An improved methodological approach for the analysis of cell-free RNA in maternal plasma

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ABSTRACT

The study of cell-free RNA in maternal plasma provides a promising tool for monitoring some pregnancy pathologies, such as preeclampsia (PE). Due to the scarcity of RNA molecules in maternal plasma we introduced substantial improvements to the protocol previously described in the literature, including a preamplification step before quantitative analysis of target genes. By applying the improved protocol for plasma RNA quantification, we obtained an increase in sensitivity of about 10 quantification cycle values in detection of both the reference and target genes in a reliable amplification range. Among mRNA markers, the corticotropin-releasing hormone (CRH) transcript has been reported to be elevated in preeclamptic patients compared to women with uncomplicated pregnancy at the same gestational age. We applied the optimized protocol to the quantitative evaluation of CRH mRNA in women with overt PE, in a small cohort of women at risk of developing this pathology and in controls. Our preliminary results confirmed the increase of CRH mRNA concentration in preeclamptic women and showed that the transcript levels were significantly elevated in the last plasma sample collected from women at risk for the disease before the PE onset.

INTRODUCTION

The presence of cell-free fetal RNA in maternal plasma was demonstrated by amplifying fetal Y-chromosome-specific zinc finger protein (ZFY) mRNA in plasma of pregnant women carrying male fetuses (1). Concerning the origin of fetal RNA in plasma of pregnant women, it was demonstrated that the placenta is the main source for releasing fetal transcripts in maternal circulation (2). The identification of fetal RNA in maternal plasma has provided a new tool for monitoring of the development of pregnancy-related pathological conditions, such as preeclampsia (PE), which results in maternal and fetal morbidity and mortality (3). Among mRNA markers, the corticotropin-releasing hormone (CRH) transcript was found to be ~10 times more elevated in maternal plasma of women with overt PE compared to controls (2). The CRH gene codes for a 41-aminoacid peptide and was found to be expressed in human placenta (4). The quantitative evaluation of circulating transcripts is, however, limited by the amount of RNA that can be extracted from plasma samples. The scarcity of these molecules in maternal circulation renders their identification particularly cumbersome. In order to improve the methodological approach for the analysis of cell-free RNA in plasma samples, we evaluated the introduction of a preamplification step before real-time quantitative polymerase chain reaction (qPCR) analysis. An optimized protocol was obtained and applied to the analysis of CRH transcript in plasma samples of women with overt PE and women at risk of this obstetrical complication compared to control pregnancies.

MATERIALS AND METHODS

Patients

Blood samples of pregnant women were collected after written informed consent, as approved by the local Ethical Review Boards. We collected plasma samples from 12 women with overt PE (sampling range: 24-36 weeks), 52 women at risk of developing this pathology (sampled at 24, 28, 32, and 36 weeks, when possible), and 12 women with uncomplicated pregnancy (sampled at 24, 28, 32, 36 weeks, and post partum, when possible). Plasma samples collected from women at risk at the clinical manifestation of PE were included in the group of women with overt pathology.

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PE was defined by elevated systolic (≥140 mmHg) or diastolic (≥90 mmHg) blood pressure on at least two consecutive occasions in a woman who was normotensive before 20 weeks of gestation and proteinuria (≥0.3 g/die). The risk factor for the inclusion in the study was a previous pregnancy complicated by PE.

Sample preparation

6 mL of peripheral blood from pregnant women were collected in Vacutainer tubes containing EDTA and processed within a few hours as previously reported (5, 6). Plasma samples were stored at -80 °C until further processing for plasma RNA extraction.

RNA extraction from plasma

RNA was extracted from 0.5 mL of plasma using the DSP Virus Kit (Qiagen) according to the manufacture's instructions. During extraction, DNase I treatment (RNase-free DNase Set, Qiagen) was carried out for 15 min at room temperature to remove any contaminating DNA. RNA extracted from plasma was eluted in 25 μL of elution buffer.

Reverse transcription

Reverse transcription was performed in a final reaction volume of 50 μL. An aliquot of 25 μL of eluted RNA was mixed with 2.5 μL 10X Random Hexamers (Applied Biosystems) subjected to one cycle at 65 °C for 5 min. This mixture was incubated at room temperature for 10 min to allow the extension of the random hexamers. Subsequently, 5 μL 10X buffer, 10 μL $MgCl_2$ 25 mM, 2.5 μL dNTPs 100mM, 2.5 μL 20 U/μL RNase inhibitor and 2.5 μL 50 U/μL MuLV reverse transcriptase (Applied Biosystems) were added. This reaction mixture underwent one cycle at 42 °C for 1 h and at 95 °C for 5 min.

cDNA preamplification

Due to the scarcity of plasma mRNA content, a preamplification reaction on cDNA was performed to increase the transcripts of interest by using pre-designed primer/probe sets (TaqMan Gene Expression Assays, Applied Biosystems) pooled at a 0.2X concentration each from a 20X solution. An aliquot of 12.5 μL of the assay pool was combined with 25 μL of TaqMan PreAmp Master Mix (Applied Biosystems) and 12.5 μL of cDNA into a 50 μL reaction mixture that was subjected to one cycle at 95 °C for 10 min followed by 10 or 14 cycles at 95 °C for 15 sec and at 60 °C for 4 min in a conventional thermal cycler.

All TaqMan gene expression assays used in this study have "m1" ID suffix, which indicates the primer/probe location within the gene; in particular "m1" indicates that the probe spans an exon junction and does not detect genomic DNA. Each primer/probe set was selected by targeting preferentially the 5' region of transcripts to improve the detection rate of fetal RNA in maternal plasma.

Relative quantitative real-time PCR

Before proceeding to the detection of single-target sequences, the preamplified products were diluted. The standard preamplification protocol suggests two alternative dilution factors, based on the number of preamplification cycles: 1:5 for 10 cycles and 1:20 for 14 cycles. An aliquot of 11.2 μL of the diluted preamplified product were mixed with 12.5 μL of TaqMan Universal PCR Master Mix and 1.25 μL of the primer/probe set specific for each gene of interest (Taqman gene Expression Assays, Applied Biosystems). Cycling conditions were: 50 °C for 2 min for uracil-N-glycosylase (UNG) activation, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s (ABI PRISM 7700 Sequence Detection System, Applied Biosystems). Each sample was analyzed in duplicate and multiple blanks (no preamplified cDNA) were included in the 96 well plate. For the analysis of the amplification of mRNA species, a specific quantification cycle (C_q) was used. The ratio of target mRNA levels to the reference gene levels was calculated by subtracting C_q logarithmic values (ΔC_q), taking the $antilog_2$ and multiplying by 10^3.

Statistical analysis

Groups were compared with a nonparametric one-way ANOVA with P-values computed through permutation. Multiple comparison correction applied for paired tests.

RESULTS

To evaluate the feasibility of the study, plasma cell-free RNA was first extracted from 5 women with uncomplicated pregnancy. The extracted RNA was reverse transcripted and evaluated by real-time amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and CRH mRNAs. In these experiments amplification signals for GAPDH, used as reference gene, were obtained at about 30 C_q, whereas for the CRH transcript we were able to detect the amplification products at about 37-38 C_q. This condition is not useful for quantitative analysis of mRNA markers and demands a procedure that allows the quantification of mRNA species of interest at low C_q values (<35) in an amplification range that provide consistent results. For this reason, we introduced a preamplification reaction to increase the number of specific cDNA target molecules. We first performed the preamplification reaction according to one of the protocols suggested by kit: 14 cycles of preamplification and 1:20 dilution of preamplified products before analyzing by qPCR. The introduction of this step in the presence of primer/probe specific for the detection of GAPDH and CRH mRNA before real-time PCR resulted in a substantial increase in sensitivity by approximately 10 C_q for both reference and target genes.

Evaluation of housekeeping genes

In order to evaluate a reference gene for the analysis of CRH (Hs 00174941_m1) mRNA concentrations in plasma of pregnant women, we performed quantitative evaluation of 7 different housekeeping genes. In particular, we used the TaqMan gene expression assays for GAPDH (Hs 99999905_m1), human actin β (ACTB, Hs 99999903_m1), human ubiquitin C (UBC, Hs 00824723_m1), human DNA-direct RNA polymerase II subunit A (POLR2A, Hs 01108291_m1), human trasferrin receptor (TFRC, Hs 00951985_m1), human TATA binding protein (TBP, Hs 99999910_m1), human succinate dehydrogenase complex (SDHA, Hs 00188166_m1), and human tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YMHAZ, Hs 00237047_m1) mRNA.

We analyzed the reference gene levels in 5 plasma samples from women with uncomplicated pregnancy at different gestational ages and post partum, using the preamplification reaction standard protocol (14 amplification cycles and 1:20 preamplified products dilution). Our results showed that GAPDH mRNA was present in maternal plasma with stable levels (ΔC_qmax-$C_q$min ranges from 1.46 to 3.37) during the whole gestation in all plasma samples (Table 1). Therefore, GAPDH was used as internal control for relative quantification of CRH mRNA.

Evaluation of protocol reproducibility using different dilution factors of preamplified products

Initially, plasma samples from 5 women with overt PE and 5 women with uncomplicated pregnancy, matched for gestational age, were extracted, reverse transcripted, preamplified, diluted 1:20 and then analyzed by qPCR. To evaluate whether the protocol was reproducible, we analyzed the same plasma samples in two different sessions. This study showed that the CRH mRNA amplification did not generate reproducible results. Thus, to improve the protocol, we decided to increase the concentration of the preamplification products to be subjected to qPCR, by using different lower dilution factors (1:10 and 1:5). To compare the results obtained with 1:20 dilution of preamplification products with those obtained at 1:10 and 1:5 dilution factors, we performed different experiments.

Comparison between 1:10 and 1:20 dilution factors

First, a 1:10 and 1:20 dilution factors were evaluated in 4 different plasma samples to analyze GAPDH and CRH mRNAs. To evaluate whether a lower dilution factor allowed the detection of the amplification signals at lower C_q values, we selected 2 positive and 2 negative plasma samples for the target mRNA; these samples had already been found to be either positive or negative at the previous analysis with 1:20 dilution factor. The results, reported in Table 2, showed that with the 1:10 dilution factor of preamplified products the amplification signals of both the reference and target mRNA anticipated of ~1 C_q with respect to the same preamplified products diluted 1:20.

Comparison among 1:5, 1:10 and 1:20 dilution factors

Due to the scarcity of mRNA of interest, we also evaluated the 1:5 dilution, which is the higher dilution factor suggested by the kit, combined with 14 preamplification cycles of cDNA of interest. The results obtained with the 1:5 dilution were also compared to those obtained with 1:10 and 1:20 dilution factors. To this aim, 3 plasma samples were analyzed for the CRH transcript. Two previously analyzed positive samples and one negative sample were selected. Results showed that with the 1:5 dilution of preamplified products the detection of PCR products of CRH mRNA anticipated of ~2 C_q with respect to the same preamplified products diluted 1:20 (Table 3). Moreover, in different experimental sessions, we confirmed that this protocol generated reproducible results and was thus definitively selected for the subsequent experiments (Figure 1).

Clinical study

Women with uncomplicated pregnancy

The optimized protocol (14 preamplification cycles and 1:5 dilution factor) was first applied on plasma samples collected at different gestational ages from women with

Table 1
Plasma concentrations of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in pregnancy

Sample ID	24 weeks	28 weeks	32 weeks	36 weeks	Post partum	$\Delta(C_q$max-$C_q$min)[a]
1	23.08	24.09	23.47	20.72	21.60	3.37
2	21.39	22.36	21.58	22.07	20.76	1.60
3	21.06	20.78	22.25	ND	19.88	2.37
4	21.37	20.20	19.91	20.50	ND	1.46
5	ND	22.23	22.19	20.47	22.08	1.76

Samples were analyzed in duplicate. Mean C_q values are reported.
[a]*$\Delta(C_q$max-$C_q$min) indicates the difference between the higher and lower C_q value for GAPDH mRNA levels.*
ND, no sample available.

Table 2
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and corticotropin-releasing hormone (CRH) C_q values with 1:10 and 1:20 dilution

	Sample 1		Sample 2		Sample 3		Sample 4	
Dilution factor	1:10	1:20	1:10	1:20	1:10	1:20	1:10	1:20
GAPDH	20.03	20.99	18.88	19.87	20.78	22.02	18.56	19.59
CRH	24.34	25.32	24.26	25.28	No signal	No signal	No signal	No signal
ΔC_q	4.31	4.33	5.38	5.41	ND	ND	ND	ND

Samples were analyzed in duplicate; ΔC_q = mean C_q (CRH) - mean C_q (GAPDH).
ND, not determined because no amplification signal was detected for the target gene.

Table 3
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and corticotropin-releasing hormone (CRH) C_q values with 1:5, 1:10 and 1:20 dilution

	Sample 1			Sample 2			Sample 3		
Dilution factor	1:5	1:10	1:20	1:5	1:10	1:20	1:5	1:10	1:20
GAPDH	18.13	19.09	20.19	20.20	21.22	22.32	19.60	20.06	21.49
CRH	26.83	27.76	29.12	No signal	No signal	No signal	25.49	26.67	27.54
ΔC_q	8.70	8.67	8.93	ND	ND	ND	5.89	6.61	6.05

Samples were analyzed in duplicate; ΔC_q = mean C_q (CRH) - mean C_q (GAPDH).
ND, not determined because no amplification signal was detected for the target gene.

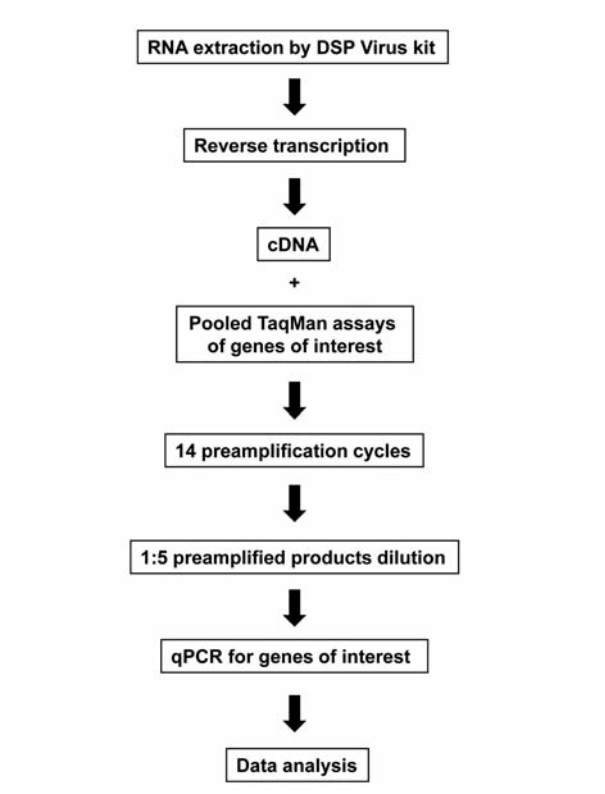


Figure 1
Schematic representation of the workflow.

uncomplicated pregnancy used as controls. Concerning the analysis of CRH, mRNA was not detectable in plasma samples of six women analyzed at 24 and 28 weeks of gestation, while it was detected in 7 out of 8 women at 32 weeks (median fold change = 3.54) and in six women analysed at 36 weeks (median fold change = 5.86).

Women with overt PE

Subsequently, we analyzed 12 plasma samples from women with overt PE. Our results confirmed that CRH mRNA concentrations were significantly increased in plasma of women sampled at the clinical manifestation of PE ($P < 0.01$) (Figure 2A).

Women at risk for developing PE

Finally, CRH transcript levels were analyzed in a cohort of women at risk for developing PE. Among these women, two developed early severe PE at 28 weeks and one developed PE post partum. In these women, CRH mRNA levels were significantly increased compared to controls matched for gestational age and this increase was detected about 2-4 weeks before the clinical manifestation of PE ($P < 0.01$). Figure 2B shows concentrations of CRH transcript detected in the last plasma sample collected before the onset of PE. In women at risk who did not developed any pathology, CRH mRNA levels remained within the physiological range.

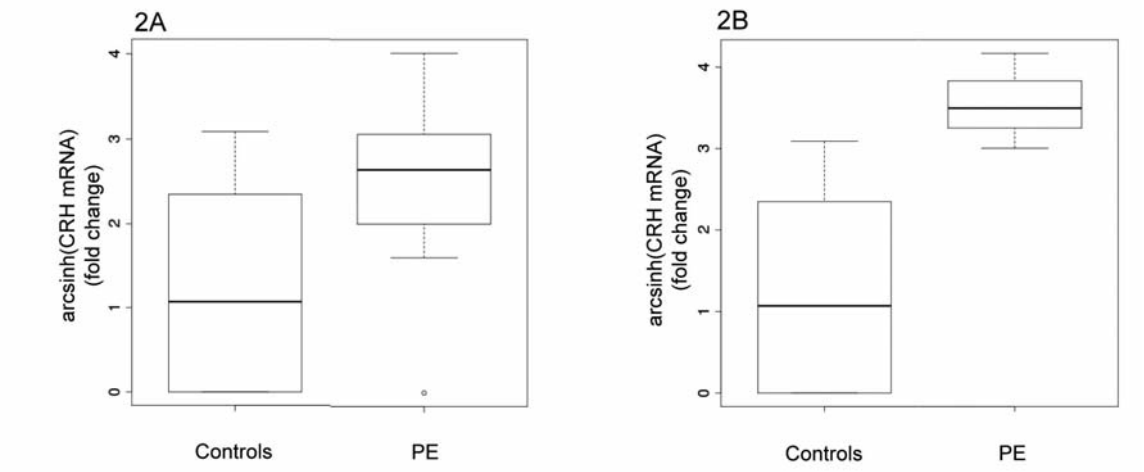


Figure 2
Box plot of corticotropin-releasing hormone (CRH) mRNA concentrations in maternal plasma of controls and women with overt preeclampsia (PE) (2A) and in women at risk for PE in the last plasma sample collected before the onset of PE (2B).
Data are plotted after arcsinh transformation. Boxes mark the interquartile range (IQR). Lines inside the boxes denote medians. The whiskers stretch to the most extreme point that is no more than 1.5 IQR from the box. A dot indicate a point more far than 1.5 IQR.

DISCUSSION

The presence of fetal RNA in plasma of pregnant women may permit the noninvasive monitoring of pregnancy complications, such as preeclampsia. Due to the scarcity of free RNA in maternal circulation, the main goal of our study was to improve the methodological approach for the quantitative analysis of these molecules in plasma samples. In the first phase of our work, we analyzed RNA molecules extracted from maternal plasma that were reverse transcripted and analyzed by qPCR for GAPDH mRNA as a reference gene and CRH mRNA. In these preliminary experiments, performed on plasma samples from women with uncomplicated pregnancy, amplification signals for the mRNA were detected at high C_q values, not reliable for relative quantification. Therefore, we evaluated the introduction of a preamplification reaction following reverse transcription and preceding qPCR in the presence of primers and probes specific for the reference and target genes. The aim of the preamplification reaction is to increase the number of specific cDNA target molecules and the use of this procedure had been previously reported in the detection of small amounts of RNA (7, 8).

In our work, we first tested the conditions suggested by the kit protocol, corresponding to 14 preamplification cycles followed by 1:20 dilution of preamplified products for qPCR analysis. This low number of amplification cycles is still within the exponential phase of PCR and is thus expected not to affect the ratio among the different mRNA species without introducing amplification bias to the samples. The introduction of the preamplification step allowed anticipating the detection of amplification signals of about 10 C_q for both reference gene and rare mRNA species, accordingly.

To select a reference gene stable during whole pregnancy, we analyzed 7 different reference genes in plasma samples of 5 women with uncomplicated pregnancy at different gestational ages and post partum by using our protocol. The results suggested that GAPDH was a good internal control since its concentrations in maternal plasma were stable throughout the whole gestation.

Subsequently, we analyzed plasma samples from 5 women with overt PE and 5 women with uncomplicated pregnancies in two independent experiments. The data obtained showed non-reproducible results for CRH mRNA and this could be ascribed to the low amount of cDNA corresponding to the rare mRNA species analyzed in our assay. To overcome this problem, we decided to increase the concentration of the preamplified products to be subjected to qPCR and evaluated the 1:10 and 1:5 dilution factors. In all samples analyzed with the 1:10 dilution, the amplification signal for both the reference and CRH genes anticipated of about 1 C_q compared to the 1:20 dilution. The ΔC_q values calculated for CRH mRNA compared to GAPDH remained unchanged for both dilutions. Similar results were obtained with the 1:5 dilution, where the amplification signals anticipated of about 2 C_q compared to the 1:20 dilution and about 1 C_q compared to the 1:10 dilution. Moreover, the 1:5 dilution provided quantitative reproducible results for CRH mRNA, when the same samples in different sessions were analysed. The final protocol thus included extraction of plasma samples by the DSP Virus Kit, reverse transcription, 14 preamplification cycles, 1:5 preamplified product dilution and qPCR. This optimized protocol provided a simple, easy workflow and was then applied to the analysis of plasma samples from women with uncomplicated pregnancy, used as controls, and women with overt PE or at risk for PE. Concerning the analysis of CRH mRNA, in controls we showed that the

transcript was detectable starting from the 32nd week of gestation with progressive increase until labor. The analysis of women with overt PE showed that, at the clinical manifestation of pathology, CRH mRNA concentrations were significantly more elevated with respect to control pregnancies. These data confirmed previous reports of about tenfold increase in fetal CRH transcript in women with overt PE compared to women with uncomplicated pregnancy matched for gestational age (2). Moreover, we wanted to evaluate the CRH mRNA levels in a cohort of women at risk for developing PE followed-up in our clinical department. Among women at risk examined in this study, in 3 subjects who developed PE, CRH concentrations were significantly increased compared to controls some weeks before the onset of the pathology. Based on these preliminary results, we plan to extend our study to investigate other RNA markers on a larger cohort of patients.

In conclusion, noninvasive analysis of RNA markers in maternal plasma has the potential to identify cases at risk of developing pregnancy complications and allow early diagnosis, providing that the protocol for analysis is accurate enough.

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