesar NS, et al. Messenger RNA of placental origin is readily detectable in maternal plasma. Proc Natl Acad Sci U S A 2003;in press.
17. Roberts JM, Redman CW. Pre-eclampsia: more than pregnancy-induced hypertension. Lancet 1993;341:1447–51.
18. Shibasaki T, Odagiri E, Shizume K, Ling N. Corticotropin-releasing factor-like activity in human placental extracts. J Clin Endocrinol Metab 1982;55:384–6.
19. Goland RS, Wardlaw SL, Stark RI, Brown LS Jr, Frantz AG. High levels of corticotropin-releasing hormone immunoactivity in maternal and fetal plasma during pregnancy. J Clin Endocrinol Metab 1986;63:1199–203.
20. McLean M, Bisits A, Davies J, Woods R, Lowry P, Smith R. A placental clock controlling the length of human pregnancy. Nat Med 1995;1:460–3.
21. Goland RS, Conwell IM, Jozak S. The effect of pre-eclampsia on human placental corticotrophin-releasing hormone content and processing. Placenta 1995;16:375–82.
22. Ahmed I, Glynn BP, Perkins AV, Castro MG, Rowe J, Morrison E, et al. Processing of procorticotropin-releasing hormone (pro-CRH): molecular forms of CRH in normal and preeclamptic pregnancy. J Clin Endocrinol Metab 2000;85:755–64.
23. Wolfe CD, Patel SP, Linton EA, Campbell EA, Anderson J, Dornhorst A, et al. Plasma corticotrophin-releasing factor (CRF) in abnormal pregnancy. Br J Obstet Gynaecol 1988;95:1003–6.
24. Perkins AV, Linton EA, Eben F, Simpson J, Wolfe CD, Redman CW. Corticotrophin-releasing hormone and corticotrophin-releasing hormone binding protein in normal and pre-eclamptic human pregnancies. Br J Obstet Gynaecol 1995;102:118–22.
25. Leung TN, Chung TK, Madsen G, Lam CW, Lam PK, Walters WA, et al. Analysis of mid-trimester corticotrophin-releasing hormone and α-fetoprotein concentrations for predicting pre-eclampsia. Hum Reprod 2000;15:1813–8.
26. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 2000;25:169–93.
27. Fournie GJ, Martres F, Pourrat JP, Alary C, Rumeau M. Plasma DNA as cell death marker in elderly patients. Gerontology 1993;39: 215–21.
28. Lo YMD, Rainer TH, Chan LY, Hjelm NM, Cocks RA. Plasma DNA as a prognostic marker in trauma patients. Clin Chem 2000;46: 319–23.
29. Hasselmann DO, Rappl G, Tilgen W, Reinhold U. Extracellular tyrosinase mRNA within apoptotic bodies is protected from degradation in human serum. Clin Chem 2001;47:1488–9.
30. Leung DN, Smith SC, To KF, Sahota DS, Baker PN. Increased placental apoptosis in pregnancies complicated by preeclampsia. Am J Obstet Gynecol 2001;184:1249–50.
31. Lau TW, Leung TN, Chan LY, Lau TK, Chan KC, Tam WH, et al. Fetal DNA clearance from maternal plasma is impaired in preeclampsia. Clin Chem 2002;48:2141–6.
32. Lo YMD, Zhang J, Leung TN, Lau TK, Chang AM, Hjelm NM. Rapid clearance of fetal DNA from maternal plasma. Am J Hum Genet 1999;64:218–24.