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Combined quantification of corticotropin-releasing hormone, cortisol-to-cortisone ratio and progesterone by liquid chromatography–Tandem mass spectrometry in placental tissue

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1. Introduction

Since its discovery in placental extracts by Shibasaki et al. in 1982 [1], important functional roles of corticotropin-releasing hormone (CRH) for human reproductive physiology were identified [2,3]. The placental syncytiotrophoblast is a major source of plasma CRH in the maternal circulation in the second half of pregnancy [4]. CRH exerts local effects at the placental bed, controlling extravillous trophoblast invasion in early gestation [5] and the timing of parturition in late gestation [6]. The latter function is achieved by an interaction of CRH with progesterone, which enhances the contractile response of the myometrium [6,7] and via the regulation of feto-placental circulation through the nitric oxide (NO)/cGMP pathway [8,9].

Moreover CRH exerts systemic effects in the mother, by driving pituitary-adrenal function [10]. The majority of circulating cortisol in the fetus is of maternal origin [11,12]. Cortisol is vital for physiologic fetal organ development [13], however excessive exposure leads to intra-uterine growth restriction (IUGR) with subsequent morbiditiy (e.g. hypertension, diabetes mellitus) in adult life [14–18]. Control of transplacental transfer of maternal cortisol to the fetus is controlled by the enzyme 11βhydroxysteroid dehydrogenase type 2 (11β-HSD2) in the syncytiotrophoblast layer [19–21]. The expression of 11β-HSD2 increases across gestation [22], possibly driven by cortisol [19,23] and CRH itself [24]. 11β-HSD2 catalyzes the unidirectional conversion of cortisol to its inactive metabolite, cortisone. Importantly, maternal glucocorticoid excess in the first trimester of pregnancy is associated with a positive placental feedback loop that accelerates rates or excessive levels of placental CRH in the third trimester with consecutive induction of preterm labor [25]. We and others have previously shown that placental CRH expression and CRH in maternal plasma are significantly elevated in pregnancies complicated by IUGR [26,27], however placental 11β-HSD2 expression is reduced [28–30], possibly contributing to fetal hypercortisolism in IUGR. Collectively, these observations underscore the importance to further investigate the role of the placental cortisol metabolism to improve fetal care and postnatal outcome.

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So far, most of the experimental data is either based on quantitative measurements of plasma cortisol/cortisone, CRH and progesterone levels via immuno-assays (e.g. RIA, ELISA) at the fetal and maternal side [14,31–33], or on placental 11β-HSD2 expression at the mRNA level [23,34]. While the use of these methods brought forth our understanding of placental physiology, they share the drawback of being insufficiently selective: As a stand-alone measurement, 11β-HSD2 expression analysis does not allow the determination of 11β-HSD2 activity, as the cortisol and cortisone levels remain unknown. Quantitative immuno-assays are sufficient to determine cortisol and cortisone in tissue (each at a time), however they face possible cross-reactivity and tissue perturbations, hence they are often labor-intensive as additional purification steps are required [35]. The use of these immuno-assays to determine CRH, as a peptide hormone, in tissue is limited to the fact, that these assays detect derivates of similar antigenicity that are often formed by posttranslational modifications essential for physiological function. A detailed comparison of possible drawbacks of CRH RIA and ELISA techniques was reviewed in detail by Latendresee et al. [36].

Recently, improved robustness and sensitivity of liquid-chromatography tandem mass spectrometry (LC-Tandem MS)-based techniques have led to reliable alternatives for peptide quantification [37–40]. Hence we set out to quantify CRH simultaneously with cortisol (F)/cortisone (E) and progesterone together in the same placental sample via LC-Tandem MS.

As we introduce this method for the first time, we thoroughly analyzed for possible interfering factors such as choice of placental sampling site and sampling technique itself.

2. Experimental

2.1. Placental sample collection and sample preparation

Following selective caesarean section at term of a singleton uncomplicated pregnancy, 6 tissue samples were collected under sterile conditions across the placenta of 9 placentas, as previously described [41]. Decidual basal plate remnants were thoroughly removed and areas with calcifications avoided. 0.5 g of placental tissue were removed directly below the decidual plate and at mid-depth in a medial-to-lateral and basal-to-chorionic fashion (see Fig. 3a). Tissue samples were thoroughly rinsed with cold PBS, snap-frozen in low-bind protein Eppendorf tubes (Eppendorf, Hamburg, Germany) and stored at −80 °C. For further usage placental samples were thawed on ice (pre-cooled vials) and ice-cold 99% Ethanol (EtOH) containing protease inhibitors Aprotinin 2.0 ng/ml, Pepstatin A 13.4 ng/ml, Leupeptin 20.0 ng/ml (all from Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany). After initial manual grinding on ice, the samples were minced (Potter S homogenizer, Braun Inc. Biotech, Germany) and sonificated (UW2070, Bandelin Electronic, Berlin, Germany) for 40 s (settings: cycle 5, power 50%, 40 s). After centrifugation for 10 min (14000 U/min, 4 °C), supernatant was used for further analysis.

2.2. Ethics

The study was reviewed and approved by the Ethical Committee of the Medical Faculty of the University of Erlangen-Nürnberg (#2625 02/28/02). Written informed consent was obtained.

2.3. Liquid chromatography- tandem mass spectrometry (LC-Tandem MS)

2.3.1. CRH

2.3.1.1. Materials and chemicals. Human (CRF, H-2435) and bovine (CRF, H-3264) CRH were purchased from Bachem (Bachem AG, Bubendorf, Switzerland). Ammonium acetate, formic acid, acetonitrile, zinc sulfate and sulfosalicylic acid were supplied by Merck (Merck Chemicals, Darmstadt, Germany). All other chemicals were of the highest purity available from Sigma–Aldrich. Cortisol, cortisone, progesterone were purchased from Sigma–Aldrich (Taufkirchen, Germany). Cortisol-9,11,12,12-D_4 and progesterone-2,2,4,6,6,17,21,21,21-D_9, were purchased from C/D/N ISOTOPES (Pointe Claire, Canada), with an isotopic enrichment of 98%. We prepared 1 g/L stock solutions of all three steroids in ethanol. For steroids measurement the precipitation solution was zinc sulfate monohydrate dissolved in water 50 g/L and methanol (MeOH) 1/1 [v/v].

2.3.1.2. Standards. Stock solutions of CRH were prepared by dissolving accurately weighed standard in 3% acetic acid. Standard solutions of human CRH for a range of 0.1–50.0 μg/L were prepared with concentrations calculated based on the peptide content declared by the manufacturer. Bovine CRH in MeOH containing 1% formic acid was used as the internal standard solution at a final concentration of 80 μg/L. Stock solutions, standards and blanks were stored at −20 °C. Calibrators containing the steroids in different concentrations across the concentration range of 0.1 μg/L – 250 μg/L were prepared by spiking an isotonic saline solution with the steroid stock solution of 10 mg/L. Progesterone-D_9 and cortisol-D_4 were used as internal standards. The internal standard solutions were made with ammonium acetate (2 mM) solution and methanol (1/1 v/v) at a final concentration of 250 μg/L for cortisol-D_4 and 25 μg/L for Progesterone-D_9. The stock solutions, standards and blanks were stored at 4 °C.

2.3.1.3. Sample preparation. After homogenization of placenta tissue 300 μL of supernatant were resuspended in 900 μL MeOH containing 1% formic acid. After continuous shaking for 10 min at 4 °C, the vials were centrifuged at 14000*g* for 10 min, 4 °C. Supernatants were transferred into deepwell plates for CRH analysis and placed in the autosampler at 15 °C.

2.3.1.4. Chromatographic conditions. The LC system consisted of a binary pump, a quaternary pump (Agilent 1100, Agilent Technologies, Böblingen, Germany) and a 12-port switching valve (VICI, Valco Instruments, Houston, USA). The sample was injected by use of a HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) fitted with a 1000 μL peak sample loop, through the Valco valve onto the extraction column (Oasis HLB 2.1∗ 20 mm, 15 μm, Waters, Eschborn, Germany). The extraction column was washed for 1.2 min (flow-rate 3 mL/min) with water/acetic acid/MeOH (92:3:5 by volume). The valve position was then switched to allow the bound material to be eluted from the extraction cartridge in back-flush mode. After 2.0 min, the Valco valve position was again switched to allow the extraction column to be purged by 1% sulfosalicylic acid (3 ml/min) and reequilibrated at a flow rate of 3 mL/min with water/acetic acid/MeOH (92:3:5 by volume). LC was performed with a Chromolith column (RP-18e, 100∗4.6 mm, Merck) at a flow rate of 1 ml/min. Mobile phase A consisted of ammonium acetate (10 mmol/L) and methanol (97:3 by volume), mobile phase B consisted of ammonium acetate (2 mmol/L) and methanol (5:95 by volume, pH 2.0). After 1.2 min the initial conditions (55% A) were increased to 100% A in 1.8 min via a linear gradient, followed by a linear return to initial conditions within 1.0 min after 4 min. Total analysis time was 6.0 min.

2.3.1.5. MS/MS conditions. An API 4000 TM (Applied Biosystems, MDS Sciex, Analyst software, version 1.5, Darmstadt, Germany) mass spectrometer fitted with a turbo ion spray source was operated in positive ionization mode without a split. Eluates were analyzed in the tandem mass spectrometry (MS/MS) mode,

fragmenting the 4-fold–charged parent ions at m/z 1190.3 for human CRH and m/z 1175.0 for bovine CRH under optimized settings determined by previous flow-injection analysis. MS conditions were as follows: declustering potential (DP) of 116 V for human and 120 V for bovine CRH, collision energy (CE) of 57–61 eV for human CRH and 52–53 eV for bovine CRH, temperature of the ESI source 450 °C, ion spray voltage 5500 V, collision gas: high-purity nitrogen.

2.3.2. Steroid profiling

CRH, cortisol, cortisone and progesterone were determined in the same placental sample. LC-Tandem MS was performed using a modified online SPE-HPLC-MS/MS assay developed by Rauh et al. [42] and Koal et al. [43] allowing quantitative analysis of steroid hormones in 100 μL serum with atmospheric pressure chemical ionization in the positive ion mode.

In brief, the assay consists of a protein precipitation based sample preparation with methanol/zinc sulfate (50 g/L, 1/1, v/v). Following tissue preparation (see "Placental sample collection and preparation") 100 μL of the tissue supernatant and calibrators were deproteinized with 200 μL MeOH/zinc sulfate (50 g/L, 1/1 v/v) and 100 μL internal standard solution.

The online SPE was performed by a Chromolith extraction column (4.6 × 50 mm), which was coupled to a Chromolith HPLC column (RP-18e, 100 × 4.6 mm, Merck). The autosampler was a HTC PAL (CTC Analytics) fitted with a 250 μL sample loop. The HPLC-MS/MS system consisted of a Shimadzu LC-20AD HPLC unit and a quaternary pump (HPLC1200 series, Agilent Technologies, Waldbronn, Germany). The samples were washed with 5% methanol and eluted in back-flush with 2 mM ammonium acetate/MeOH (50:50, v/v) onto the analytical column. Mobile phase A was a mixture of MeOH and water (containing 2.0 mM ammonium acetate) in a ratio of 90:10 (v:v). Mobile phase B was a mixture of MeOH and water (containing 2.0 mM ammonium acetate; pH value adjusted to 4.5 with acetate acid) in a ratio of 5:95 (v:v). The total flow rate of the binary gradient module was maintained at 1 ml/min. After 1.0 min the initial conditions (50% A) were increased to 56% A in 3.8 min via a linear gradient, followed by linear gradient to 90% A in 1.7 min and a return to initial conditions after 7.5 min. The total online SPE-HPLC-MS/MS analysis time per sample was 8 min. The column temperature was set at 40 °C. The injection volume was 200 μL.

For MRM based mass spectrometric detection a 4000QTrap® triple quadrupole mass spectrometer (AB Sciex, Toronto, Canada) equipped with an APCI source in positive mode was used. Sample analysis was performed in the multiple-reaction monitoring mode with a dwell time of 150 ms per channel using the following transitions for quantification: m/z 363.2/121.2(309.4) cortisol, m/z 361.1/162.9(239.0) cortisone, m/z 315/97(109.1) progesterone, cortisol D_4 367.3/121.2, and progesterone D_9 324.3/100.4. The parameters of the mass spectrometer are summarized as follows: Curtain Gas (CUR) at 15, collision assisted dissociation Gas (CAD) at 7, nebulizer current (NC) at 5.0, temperature (TEM) at 450 °C, sheath Gas (GS1) at 55 and resolution at unit.

2.3.3. Assay validation

The linearity of the method was evaluated across the concentration range of 0.1–50 μg/L for CRH, and 0.1–1000 μg/L for the steroid hormones. The assay acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except at the lower limit of quantification which was set at 20%. The lower limit of quantification (LLOQ) was defined as the lowest concentration with a RSD of ⩽20%. The calibration curve was required to demonstrate a correlation coefficient of 0.990 or higher.

We defined the lowest limit of detection as a signal at least three times greater than baseline noise. The lower limit of detection (LLOD) was ascertained by the analysis of 5.0 μg/L calibrator and was established as the concentration at which a S/N ratio of 3.0 was obtained.

The interassay imprecision was calculated using the mean and coefficient of variation [RSD]. To determine interassay variability, we analyzed six different extracts of one placenta tissue sample, which was homogenized in one single step. Additionally, we sliced one placenta sample in six parts, to estimate the impact of tissue homogenization. The interassay imprecision was calculated using the mean coefficient of variation [RSD] of the six replicates, two of which were measured on three separate days in duplicate.

To investigate the possible interference of the tissue matrix with MS signals, we compared the peak area of the internal standard in calibrator solutions and in the presence of matrix. Recovery rates were determined using four processed tissue samples which had been spiked with standard solution to give a nominal concentration of 10, 20, 30 and 40 μg/L. The recoveries were calculated by linear regression.

3. Statistical analysis

Data processing and graphic presentation were performed with Sigmaplot 200 (Systat Software GmbH, Erkrath, Germany), Origin software (Originlab Corp., Northampton, MA, USA) and GraphPad Prism Version 4.0c (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± standard error (SEM), unless otherwise stated.

4. Results

4.1. Assay characteristics

Tandem MS characteristics for steroid analysis using a modified online SPE-HPLC-MS/MS assay have been described in detail previously [42–44]. Hence, we focused on the description of CRH assay characteristics. Adaptions to the established steroid protocol were outlined, where needed.

For CRH detection an online extraction method with a column-switching technique was used. Representative ion chromatograms are shown in Fig. 2. At a flow rate of 1 mL/min, the total run time was 6 min. Under the chromatographic conditions described, the mean (SD) retention time for CRH was 4.53 min. As demonstrated in Fig. 1a CRH was detected as its 3- to 5-fold–charged ions, $(M + 3H)^{3+}$, $(M + 4H)^{4+}$ and $(M + 5H)^{5+}$, showing the predominant intensity at m/z for the 4-fold–charged analyte (M, molecular mass; z, the charge on the molecule caused by the uptake of protons during ionization). The most abundant 4-fold–charged ions were fragmented by collision-induced dissociation, yielding a typical series of *y*- and *b*-ions assignable to the fragments indicated in Fig. 1b. The most intensive product ion signals were chosen for the multiple reactions with $m/z > m/z$ parent ion [45]: 1190.3/1439.3, 1190.3/1471.6, 1190.3/1103.9, 1190.3/1099.6 -for human CRH and 1175.0/1451.5, 1175.0/1419.0 -for bovine CRH. The total signal intensity of all respective product ions was used to display the chromatogram, allowing the quantification of samples and standards. Bovine CRH was used as an internal standard, because of its chemical and structural similarity to human CRH (Supplementary Fig. 1). The amino acid sequences of human and bovine CRH differ by only 8 amino acids at positions 2, 22, 23, 25, 33, 38, 39, 41 (81% sequence homology). As a result, the molecular weights (MW) (human MW 4757.52, bovine MW 4697.40) and isoelectric points are also quite similar.

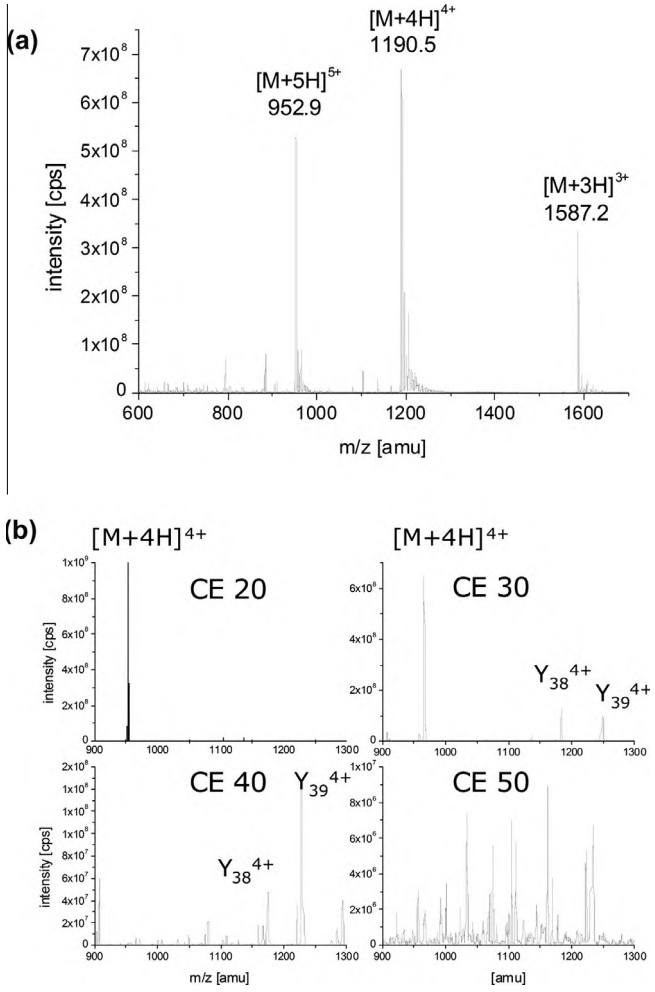

Fig. 1. (a) ESI Q1 full scan mass spectra of human (MW 4757.52) CRH. Full-MS analysis demonstrates triply charged $[M + 3H]^{3+}$, 4- $[M + 4H]^{4+}$ and fivefold–charged $[M + 5H]^{5+}$ ions. (b) ESI mass spectra of human CRH following collision-induced dissociation: Product ion scan spectrum of the fourfold–charged ion signal $[M + 4H]^{4+}$, resulting product ions are assigned to the amino acid sequence.

4.2. Assay validation

The precipitation method allows rapid, easy and effective tissue preparation. The results indicated that a precipitation with formic acid and methanol achieved the optimal extraction recovery of analytes. The mean recovery of extraction for bovine CRH added to placental tissue lysates ($n = 4$) was 108 ± 2% with internal standard correction. To investigate the possible interference of the tissue matrix with MS signals, we compared the peak area of the internal standard in calibrator solutions and in the presence of matrix. On average, in these cases the signals were 105 ± 8% for CRH, 74 ± 13% for progesterone and 79 ± 9% for cortisol, indicating only weak matrix effects for CRH, and moderate matrix effects for steroids.

The method proved linear from 0.1 to 50 μg/L ($r > 0.99$, injection volume 900 μL) for CRH and up to 1000 μg/L for the specified steroids. Calibration curves were calculated using linear least squares regression according to the equation $y = a + bx$, where y is the peak-area ratio of substance to internal standard and x is the analyte concentration of the calibrator sample. We used $1/x$ weighting to ensure maximum accuracy at lower concentrations. The mean y-intercept was 0.0002 and the mean slope was 0.019 for CRH.

The lowest limit of detection was 0.1 to 0.2 μg/L, when analyzing calibrator samples. The limit of quantification derived from the precision profile curve of CRH was 0.5 μg/L. The mean interassay coefficients of CRH were 6.3% ($n = 12$) for supernatant processing and 15.1% ($n = 12$) for processing including tissue homogenization. Similar results were found for cortisone (4.0% and 13.2%, respectively) and progesterone (8.3% and 12.2%, respectively). For cortisol the interassay coefficient for supernatant processing was 34%, compared to a coefficient of only 3.6% after homogenization, indicating a higher variability of cortisol in placental tissue.

4.3. LC-Tandem MS detection of placental CRH

We analyzed the variation of CRH content in proximal, intermediate and peripheral placental regions (medial-to-lateral sampling, Fig. 3a and b) and CRH content in samples from the placental core vs. samples below the decidual basal plate at the villous surface (basal-to-chorionic sampling, Fig. 3a and b).

Medial-to-lateral sampling in the placental core region showed a significantly ($p < 0.05$) higher variability than horizontal sampling at the placental surface ($n = 6$ for all):

Placental CRH content at the core region in relation to the sample distance to the umbilical cord was: proximal 14.7 ± 2.6 ng/g (range 31.5 ng/g), intermediate 17.0 ± 2.1 ng/g (range 36.2 ng/g), peripheral 10.9 ± 1.1 ng/g (range 15 ng/g). Placental CRH content at the villous surface in relation to the sample distance to the umbilical cord was: proximal 14.1 ± 0.2 ng/g (range 1.6 ng/g), intermediate 13.2 ± 0.9 ng/g (range 10.6 ng/g), peripheral 13.0 ± 0.9 ng/g (range 6.9 ng/g). There was no significant difference in CRH content when comparing basal to chorionic samples (Fig. 4a): the mean CRH content at the placental core was 14.5 ± 1.2 ng/g versus 13.4 ± 0.5 ng/g CRH at the placental surface.

4.4. LC-Tandem MS detection of placental cortisol and cortisone

We analyzed the variation of cortisol (F) and cortisone (E) content in proximal, intermediate and peripheral placental regions (medial-to-lateral sampling, Fig. 3a and b), as well as F and E content in samples from the placental core vs. samples below the decidual basal plate at the villous surface (basal-to-chorionic sampling, Fig. 3a and b). In the placental core region (chorionic sampling, Fig. 3a) both F and E content was low, at 0.9 ± 0.1 ng/g – 1.2 ± 0.2 ng/g. There was a significant difference ($p < 0.001$) in F and E content when comparing basal to chorionic samples (Fig. 3a and b): Medial-to-lateral sampling at the basal placental surface returned significantly higher F and E contents ($p < 0.001$, $n = 6$ for all) ranging from 3.6 ± 0.4 ng/g – 5.6 ± 0.4 ng/g for F and 73.3 ± 6.8 ng/g to 80.3 ± 3.0 ng/g for E. This resulted in a significantly ($p < 0.001$) higher F/E–ratio of 0.063 ± 0.006 at the placental surface when compared to the F/E ratio at the chorionic placental core 0.013 ± 0.001 (Fig. 4b). Medial-to-lateral sampling at the basal placental surface only slightly influenced F/E–ratios, with a significantly lower F/E–ratio of 0.05 ± 0.01 ($p < 0.05$) in the intermediate region (Fig. 3b), when compared to proximal (0.08 ± 0.012) and peripheral (0.08 ± 0.007) sampling site F/E–ratios.

4.5. LC-Tandem MS detection of placental progesterone

We analyzed the variation of progesterone content in proximal, intermediate and peripheral placental regions (medial-to-lateral sampling, Fig. 3a and b), as well as progesterone content in samples from the placental chorionic core vs. samples below the decidual basal plate at the villous surface (basal-to-chorionic sampling, Fig. 3a and b).

Basal-to-chorionic sampling revealed, that progesterone tissue levels (907.4 ± 36.1 ng/g, range 684.0 ng/g – 1120.0 ng/g) at the basal placental surface were significantly ($p < 0.005$) higher than the

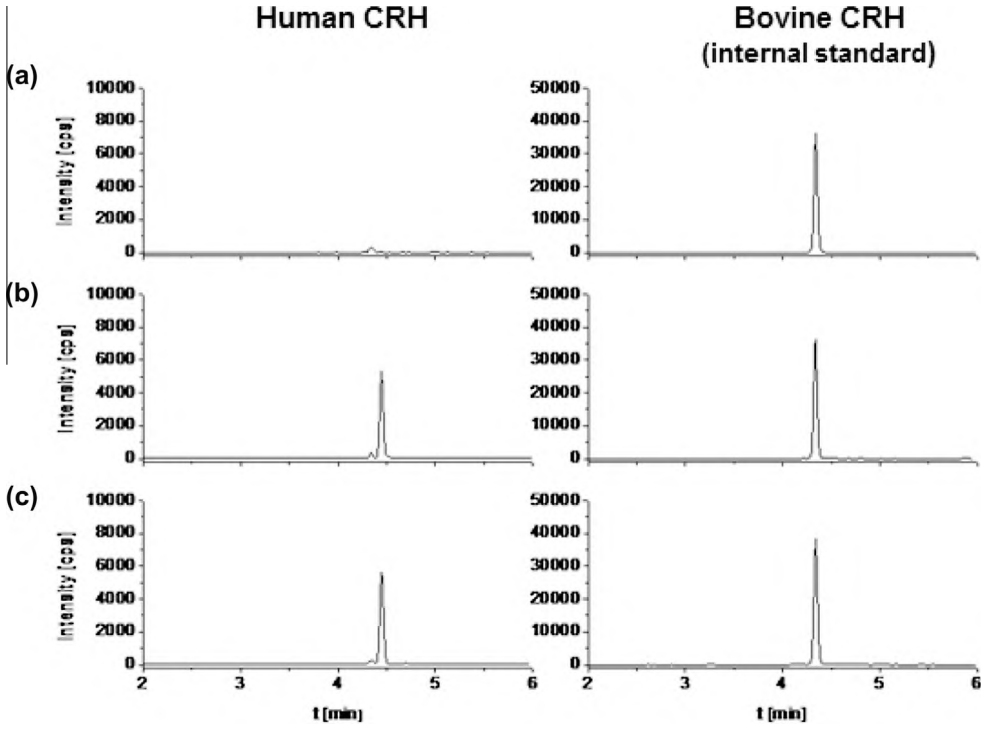

Fig. 2. Representative HPLC-MS/MS chromatograms of human CRH (left) and bovine CRH (internal standard, right), detected by fragmentation of the fourfold-charged precursor ions $[M + 4H]^{4+}$. The retention time of CRH was 4.5 min. The total chromatographic analysis time was 6 min per sample. MRM transitions (sum of the respective transitions). Legend: (a) blank value, (b) calibrator sample (10 μg/L), (c) placental sample (human CRH 19.2 ng/g).

progesterone levels at the placental core (749.7 ± 33.3 ng/g, range 556.0 ng/g–904.0 ng/g), as shown in Fig. 4c.

Medial-to-lateral sampling showed no significant difference for basal placental samples (range 887.8 ± 117.4 ng/g – 919.3 ± 25.5 ng/g), while there was a significantly lower progesterone content in chorionic placental samples from the periphery (607.0 ± 26.12 ng/g), when compared to proximal (818.5 ± 28.6, $p < 0.002$) and intermediate (700.0 ± 47.8, $p < 0.02$) chorionic samples from the villous core region (Fig. 3a and b).

5. Discussion

The focus of our study was the evaluation of LC-Tandem MS measurement of CRH with its signaling partners in placental tissue. As the architecture and perfusion varies greatly across the placental disk, special care has to be applied to sample preparation. Sampling parameters, such as proximity to the umbilical cord, or to the basal- or chorionic- plate may strongly influence gene and protein expression [46]. Interestingly, placental samples from pregnancies complicated by IUGR and SGA, show different mRNA expression rates than samples from AGA placentas (all placental core samples), depending on the sampling site relative to the umbilical cord, as recently shown for CRH expression [41]. We show that the optimal read-out for cortisol turnover to cortisone is seen beneath the decidual surface of the organ, while sampling at proximal, intermediate and peripheral placental regions had no influence. This finding supports the function of 11β-HSD2 as feto-maternal enzymatic barrier [21], with placental tissue at close proximity to the maternal environment showing a greater cortisol/cortisone–ratio. Progesterone was measured at higher levels in the placental surface area, while CRH was readily detectable at stable levels throughout the whole placenta. We were not able to identify a significant correlation of CRH with any of the other placental factors tested, however this might be related to the limited number of samples studied. As a result, we introduce LC- Tandem MS as a reliable, quantitative and multi-modal method for the analysis of CRH and its signaling partners in placental tissue. Because the placental interface links the maternal and fetal HPA-axis via CRH, multimodal analysis of CRH-interacting endocrine factors at the placental level was another aim of our study. Importantly, we were able to measure progesterone in the same tissue sample with CRH. The interaction of CRH and progesterone in promoting myometrial contractility or quiescence at the feto-maternal interface is important for the timing of labor [47] (reviewed in detail by Vrachnis et al. [48]). The onset of labor is thought to be secondary to a functional progesterone withdrawal [49,50]. Progesterone differentially controls placental CRH promoter activity via its receptor isoforms A and B [51] and fosters CRH-induced uterine quiescence via promoting CRH receptor availability. Interestingly, CRH negatively feeds back to progesterone by decreasing enzymatic progesterone production via PKC-dependent inhibition of placental CYP11A1 and HSD3B1 expression [52]. The effect of functional progesterone withdrawal in late pregnancy on the above receptors might enable myometrial contractions during labor [53], possibly by reduction of the relaxant effect of CRH [7]. Lately, it became evident that CRH and progesterone are not only involved in changes in the endocrine environment at the feto-maternal interface, but modulate local inflammatory immune responses, including the production of cytokines (IL-1 β, IL-6) that control significant mechanisms of labor. Cytokines, such as TNF and IL-1 β, not only induce CRH production directly [54], but also indirectly via inhibition of placental 11b-HSD 2 [55]. The consecutive increase of cortisol levels could increase placental CRH production [23]. In this respect, LC-Tandem MS enabled us to additionally quantify the CRH proxys cortisol/cortisone in the same tissue sample with CRH and progesterone.

Although we focused on the analysis of healthy placentas at term, the presented multi-modal method of placental hormone analysis might be of use for the future analysis of placental processes in pregnancies complicated by intra-uterine growth

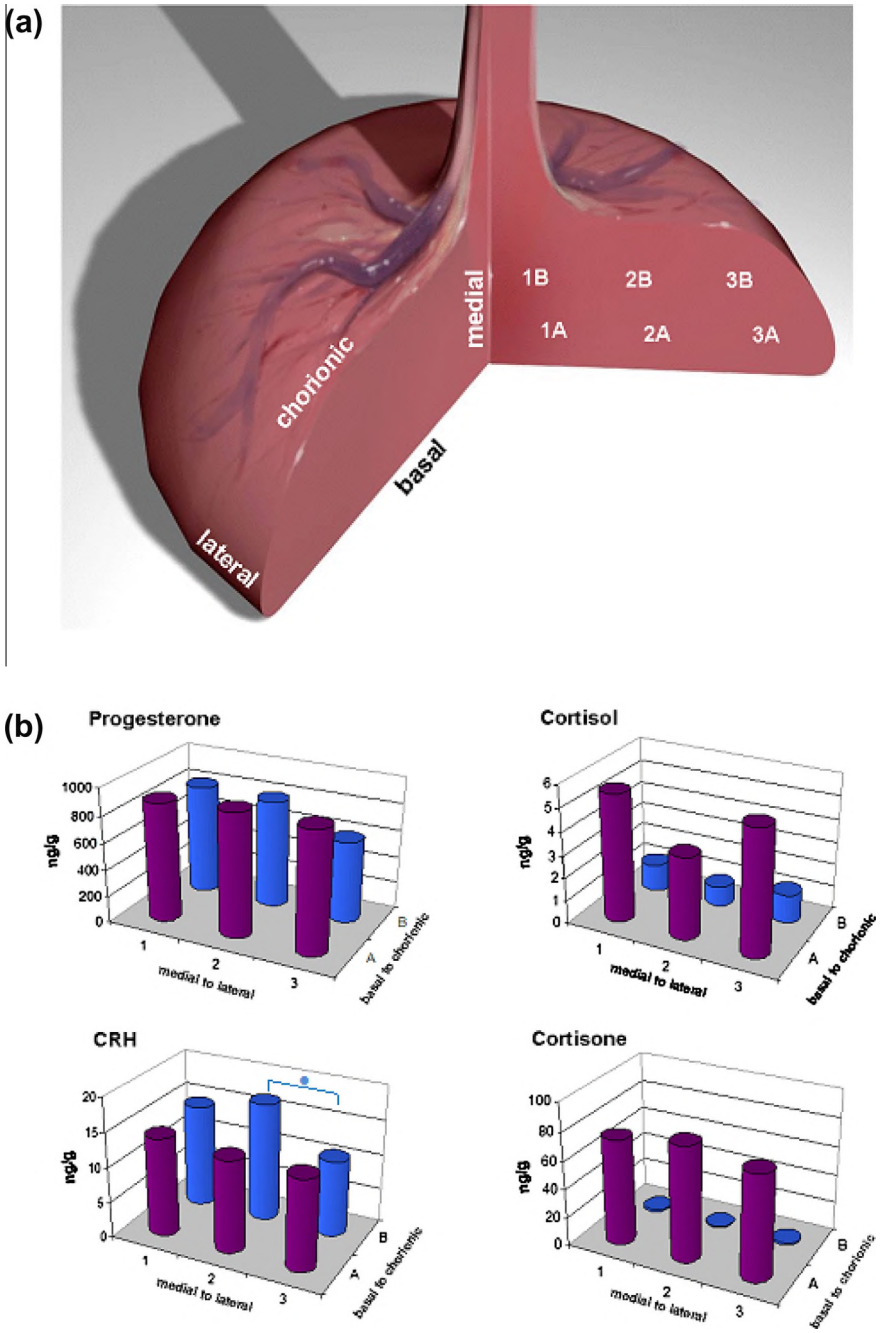

Fig. 3. The correlation between gene expression level and location of placental sampling site. Layout adopted from Wyatt et al. [46]. (a) Location of sampling sites within the placental disk. (b) The placental hormone expression in ng/g according to sample location ($n = 6$, for all). Legend: A = basal, B = chorionic; 1 = proximal, 2 = intermediate, 3 = peripheral sampling sites in relation to the umbilical cord stem. ● = $p < 0.05$.

restriction (IUGR). IUGR is characterized by increased placental CRH [26,27], reduced 11β-HSD2 activity and hence fetal hypercortisolism [16,30,56]. So far most studies analyzing placental 11β-HSD2 activity in human cohorts use either placental tissue lysates [56] or try to indirectly asses its activity by comparing cortisol cord blood levels of newborns to maternal levels [14,31].

The role of serum progesterone in IUGR is controversially discussed. Older studies in Japanese cohorts were indicative of lower levels of progesterone in IUGR, following a multifactor analysis [57,58]. However, low oxygen pressure at high altitudes (an in vivo model resembling IUGR and preeclampsia) has been shown to increase progesterone levels and concomitantly lower estradiol levels [59]. To our knowledge, there are no reports on placental progesterone tissue levels in IUGR.

Beyond its role at the maternal side, placental CRH also acts on the fetal pituitary-adrenal axis [60]: via induction of adrenocorticotropic hormone (ACTH) in the fetal pituitary and in the placenta CRH indirectly stimulates the fetal adrenals to produce cortisol. As CRH increases exponentially at the end of gestation and placental CRH production is induced via cortisol, CRH exerts a positive feed-back loop on its own placental production. CRH also fosters negative feed-back loops: CRH induced ACTH additionally drives adrenal dehydroepiandrosterone(-sulfate) (DHEA/DHEA-S) secretion, which is converted into estrogens at the placental level.

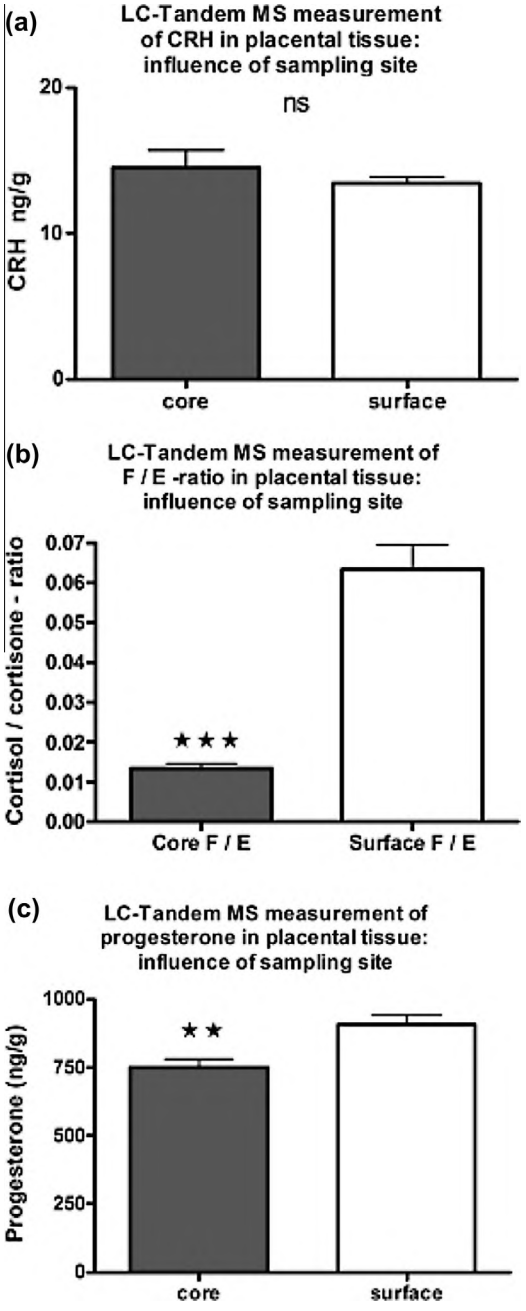

Fig. 4. LC-Tandem MS measurement of (a) CRH (ng/g), (b) cortisol / cortisone–ratio and (c) progesterone in placental tissue: influence of sampling site (core vs. surface). Legend: "Core" equals the pooled "A" samples, "surface" equals the pooled "B" samples of Fig. 3b; F = Cortisol, E = Cortisone, ns = not significant, ★★ = $p < 0.005$, ★★★ = $p < 0.0001$.

Estrogen, in turn, is known to inhibit CRH production, hence facilitating parturition [47,61,62]. Interestingly, we were previously able to successfully measure estrogen and DHEA/S via LC-Tandem MS in serum [43] together with progesterone and cortisol/cortisone. Therefore it seems intriguing for future studies to include the placental metabolism of these hormones in our presented LC-Tandem MS tissue analysis. We hope that our methodical concept of multi-modal placental tissue analysis via LC-Tandem MS will strengthen the predictive value of placental endocrine biomarkers for a more efficient prevention of fetally programmed post-IUGR morbidities, as already shown for single placental biomarkers [14,34,63].

Taken together the multi-modal LC-Tandem MS approach helps to effectively link the above placental feedback loops at the level of the placenta, providing a deeper insight into the placental glucocorticoid metabolism and hence into the regulation of fetal development and maternal parturition.

6. Authors' contributions

MR contributed to conception and design of the study, to data acquisition and critically revised the manuscript for important intellectual content. WR and MRue critically revised the manuscript for important intellectual content. FBF analyzed and interpreted the data and drafted the manuscript.

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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.steroids.2013.04.015.

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