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A novel radioimmunoassay of 16α-hydroxy-dehydroepiandrosterone and its physiological levels

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Abstract

16α-Hydroxy-dehydroepiandrosterone (16α-OH-DHEA) belongs to the products of extensive DHEA metabolism in mammalian tissues. It is a precursor of 16α-hydroxylated estrogens, increased levels of which are associated with autoimmune disorders. A highly specific radioimmunoassay of unconjugated 16α-OH-DHEA was developed and evaluated. Polyclonal rabbit antisera were raised against 3β,16α-dihydroxy-17,19-dione-19-*O*-(carboxymethyloxime) and 3β,16α-dihydroxy-7,17-dione-7-*O*-(carboxymethyloxime) BSA conjugates. Two methods were used for preparation of the conjugates. Homologous radioiodinated derivatives with tyrosine methyl ester were prepared as tracers. While antisera to 7-CMO cross-reacted with DHEA as much as by 58%, the cross-reaction of the chosen antiserum prepared via 19-oxogroup by micellar conjugation technique with 16β-OH-DHEA was only 0.13% and with all other structurally related steroids, including DHEA were lower than 0.01%. The detection limit was 0.017 pmol (5.7 pg)/tube, the average intra- and inter-assay coefficients of variation were 8.2 and 11.4%, respectively. Mean recovery of serum spiked with 16α-OH-DHEA varied between 80 and 110%, the results were independent on sample dilution. 16α-OH-DHEA concentrations in 18 randomly selected sera, including 6 samples from patients with thyroid cancer were compared with results obtained by earlier GC–MS method. Physiological levels of 16α-OH-DHEA in 316 sera (184 females and 132 males) analyzed so far varied between 0.0 and 1.86 nmol/l.

Keywords: 16α-Hydroxy-dehydroepiandrosterone; Radioimmunoassay; Physiological levels

1. Introduction

Dehydroepiandrosterone (DHEA) produced from cholesterol in the adrenals as a sulfate is the most abundant steroid in human circulation. It undergoes an intensive metabolism in many mammalian tissues. The main site of its metabolism is liver, but it proceeds in many other tissues, including brain. One of its metabolites is also 16α-hydroxy-dehydroepiandrosterone (16α-OH-DHEA) [1,2].

16α-OH-DHEA is a precursor of fetal 16α-hydroxylated estrogens. As early as in 1950s, it had been detected in relatively large concentrations in umbilical blood of newborns and during the early neonatal life, as well as in amniotic fluid; for literature see Ref. [3].

The enzymes responsible for 16-hydroxylation of steroids belong to non-specific monooxidases of cytochrome P-450 family, commonly denoted EC 1.14.14.1. Not less than 200 genes encoding for various isoenzymes of this group are known, of which more than 20 occur in humans. For steroid 16α-hydroxylation in humans of special importance are two enzymes, CYP3A4 from liver microsomes and CYP1A1, present in various tissues [4].

Since, development of its first immunoassay [5], it has been known that it is a normal constituent of human serum. In adults, increased levels of 16-hydroxylated estrogens were put in relation to the risk of cancer of female reproductive system [6] and more recently, to some systemic autoimmune diseases [7,8]; for review, see also Ref. [2]. Because 16α-OH-DHEA is a precursor of the latter steroids, we have raised a hypothesis whether increased formation of 16α-OH-DHEA could not be a risk factor in development of these diseases. Here, a novel specific immunoassay for its determination in

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human serum is presented, together with its physiological levels in a representative sample of population.

2. Materials and methods

All chemicals were of analytical grade from Sigma (St. Louis, MI, USA), 16α-OH-DHEA and other steroids were purchased from Steraloids (Newport, RI, USA). Solvents used for HPLC and GC–MS were of HPLC grade, Sylon B was from Supelco (Bellefonte, PA, USA).

Development of the method included synthesis of the haptens, their conjugation with bovine serum albumin (BSA) by two different techniques, immunization of rabbits, collection and characterization of antisera, synthesis of tracer and optimization of the radioimmunoassay.

2.1. Hapten synthesis

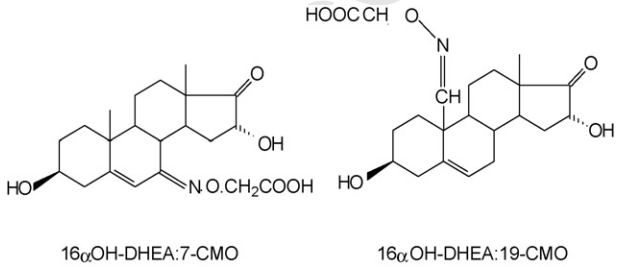
The haptens 3β,16α-dihydroxy-5-androstene-17,19-dione-19-*O*-(carboxymethyloxime) (16α-OH-DHEA-19-CMO) and 3β,16α-dihydroxy-5-androstene-7,17-dione-7-*O*-(carboxymethyloxime) (16α-OH-DHEA-7-CMO) were synthesized by Pouzar et al. by recently published method [9]. Their formulas are shown in Fig. 1.

2.2. Preparation of immunogens

The common mixed anhydride, and the micellar method of Yatsimirskaya et al. [10] have been used for conjugation of each hapten to bovine serum albumin (BSA). The starting hapten–protein molar ratio was 50, the resulting conjugates contained 10–12 and 11–14 mol of the hapten in 1 mol of the conjugates, respectively, as determined by spectrophotometry with trinitrobenzene sulfonic acid.

2.3. Immunization of rabbits and collection of antisera

Female rabbits were immunized by a standard procedure [11] using 100 μg doses of the immunogens emulsified in a mixture of complete Freund's adjuvants–saline 1:1 (100 μl) in 3 weeks intervals. Three rabbits were immunized with each immunogen. After the third immunization, the sera were collected from the ear vein and lyophilized.



16αOH-DHEA:7-CMO 16αOH-DHEA:19-CMO

Fig. 1. 16α-OH-DHEA derivatives used as haptens for conjugation with BSA.

2.4. Synthesis of the tracer

Position homologous [^{125}I]iodo tyrosine methyl ester (TME) conjugates with both haptens were prepared by a convenient radioiodination using chloramine-T [11]. The conjugate itself was prepared by a modified *N*-hydroxysuccinimide method [12]. The conjugate and the tracer were purified on TLC plates (DC-Alufolien Art. 5593, Merck, FRG) in system dichloromethane-2-propanol 92:8 (v/v). The distribution of radioactivity on the plate was measured by scanning with automatic TLC linear analyzer Tracemaster 40 with counting tube LB 2821 (Berthold, Wildbad, FRG).

2.5. Radioimmunoassay

Serum (100 μl) was diluted with 400 μl of saline and extracted twice with 2 ml of diethyl ether. The water phase was left frozen in solid carbon dioxide bath and ether extracts were combined and evaporated to dryness. The standard solutions contained 0.032–16.4 pmol (9.75–5000 pg) of 16α-OH-DHEA in 100 μl RIA buffer (20 mmol/l sodium phosphate–saline, pH 7.2 containing 1 g/l BSA). To the dry residues of samples or to the tubes for standard curve, the buffer or standard solution, radioligand and antiserum, 100 μl, each were added. The tubes for determination of unspecific binding contained radioligand and buffer only, final volume in each tube was 300 μl. The content of tubes was mixed in vortex, and after overnight incubation at 4 °C, 500 μl of dextran coated charcoal (0.25 and 0.025 g/100 ml) was added to each tube except the total, the content was vortexed briefly and after 10 min staying at 4 °C, the tubes were centrifuged at 4 °C and 3000 rpm for 10 min. The supernatants were decanted into another set of tubes in which the radioactivity of ^{125}I was measured on Berthold gamma counter. The concentration of 16α-OH-DHEA was calculated from log–logit plot.

2.6. HPLC separation of 16α-OH-DHEA and its determination by GC–MS

Eighteen randomly selected sera, including six samples from patients with thyroid cancer were analyzed by the gas chromatography–mass spectrometry (GS–MS) method previously described for detection and quantification of 16α-OH-DHEA in human seminal plasma [13]. In brief, the procedure was as follows: serum (1 ml), spiked with [^{3}H] 11β-androstenedione (for monitoring of losses during procession) was extracted twice with diethyl ether and, after evaporation of the solvent, the extract was partitioned between water–methanol and *n*-pentane, the methanol–water phase was evaporated and the dry residue applied on reverse phase C18 column of the HPLC sytem (Gilson, Villiers le Bel, France). Isothermal elution (40 °C) followed using a binary linear gradient consisting of 15% water solution of acetonitrile containing 1 g/l ammonium bicarbonate–methanol.

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Standards were detected at 205 nm, 16α-OH-DHEA was collected at 12.4–12.9 min (peak 12.65). Following evaporation, the analyte-containing fraction was derivatized with O-(2,3,4,5,6-pentafluoro-benzyl)-hydroxylamine hydrochloride (PFBOX-HCl)-pyridine mixture (2:98, v/v) at 80 °C and, after further evaporation, pyridine and Sylon B (bis(trimethylsilyl)trifluoracetamide) were added and the incubation was repeated. The content was then extracted with acetonitrile–isooctane (1:4, v/v), the lower phase was removed and the upper phase containing pentafluorobenzyloxime trimethylsilyl derivative was evaporated again. The dry residue was dissolved in isooctane solution containing internal standard (17α-methylandrostenediol, previously derivatized in the same way). 16α-OH-DHEA (external standard) was derivatized in the same way and used for calibration. The samples were then analyzed by GC–MS. The system from Shimadzu (Kyoto, Japan) consisted of the gas chromatograph GC-17A, the simple quadrupole analyzer QP 5050 A equipped with electron impact ionization on 70 eV, with the mass range from 10 to 900 amu. The medium-polar capillary column ZEBRON ZB-50 from Phenomenex (Torrance, CA, USA) was used with helium as a mobile phase. The temperature at the injection port was 300 °C of the interface 320 °C. Analyses were performed according to temperature and pressure program described in detail under Ref. [13]. Splitless mode was used; the detector voltage was 2 kV. The detection was performed by selected ion monitoring (SIM) mode. For checking the reproducibility, the internal standard fragments *m/z* 253.3 and 268.3 were monitored, while fragments *m/z* 266, 256 and 446 were followed in the case of calibration and samples.

2.7. Samples

The rests of serum samples collected during the screening programme of iodine deficiency in the Czech Republic within the years 2003–2005 have been used for determination of physiological levels of 16α-OH-DHEA. The samples of patients with overt endocrinopathies were excluded. In addition, six sera from patients operated for thyroid carcinomas were analyzed by both RIA and GC–MS methods. The use of the samples was approved by the Ethical Committee of the Institute of Endocrinology (Prague).

2.8. Statistics

Statistical software GraphPad Prism (Aurora, CO, USA) was used for linear regression analysis. Robust Kruskal–Wallis ANOVA followed by Kruskal–Wallis multiple comparisons was used for evaluation of differences between individual age groups. Statistical software Statgraphics Plus 3.0 (Manugistics Inc., Rockville, MA, USA) was used.

3. Results

3.1. Specificity of the antisera

The cross-reactions of three polyclonal rabbit antisera obtained after immunization with immunogens to 16α-OH-DHEA, synthesized via 7-oxo- or 19-oxo-groups and by using two different conjugation techniques, with 12 chemically related steroids occurring in human serum are shown in Table 1. The antiserum prepared via 19-CMO and by mixed anhydride technique was further used in the method.

3.2. Reliability criteria of the method

3.2.1. Precision

The average intra- ($n = 10$) and inter- ($n = 8$) assay coefficients variation of mixed serum pool containing 0.865 nmol/l of the analyte were 8.2 and 11.4%, respectively.

Table 1
Cross-reactions of three polyclonal rabbit antisera to 16α-OH-DHEA prepared via 7-CMO- and 19-CMO-groups using two conjugation techniques with selected steroids present in human serum

Steroid	Via 7-CMO[a] Micellar[b,c]	Via 19-CMO[a] Micellar[b,c]	Via 19-CMO[a] Mixed anhydride[b]
16α-OH-DHEA	100	100	100
16β-OH-DHEA	43.8	3.8	0.13
DHEA	58.0	0.28	<0.01
3β,16α-Dihydroxy-5-pregnen-20-one	6.27	<0.01	<0.01
5-Androstene-3β,17β-diol	3.95	<0.01	<0.01
3β-Hydroxy-5-pregnen-20-one	1.87	<0.01	<0.01
3β-Hydroxy-5α-androstan-17-one	7.72	0.13	<0.01
Estriol	<0.01	<0.01	<0.01
DHEAS	0.02	0.07	<0.01
Progesterone	0.28	0.03	<0.01
4-Androstene-3,17-dione	0.73	0.10	<0.01
Testosterone	0.83	0.02	<0.01

[a] Antiserum.
[b] Method.
[c] Conjugation of the hapten derivative with BSA was performed by the "micellar" technique of Yatsimirskaya et al. [10].

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Table 2
Recovery of 16α-OH-DHEA added to normal pooled human serum

Added steroid	Expected	Found (mean ± S.D.)	CV (%)	%Recovery
0		0.865 ± 0.071	8.21	100
0.657 nmol/l (20 pg/tube)	1.522	2.074 ± 0.128	6.17	136
2.628 nmol/l (80 pg/tube)	3.493	4.859 ± 0.288	5.93	139
10.512 nmol/l (320 pg/tube)	11.377	10.501 ± 1.201	11.4	92.3

Mean ± S.D. from five experiments.

Table 3
Independence on dilution

Serum volume (μl)	Found: mean ± S.D. (pmol/tube)	Recalculated (nmol/l)	CV (%)
12.5	0.0232 ± 0.0052	1.856	22.5
25	0.0459 ± 0.00652	1.836	14.2
50	0.0720 ± 0.004327	1.440	6.01
100	0.1728 ± 0.09487	3.728	5.49
200	0.2838 ± 0.02497	1.419	8.80

3.2.2. Sensitivity

The detection limit, expressed as the minimal amount of 16α-OH-DHEA distinguishable from the zero sample with 95% probability was 0.017 pmol (5.7 pg)/tube.

3.2.3. Accuracy

Pooled sample of human serum was spiked with increasing amounts of the analyte (0.657, 2.628 and 10.512 nmol/l, i.e. 20, 40 and 320 pg/100 μl). The recovery varied from 92.3 to 139% (Table 2).

3.2.4. Independence on dilution

12.5–200 μl samples of serum were processed. The recalculated levels did not depend on dilution within this range (Table 3).

3.3. Comparison with GC–MS method

Eighteen randomly selected sera, including six sera from patients operated for thyroid carcinoma were analyzed by presented RIA and previously published GC–MS technique [13]. The linear regression line with 95% confidence limits is shown in Fig. 2.

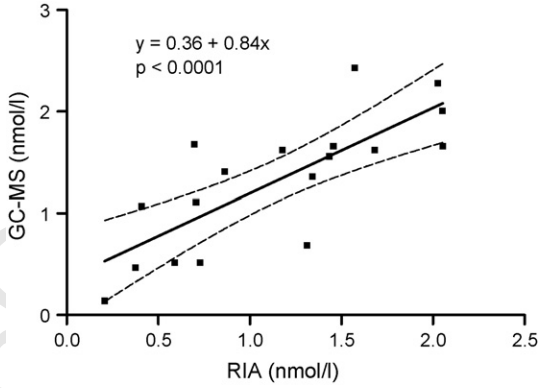

Fig. 2. Comparison of radioimmunoassay (RIA) and gas chromatography–mass spectrometry (GC–MS) for determination of 16α-OH-DHEA in 18 human sera. The full straight line shows linear regression, the dotted lines 95% confident limits.

3.4. Physiological levels

Physiological levels of 16α-OH-DHEA were determined in 316 sera (184 females and 132 males). The results are summarized in Fig. 3(a, females; b, males). The individual data are listed according to nine age groups from children below 10 years up to adults above 60 years. They cover a broad range from 0 to 1.864 nmol/l in males and 0–1.438 nmol/l in females, respectively. Full squares depict medians, vertical error bars 25 and 75% percentiles.

The differences between individual pairs of age groups are shown in Table 4. The matrix provides the *Z*-values obtained by Kruskal–Wallis multiple comparison test. The medians (see Fig. 3) differ significantly if *Z*-values are higher than 1.96 (bold letters). The upper right part of the matrix shows the data from females, the lower left part show those of males.

Table 4
Significance of differences between individual pairs of age groups

Age group (years)	Below 10	10–12	13–15	16–20	21–30	31–40	41–50	51–60	Above 60
Below 10	0	1.6657	**4.3501**	**3.6162**	**5.1154**	**4.3082**	**4.2677**	**3.2731**	**3.9937**
10–12	1.4637	0	**2.8487**	**2.087**	**3.4529**	**2.5393**	**2.4033**	1.4296	**2.4788**
13–15	**3.1586**	**2.2944**	0	0.734	0.1667	0.839	1.1578	1.9263	0.3564
16–20	**4.3649**	**3.794**	**2.2246**	0	1.0016	0.0295	0.2424	1.049	0.3775
21–30	**5.0925**	**4.7333**	**2.9342**	0.273	0	1.226	1.6673	**2.5572**	0.5722
31–40	**4.8211**	**4.3949**	**2.5913**	0.0108	0.2931	0	0.3669	1.4023	0.4172
41–50	**2.8103**	1.8499	0.2022	**2.2449**	**2.8832**	**2.5658**	0	1.167	0.7133
51–60	1.8558	0.6233	1.4109	**3.1124**	**3.8554**	**3.5421**	1.106	0	1.5003
Above 60	1.8156	0.7982	0.72	**2.3302**	**2.7977**	**2.5462**	0.5423	0.3127	0

The matrix shows the *Z*-values obtained by Kruskal–Wallis multiple comparison test. The medians (see Fig. 3) differ significantly if the *Z*-values are higher than 1.96 (bold letters). The upper right part of the matrix gives the data from females, the lower left part those of males.

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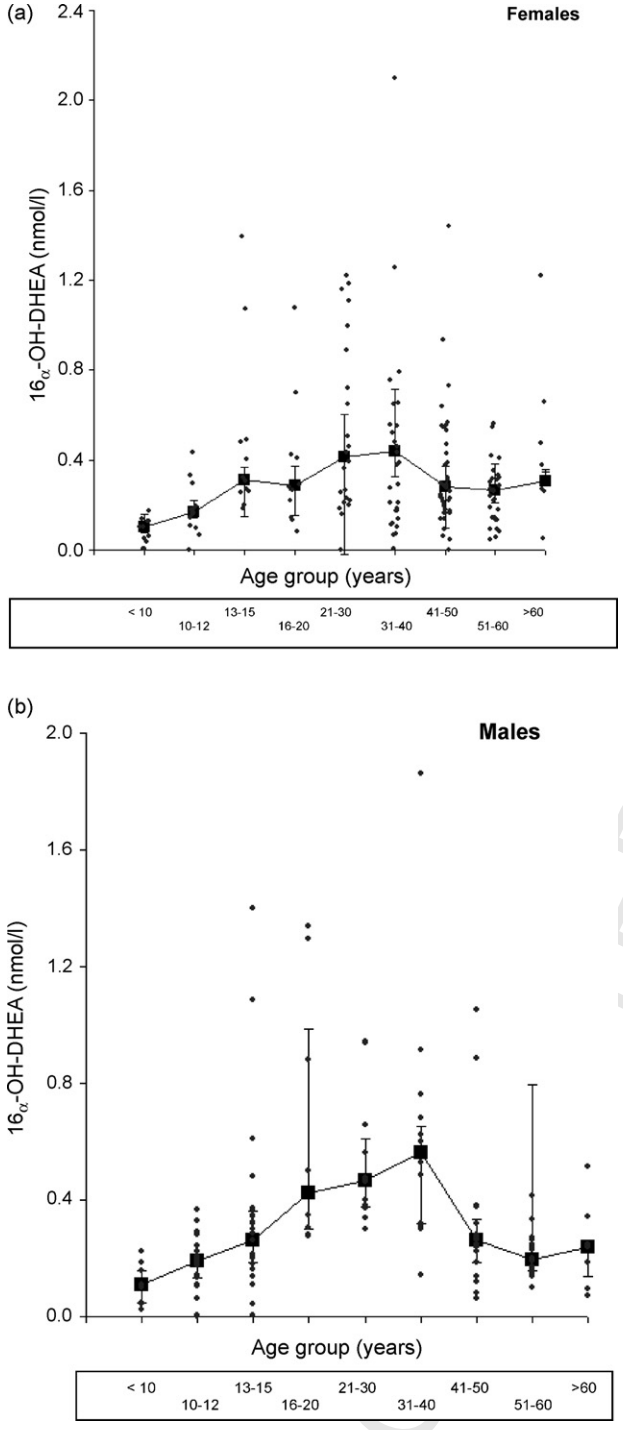

Fig. 3. (a) Physiological levels of 16α-OH-DHEA in female sera ($n = 184$) according to age. The full squares show medians, the vertical error bars 0.25 and 0.75 percentiles and (b) physiological levels of 16α-OH-DHEA in males ($n = 132$) according to age. The full squares show medians, the vertical error bars 0.25 and 0.75 percentiles.

4. Discussion

The first reports on 16-hydroxylated DHEA metabolites in umbilical blood of newborns come from early 1950s. A series of papers appeared soon; later on isolation and identification of various 16α- or 16β-hydroxysteroids in urine of children and adults with various endocrine disorders as, e.g. Ref. [14]; for literature, see Ref. [2]. GC–MS technique was the method of choice. Determination of 16α-OH-DHEA was used mostly for monitoring of the fetoplacental unit function. We have used recently this analytical approach for detection and quantification of this steroid in human seminal plasma [13]. The first successful RIA of the Japanese authors [5] concerned pregnancy as well, including measurement of this steroid in umbilical blood.

Here, we report a rapid and sensitive immunoassay of 16α-OH-DHEA, suitable for screening purposes. The high specificity has been achieved by using antiserum raised against hapten prepared via position 19 of the steroid molecule. The method was compared with previously described GC–MS [13]: the results may be considered still satisfactory, the higher values, especially at the low analyte concentration obtained by GC–MS, should be ascribed to a lower sensitivity of the latter method, requiring much larger volume (1 ml) of serum.

For the first time, the data are given of its levels in a representative sample of human population of both sexes, sorted according to age. The levels are by order of magnitude lower than those measured by Furuya et al. [5] in the late pregnancy (11.04 ± 6.27 nmol/l in 38–40th week) and more than 20 times lower than in umbilical blood. On the other hand, they are comparable with physiological concentrations of the products of a concurrent metabolic reaction leading to 7-hydroxylated DHEA metabolites [15]. The age dependence of 16α-OH-DHEA levels resembeled that of its precursor DHEA, but the peak was reached as late as after 30th year in both sexes, i.e. by almost 10 years later than in the case of DHEA [16]. A trend to an increase of its levels since, childhood to adulthood, followed by a decrease after the fourth decade was more distinct in males.

Using this method, we would like to test the hypothesis, whether this metabolite is increased in sera from patients with various endocrine autoimmune disorders, especially in thyreopathies.

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A novel radioimmunoassay of 7-oxo-DHEA and its physiological levels

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ABSTRACT

A novel radioimmunoassay (RIA) of unconjugated 7-oxo-dehydroepiandrosterone (7-oxo-DHEA) in human serum was developed for the first time. This steroid is an intermediate in the biosynthesis of immunomodulatory 7-hydroxylated DHEA metabolites, and has been shown to possess thermogenic properties. The method employs polyclonal rabbit antiserum to (19E)-3β-hydroxy-7,17,19-trione-19-O-(carboxymethyloxime):BSA conjugate and a homologous radioiodinated derivative with tyrosine methyl ester. The cross reactivity of the antiserum with structurally closest 7-hydroxyepimers of DHEA was lower than 1.7%, with DHEA 0.4%, with all other related steroid less than 0.4%. The method includes ether extraction of serum (0.5 ml), followed by RIA. Its detection limit was 0.06 pmol (18 pg)/tube, the average intra- and inter-assay coefficients of variation were 4.1% and 8.3%, respectively. Mean recovery of serum spiked with 7-oxo-DHEA varied between 78.8% and 112%. Its levels in three serum pools were compared with a low-resolution gas chromatography–mass spectrometry method with satisfactory results. The method has been used for determination of 7-oxo-DHEA in serum samples of 215 subjects (91 males and 124 females) without overt endocrine disorders, aged 5–71 years. The over-all mean ± S.D. was 0.280 ± 0.227, the median 0.239 nmol/l. No significant sex differences were recorded. The only group which differed significantly from all other ones were males below 10 years, significantly lower values than in other age groups were found also in the first two age groups of females.

1. Introduction

3β-Hydroxy-5-androstene-7,17-dione (7-oxo-dehydroepiandrosterone,7-oxo-DHEA) is a natural constituent of human blood present in serum in a low nanomolar range [1]. It is an intermediate in the biosynthesis of 7-hydroxylated DHEA metabolites, believed to act as local immunoprotective agents [2,3]. Its formation in the liver, brain and other tissues have been demonstrated in many species including humans; for the literature see, e.g. [4–6] and other citations therein. In addition, it has been shown to possess thermogenic properties comparable with those of thyroid hormones and therefore it was named ergosteroid [7]. Since the latter pioneering work, a number of studies from Lardyïs group appeared about its thermogenic properties and possible sites of its action.

There is, however, one more interesting feature of 7-oxo-DHEA: as demonstrated by Robinzon et al. [8,9], the enzyme responsible for the interconversion of 7-hydroxylated-DHEA epimers via 7-oxo-DHEA is identical with peripheral 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), catalyzing

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the reduction of inactive cortisone to cortisol. In the enzyme-kinetic study with recombinant human 11β-HSD1 expressed in yeast [10] the authors demonstrated that 7-oxygenated DHEA metabolites (7α-, 7β- and 7-oxo-DHEA) can serve substrates for this enzyme under both oxidative and reductive conditions. They concluded that though circulating concentrations of cortisol and cortisone are by orders of magnitude higher than those of 7-oxygenated-DHEA metabolites, in tissues such as liver where 11β-HSD1 is colocalized in endoplasmic reticulum membrane with hexose-6-phosphate dehydrogenase generating NADPH, and where 11β-HSD1-mediated cortisone reduction is a privileged reaction, 7-oxo-DHEA may compete with this reaction. This concept was further developed in the recent work of the same authors, with the conclusion that 7-oxygenated-DHEA metabolites may serve as natural antiglucocorticoids, tuning their local excessive effects [11].

In some countries 7-oxo-DHEA is commercially available under the trademark 7-keto-DHEA and belongs to steroid prohormones used as nutritional supplements in sports [12]. In another paper of Lardy's group, the authors tested the effect of 7-oxo-DHEA-3-acetate administration to male volunteers on major hormonal parameters, in a placebo-controlled study. They have found only minor changes in hormonal spectrum even at large doses and concluded that this steroid is safe and well tolerated by normal men [13]. Also our group tested the effects of short-term transdermal application of 7-oxo-DHEA to healthy male volunteers on various hormone and lipid parameters. The main finding was the drop of atherogenic index after treatment indicating that this steroid may be suitable for improving lipid parameters in elderly men [14]. In contrast to DHEA, 7-oxo-DHEA is not a precursor of biologically active androgens and estrogens known to enhance the risk of cancer of prostate or female reproductive organs. For its thermogenic properties, 7-oxo-DHEA was even suggested for prevention of Raynaud's attacks (abnormal digital vasoconstriction in response to cold) [15].

All things considered, 7-oxo-DHEA is an interesting endogenous steroid and a potential candidate for steroid supplement therapy instead of DHEA. In contrast to many reports concerning identification and quantification of 7-oxo-DHEA as a metabolite, to the best of our knowledge there is only one paper on its physiological levels in human blood [1]. The latter authors have used a high-resolution GC–MS technique. Here, an inexpensive radioimmunoassay alternative is described and used for determination of physiological levels of 7-oxo-DHEA in a representative group of healthy subjects.

2. Experimental

2.1. Materials

All chemicals were of analytical grade from Sigma (St. Louis, MI, USA), 7-oxo-DHEA and other steroids were purchased from Steraloids (Newport, RI, USA). Solvents used for GC–MS were of HPLC grade, methoxyamine hydrochloride and Sylon B were from Supelco (Bellefonte, PA, USA).

2.2. Hapten synthesis

2.2.1. Synthetic pathway

The synthesis of the hapten, (19*E*)-(3β-hydroxy-5-androstene-7,17,19-trione-19-*O*-(carboxymethyloxime) (**4**, 7-oxo-DHEA-19-CMO) started from known (19*E*)-3β-acetoxy-5-androstene-17,19-dione-19-*O*-(methoxycarbonylmethyl)oxime (**1**) [16]. For introduction of 7-oxo group into the molecule, an oxidation with a complex of chromium trioxide and 2,5-

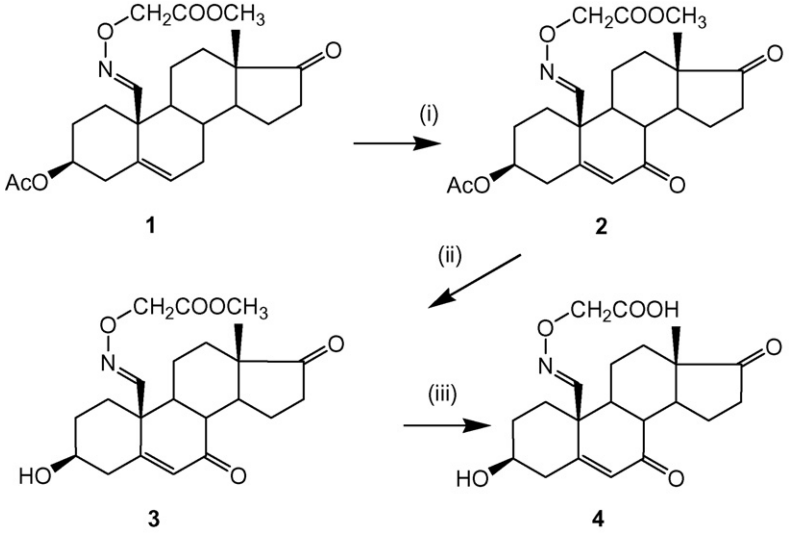

Fig. 1 – Scheme of preparation of (19*E*)-3β-hydroxy-5-androstene-7,17,19-trione-19-*O*-(carboxymethyl)oxime used as a hapten for immunogen synthesis.

dimethylpyrazole [17] was used. Removal of protective groups from this compound in alkaline medium yielded an impure hapten. This raw product was firstly methylated by diazomethane and the resulting methyl ester was purified by preparative TLC. Mild hydrolysis with sodium hydroxide at room temperature gave required 19-CMO derivative in 20% overall yield. Its structure was confirmed by IR and NMR spectra. The synthetic scheme is shown in Fig. 1.

2.2.2. *General*

Melting points were determined on a Boetius micro melting point apparatus (Germany). Optical rotations were measured at 25 °C on AUTOPOL IV polarimeter (Rudolph Research Analytical, USA), and $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. Infrared spectra (wavenumbers in cm^{-1}) were recorded on a Bruker IFS 88 spectrometer. ^{1}H NMR spectra were taken on Varian UNITY-200 instrument (200 MHz, FT mode) at 23 °C in $CDCl_3$ with tetramethylsilane as the internal standard. Chemical shifts are given in ppm (delta-scale); coupling constants (*J*) and widths of multiplets (*W*) are given in Hz. Thin-layer chromatography (TLC) was performed on silica gel G (ICN Biochemicals), detection was accomplished by spraying with concentrated sulfuric acid followed by heating. Preparative TLC was carried out on 200 mm × 200 mm plates (layer thickness 0.4 mm), detected by spraying with morin (0.2% solution in methanol) followed by UV visualization (365 nm). For column chromatography, neutral silica gel Kieselgel 60 (Merck) was used. Solutions in organic solvents were dried over anhydrous sodium sulfate and concentrated on a rotary evaporator in vacuo (0.25 kPa, bath temperature 40 °C).

2.2.3. *(19E)-3β-Acetoxy-5-androstene-7,17,19-trione-19-O-(methoxycarbonylmethyl)oxime (2)*

A suspension of chromium trioxide (1.65 g, 16.5 mmol) in dichloromethane (12 ml) was stirred at −25 °C under argon with 3,5-dimethylpyrazole (1.65 g, 17.2 mmol). After 15 min, a solution of (19E)-3β-acetoxy-5-androstene-17,19-dione-19-*O*-(methoxycarbonylmethyl) oxime [16] (**1**, 389 mg, 1.0 mmol) in dichloromethane (2 ml) was added dropwise, and stirring was continued at −20 °C for 4 h. The reaction mixture was then diluted with a mixture of benzene/ethyl acetate (7:3, 30 ml) and filtered through a short column of silica gel (10 g) layered with celite. The column was washed with the same solvent mixture, and the solvents were evaporated in vacuo. Chromatography on a column of silica gel (30 g) in a mixture of benzene/ethyl acetate (90:10 to 80:20) afforded 278 mg (62%) of title compound **2**, mp 128–131 °C (ether), $[\alpha]_D$ −145 (c 0.63, chloroform). IR spectrum (tetrachloromethane): 1763 (C=O, $COOCH_3$); 1743 (C=O, 17-ketone and acetate); 1681 (C=O, 7-ketone); 1640 (C=C); 1 240, 1030 (C–O, acetate) 1 210 (C–O, $COOCH_3$). ^{1}H NMR (200 MHz): 7.55 (1 H, s, H-19); 5.91 (1 H, d, *J* = 1.7, H-6); 4.76 (1 H, m, *W* = 32, H-3); 4.64 (2 H, s, OCH_2COO); 3.76 (3 H, s, $COOCH_3$); 2.05 (3 H, s, $OOCCH_3$); 0.89 (3 H, s, 3 x H-18). Analysis calculated for $C_{24}H_{31}NO_7$ (445.5): C, 64.70; H, 7.01; N, 3.14. Found: C, 64.53; H, 6.85; N, 3.25.

2.2.4. *(19E)-3β-Hydroxy-5-androstene-7,17,19-trione-19-O-(methoxycarbonylmethyl) oxime (3)*

To a solution of (19E)-3β-acetoxy-5-androstene-7,17,19-trione-19-*O*(methoxycarbonyl-methyl)oxime (**2**, 267 mg, 0.60 mmol) in a mixture of tetrahydrofurane (6 ml) and methanol (1 ml), 0.4 M aqueous sodium hydroxide (3.75 ml) was added dropwise, and the mixture was stirred at 42 °C for 3 h. The solution was neutralized with 5% hydrochloric acid, concentrated in vacuo, diluted with water (50 ml), and extracted with ethyl acetate (2 × 70 ml). The extract was washed with water, dried and the solvent was evaporated. The residue was dissolved in ether (10 ml) and methanol (5 ml) and treated with an etheral solution of diazomethane at 0 °C for 5 min. The excess diazomethane and the solvents were evaporated in vacuo, and the residue was chromatographed of three preparative silica gel plates in benzene/acetone (80:20). The yield of title compound (**3**) was 146 mg (60%), mp 135–136 °C (ether), $[\alpha]_D$ −169 (c 0.94, chloroform). IR spectrum (chloroform): 3 608 (O–H); 1756 shoulder (C=O, $COOCH_3$); 1736 (C=O, 17-ketone); 1672 (C=O, 7-ketone); 1638 (C=C); 1026 (C–O). ^{1}H NMR (200 MHz): 7.54 (1 H, s, H-19); 5.90 (1 H, d, *J* = 1.7, H-6); 4.63 (2 H, s, OCH_2COO); 3.75 (3 H, s, $COOCH_3$); 3.73 (1 H, m, *W* = 32, H-3); 0.89 (3 H, s, 3 x H-18). Analysis calculated for $C_{22}H_{29}NO_6$ (403.5): C, 65.49; H, 7.24; N, 3.47. Found: C, 65.33; H, 7.07; N, 3.62.

2.2.5. *(19E)-3β-Hydroxy-5-androstene-7,17,19-trione-19-O-(carboxymethyl)oxime (4)*

Aqueous sodium hydroxide (0.4 M, 2.5 ml) was added to a stirred solution of (19E)-3β-hydroxy-5-androstene-7,17,19-trione-19-*O*-(methoxycarbonylmethyl)oxime (**3**, 141 mg, 0.35 mmol) in a mixture of THF (2.5 ml) and methanol (0.5 ml). After 30 min of stirring, the reaction mixture was cooled on ice, ice water was added (5 ml) and most of organic solvents were removed in vacuo at 20 °C. Ice water (40 ml) was added, the mixture was extracted with ether (10 ml) and the organic layer was discarded. Aqueous phase was cooled on ice and 10% H_2SO_4 (1 ml) was added. The mixture was extracted with ethyl acetate (2 × 20 ml), and the combined extracts were washed with ice water (3×), dried and evaporated. The residue was crystallized from a mixture of methanol and water affording 75 mg (55%) of CMO derivative **4**, mp 193–196 °C, $[\alpha]_D$ −197 (c 1.1, chloroform). IR spectrum (KBr pellet): 3405 (O–H); 2742, 2626, 2524 (COOH, dimer); 1736 (C=O, 17-ketone); 1715 (C=O, COOH dimer); 1672 (C=O, 7-ketone); 1640 (C=C); 1028 (C–O). ^{1}H NMR (200 MHz): 7.54 (1 H, s, H-19); 5.89 (1 H, d, *J* = 1.4, H-6); 4.60 (2 H, s, OCH_2COO); 3.46 (1 H, m, *W* = 32, H-3); 0.86 (3 H, s, 3 x H-18). Analysis calculated for $C_{21}H_{27}NO_6$ (389.4): C, 64.77; H, 6.99; N, 3.60. Found: C, 64.58; H, 7.07; N, 3.69.

2.3. *Preparation of immunogens*

The micellar method of Yatsimirskaya et al. [18] has been used for conjugation of the hapten to bovine serum albumin (BSA). The starting hapten-protein molar ratio was 50, the resulting conjugates contained 11–13 mol of the hapten in 1 mol of the conjugates, as determined by spectrophotometry with trinitrobenzene sulfonic acid.

2.4. *Immunization of rabbits and collection of antisera*

Six female rabbits were immunized by a standard procedure [19] using 100 μg doses of the immunogens emulsified in a mixture of complete Freund's adjuvans-saline 1:1 (100 μl) in 3 weeks intervals. After the third immunization the sera

Table 1 – Cross reactions of two polyclonal rabbit antisera to 7-oxo-DHEA-19-CMO:BSA with 11 chemically related steroids occurring in human serum

Steroid	Cross-reactions in per cents	
	Antiserum No. 1	Antiserum No. 4
7-oxo-DHEA	100	100
7α-OH-DHEA	0.45	1.72
7β-OH-DHEA	2.27	0.81
DHEA	3.77	0.45
DHEAS	0.55	0.19
3β-Hydroxy-5α-androstan-17-one	1.14	0.36
5-Androstene-3β,17β-diol	0.03	<0.01
3α-Hydroxy-5α-androstan-17-one	0.14	0.13
4-Androstene-3,17-dione	1.65	0.17
Testosterone	0.04	<0.01
3β-Hydroxy-5β-androstan-17-one	<0.01	<0.01
3β-Hydroxy-5-pregnen-20-one	0.02	<0.01

Antiserum No. 4 has been further used in the assay.

were collected from the ear vein and lyophilized. The antiserum No. 4 with the lowest cross-reaction with structurally related steroids (see Section 3, Table 1) was chosen for assay.

2.5. *Synthesis of the tracer*

Position homologous [^{125}I]iodo tyrosine methyl ester (TME) conjugate with the hapten was prepared by a convenient radioiodination using chloramine-T [19]. The conjugate itself was prepared by a modified *N*-hydroxysuccinimide method [20]. The product was chromatographed on thin layer plate of silica (Kieselgel 60 F_{254}, Merck, Darmstadt, FRG) in system dichloromethane-2-propanol 92:8 (v/v). The distribution of radioactivity on the plate was measured by scanning with automatic TLC linear analyzer Tracemaster 40 with counting tube LB 2821 (Berthold, Wildbad, FRG). Specific radioactivity of the product assessed by self-displacement method [21], calculated for unlabeled analyte, was 32 Ci/mmol.

2.6. *Radioimmunoassay*

Serum (500 μl) was extracted twice with 2 ml of diethyl ether. The water phase was left frozen in solid carbon dioxide bath and ether extracts were combined and evaporated to dryness. The standard solutions contained 0.064–33.1 pmol (19.5–10,000 pg) of 7-oxo-DHEA in 100 μl RIA buffer (20 mmol/l sodium phosphate-saline, pH 7.2 containing 1 g/l BSA). To the dry residues of samples or to the tubes for standard curve the buffer or standard solution, radioligand and antiserum (No. 4, working dilution 1:4000), 100 μl, each were added. The tubes for determination of unspecific binding contained radioligand and buffer only, final volume was 300 μl. The content of tubes was mixed on vortex, and after overnight incubation at 4 °C, 500 μl of dextran-coated charcoal (0.25 and 0.025 g/100 ml) was added to each tube except the total, the content was vortexed briefly and after 10 min staying at 4 °C the tubes were centrifuged at 4 °C and 3000 rpm for 10 min. The supernatants were decanted into another set of tubes in which the radioactivity of ^{125}I was measured on Berthold gamma counter. The concentration of 7-oxo-DHEA was calculated from the log–logit plot.

2.7. *Determination of 7-oxo-DHEA in serum pools by GC–MS*

2.7.1. *Extraction and procession of samples*

Three serum pools from adult males, females and children, 5 ml each, were spiked with [^{3}H] 11β-hydroxyandrostenedione (167 Bq) (for monitoring of losses during procession), and extracted twice with diethyl ether (15 ml). After evaporation of the solvent, the extracts were partitioned between *n*-pentane (2.5 ml)-methanol (2.0 ml) and water (0.5 ml). Upper *n*-pentane–methanol phase was removed by sucking with a Pasteur pipette and the methanol–water phase was evaporated on vacuum centrifuge evaporator. The recovered radioactivity of [^{3}H]11β-androstenedione amounted 90 ± 8%.

2.7.2. *Derivatization*

Methoxyamine hydrochloride MOX-pyridine mixture (2:98, 25 μl) was added to dry residues from sample extracts or to the empty tubes (blank) and the content was incubated at 80 °C for 2 h. Following evaporation, pyridine (30 μl) and Sylon B (10 μl) were added, and the incubation was repeated for 45 min. Acetonitrile (50 μl) and isooctane (200 μl) were then added and the mixture was extracted by shaking for 1 min. The lower phase was removed carefully and the upper was transferred quantitatively into another vial and evaporated again. The dry residues of methoxyamine trimethylsilyl derivative were dissolved in 20 μl of isooctane. 7-oxo-DHEA (external standard) in concentration 20 pg/μl was derivatized in the same way and used for calibration. 5 μl of Isooctane solutions, corresponding to 1.25 ml of serum pools or standard (100 pg) were applied into GC–MS system.

2.7.3. *GC–MS*

The GC/MS system from Shimadzu (Kyoto, Japan) consisted of the gas chromatograph GC-17A, the simple quadrupole analyzer QP 5050A equipped with electron impact ioniza-

Table 2 – Recovery of 7-oxo-DHEA added to normal pooled human serum

Added steroid	Found		% Recovery	c.v. (%)
	nmol/l	pg/tube		
None	0.25	38.2	–	5.1
0.33 nmol/l (100 pg/tube)	0.72	109	78.8	7.3
0.66 nmol/l (200 pg/tube)	1.77	267	112	8.1
0.99 nmol/l (300 pg/tube)	2.07	314	92.8	8.0

Given means and coefficients of variation from five experiments.

Table 3 – Independence on dilution

Serum volume (μl)	Found (pg/tube)	Recalculated (nmol/l)	c.v. (%)
800	152	0.63	8.9
400	93.4	0.77	6.6
200	41.6	0.69	9.6

Pooled human serum of high 7-oxo-DHEA content was serially diluted with steroid-free serum and analyzed for 7-oxo-DHEA. Given means and coefficients of variation from five experiments.

tion on 70 eV, with the mass range from 10 to 900 amu. The medium-polar capillary column ZEBRON ZB-50 from Phenomenex (Torrance, CA, USA) 15 m × 0.25 mm, film thickness 0.15 μm was used with helium as a mobile phase. The temperature at the injection port was 300 °C, of the interface 320 °C. Analyses were performed according to following temperature and pressure program: 0–1 min, 120 °C, 100 kPa; 1–3.5 min, linear gradient 120–220 °C (40 °C/min), 3051 kPa (8.5 kPa/min); 3.5–10.4 min, linear gradient 220–240 °C (2.9 °C/min), 51–54.5 kPa (0.5 kPa/min); 10.4–12.15 min, linear gradient 240–310 °C (40 °C/min), 54.5–70.0 kPa (9 kPa/min); 12.15–14.2 min, 310 °C, 70.0 kPa. Splitless mode was used; the detector voltage was 1.65 kV. The detection was performed by selected ion monitoring (SIM) mode. Fragments *m*/*z* 129, 238 and 386 were followed in the case of calibration and samples. The retention time for 7-oxo-DHEA was 10.98 min. The contents of 7-oxo-DHEA in analyzed serum pools were calculated from the area under peak corresponding to effective mass fragment *m*/*z* 386.3. The results were corrected to losses during extraction and solvent partition.

2.8. *Sera*

The rests of serum samples from 215 subjects (91 male, 9–71 years and 124 females 5–65 years) collected during the screening programme of iodine defficiency in the Czech Republic within the years 2003–2006 were used for determination of physiological levels of 7-oxo-DHEA. The samples of patients with overt endocrinopathies were excluded. The use of the samples was approved by the Ethical Committee of the Institute of Endocrinology (Prague).

2.9. *Statistics*

Statistical software GraphPad Prism (Aurora, CO, USA) was used for linear regression analysis. Robust Kruskal–Wallis ANOVA followed by Kruskal–Wallis multiple comparisons were used for evaluation of differences between individual age groups. Statistical software Statgraphics Plus 3.0 (Manugistics Inc., Rockville, MA, USA) was used.

3. Results

3.1. *Specificity of the antisera*

The cross-reactions of two polyclonal rabbit antisera, obtained after immunization with immunogen raised to 7-oxo-DHEA-19-CMO:BSA with 11 chemically related steroids occurring in human serum are shown in Table 1. The antiserum No. 4 has been further used in the method.

3.2. *Reliability criteria of the method*

3.2.1. *Precision*

Intra- and inter-assay coefficients of variation, determined by repeated measurement ($n = 10$ and 8) of the analyte in the mixed serum pool containing 0.27 nmol/l of 7-oxo-DHEA were 4.1% and 8.3%, respectively.

3.2.2. *Sensitivity*

The detection limit, expressed as the minimal amount of 7-oxo-DHEA distinguishable from the zero sample with 95% probability was 0.06 pmol (18 pg)/tube, corresponding to concentration 0.12 nmol/l.

3.2.3. *Accuracy*

Pooled samples of human serum were spiked with increasing amounts of the analyte (0, 0.33, 0.66 and 0.99 pmol, i.e. 0, 100, 200 and 300 pg/tube). The recovery varied between 78.8% and 112% (Table 2).

3.2.4. *Independence on dilution*

A 800 μl of pooled human with a high content of the analyte was serially diluted with steroid-free serum and analyzed. The

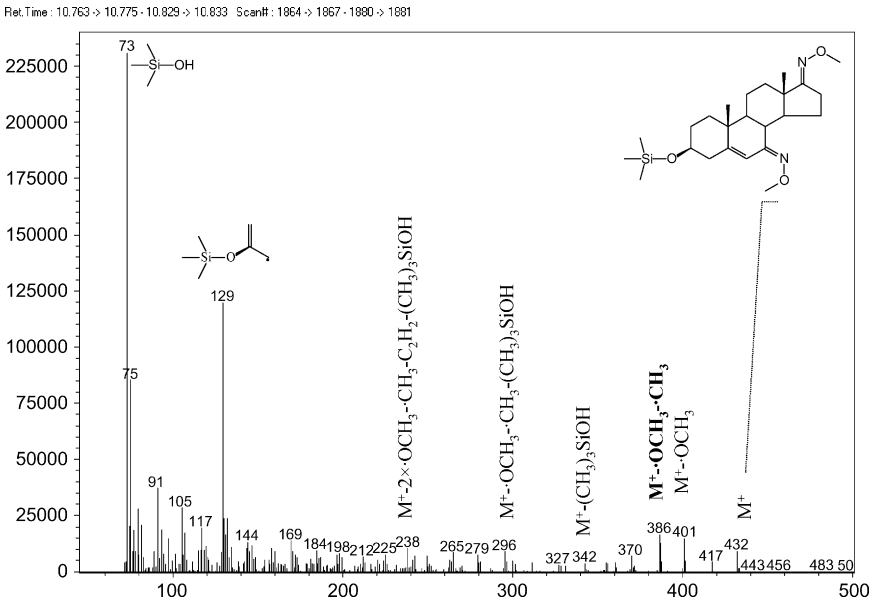

Fig. 2 – Mass spectrum of 7-oxo-DHEA-mehoxyamine-trimethylsylil derivative with depicted fragments.

recalculated levels did not depend significantly on dilution within this range (Table 3).

3.3. *Comparison with GC–MS method*

Three serum pools from children of both sexes (below 16 years), adult females and males were analyzed by both RIA and GC–MS method described in Section 2. Because of the low detection limit of the GC–MS, large volume of serum pools (5 ml each) should have been taken for analysis. Fig. 2 shows the GC–MS spectrum of authentic 7-oxo-DHEA-methoxyamine-trimethylsilyl derivative, Fig. 3 the chromatograms of serum extracts and standard at recorded effective masses *m/z* 238 and 386. Fragment *m/z* 386, corresponding to M^+–OCH_3–CH_3 was used for quantification of the analyte in selected ion monitoring (SIM) mode. The results of 7-oxo-DHEA determination by GC–MS and RIA are shown in Table 4.

3.4. *Physiological levels*

Physiological levels of 7-oxo-DHEA were determined in 215 subjects (91 males and 124 females) without overt endocrine disorders, aged 5–71 years. The over-all mean ± S.D. was 0.280 ± 0.39, the median 0.239 nmol/l.

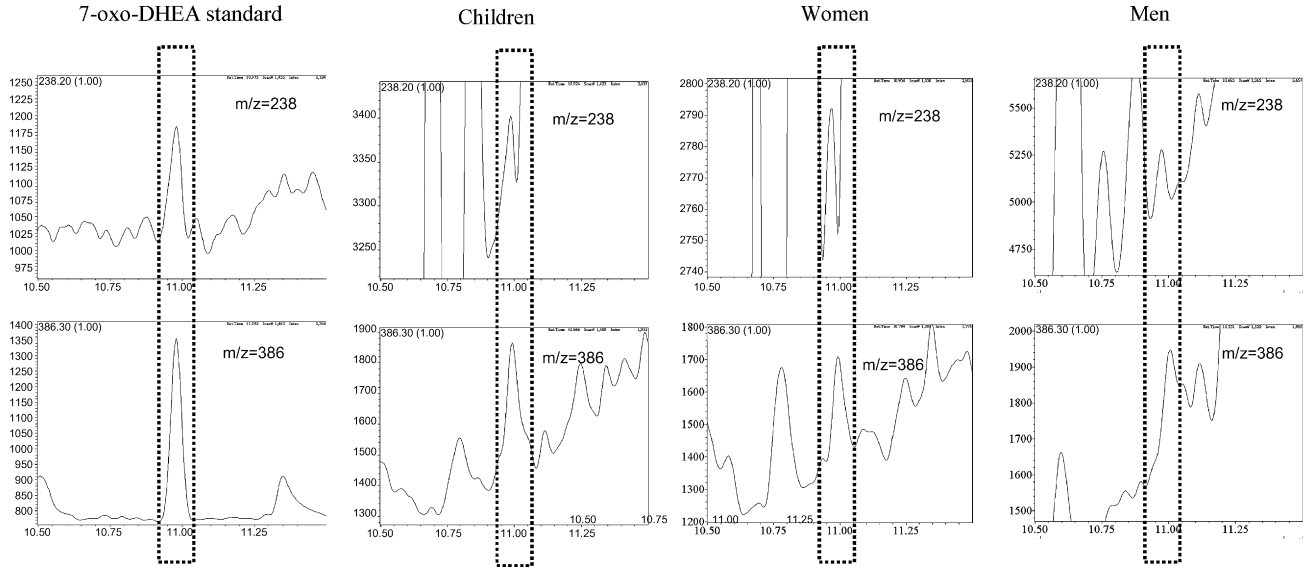

Fig. 3 – Chromatograms of serum extracts and standard 7-oxo-DHEA at recorded effective masses *m/z* 238 and 386. Fragment *m/z* 386, corresponding to M^+–OCH_3–CH_3 was used for quantification of the analyte in selected ion monitoring (SIM) mode.

Table 4 – Comparison of RIA with GC–MS

Serum pool	GC–MS		RIA	
	nmol/l	pg/ml	nmol/l	c.v. (%)
Children	0.180	55.3	0.206	5.8
Females	0.177	53.6	0.198	6.5
Males	0.208	62.9	0.283	6.0

Three serum pools from children, adult females and males were analyzed by both RIA and GC–MS method. Because of a low detection limit of the GC–MS, large volume of serum pools (5 ml each) were analyzed. In the case of RIA means and coefficients of variation from five experiments are given.

The results are summarized in Fig. 4a (females) and b (males). They cover a broad range from undetectable concentrations to 0.98 in males, and 0–1.38 nmol/l in females, respectively. Full squares depict medians, vertical error bars S.D.

The differences between individual pairs of age groups are shown in Table 5. The matrix provides the Z-value obtained by Kruskal–Wallis Multiple Comparison Test. The medians (see Fig. 4) differ significantly if Z-value are higher than 1.96 (bold letters). The upper right part of the matrix shows the data from females, the lower left part those of males. As may be seen, the only group which differred significantly from all other ones were males below 10 years. Significantly lower values than in other age groups were found also in the first two age groups of females.

4. Discussion

In contrast to the many studies about DHEA metabolism, so far only two reports concern its 7-oxo-metabolite in human blood [1,22]. The earlier HPLC method for 7-oxo-DHEA sulfate of Lardy's group [22] was not sensitive enough for detection of the unconjugated steroid. The method was developed primarily to study the behavior of 7-oxo-DHEA and/or its major metabolites in subjects who had been administered 7-oxo-DHEA acetate. Using the high-resolution GC-MS, the Japaneese authors [1] in a more recent study measured, for the first time, unconjugated 7-oxo-DHEA (along with its precursor and other 7-oxygenated metabolites) in blood from 18 healthy volunteers of both sexes. They have reported a wide range of levels, ranging from undetectable values to 0.25 nmol/l.

Here, we report for the first time an inexpensive and easy radioimmunossay alternative for 7-oxo-DHEA determination in human serum. The relatively high specificity of the method was achieved by using antiserum, raised with hapten conjugated to BSA via the 19-oxo-group of the steroid molecule. The derivative used for the immunogen synthesis 7-oxo-DHEA-19-CMO was synthesized for the first time, too. The analytical criteria of the assay, precision, sensitivity and accuracy were satisfactory. The results have been compared with a low-resolution GC–MS method. With respect to the lower sensitivity of our GC–MS when compared with the method reported by others [1], large volumes of pooled serum should have been processed. The average levels of 7-oxo-DHEA as measured by RIA were somewhat higher than by GC–MS, but

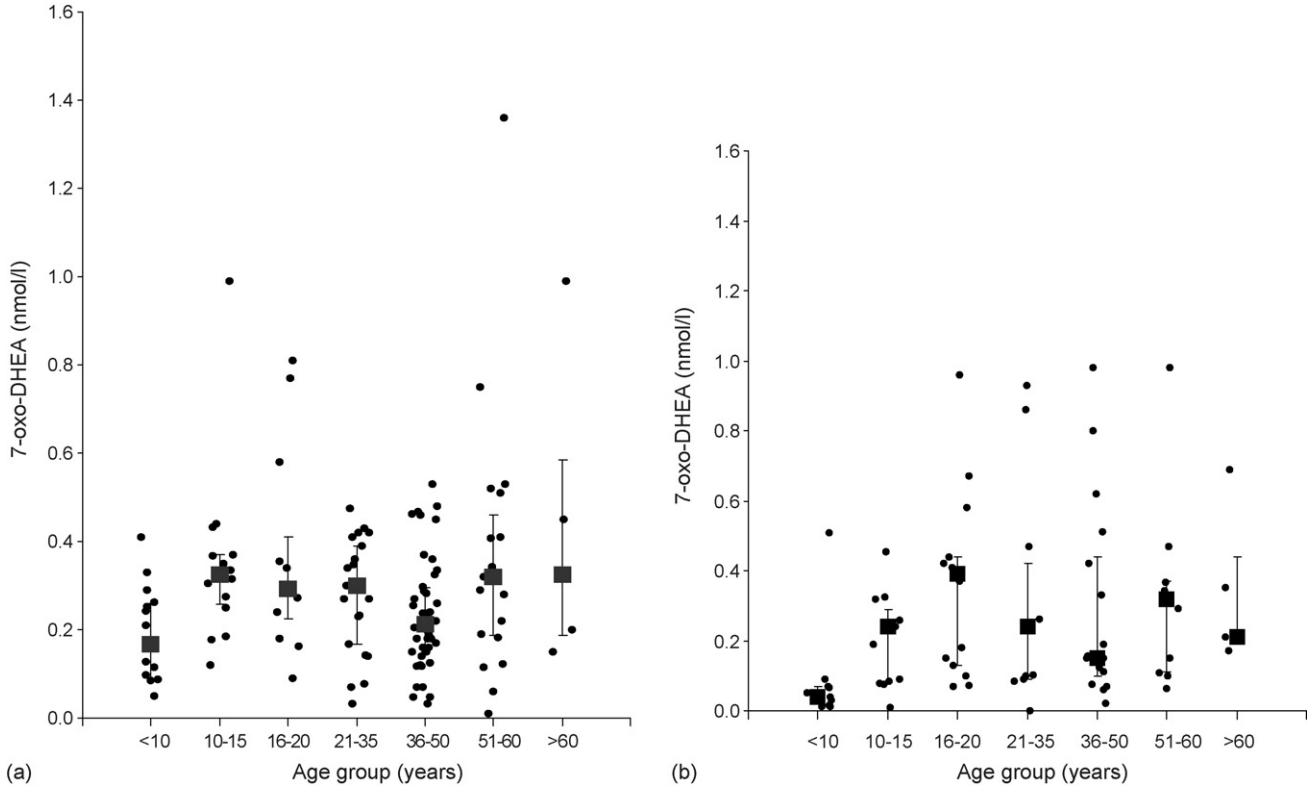

Fig. 4 – Individual levels of 7-oxo-DHEA in female (a) and male (b) serum according to age groups. Full squares depict medians, vertical error bars S.D.

Table 5 – Significance of differences between individual age groups

Age group (years)	Age group (years)						
	Below 10	11–15	16–20	21–35	36–50	51–60	Above 60
Below 10	0	**2.611**	**2.3047**	1.9465	1.0385	**2.3726**	1.6898
11–15	**2.3563**	0	0.2039	0.9137	**2.1593**	0.4292	0.0509
16–20	**3.8591**	1.3386	0	0.6496	1.7908	0.1925	0.0889
21–35	**2.8822**	0.5654	0.7164	0	1.3138	0.5182	0.525
36–50	**3.3378**	0.7174	0.7161	0.0843	0	1.8634	1.2184
51–60	**3.2193**	0.9582	0.2714	0.3997	0.3593	0	0.2224
Above 60	**3.0114**	1.2957	0.364	0.8612	0.8506	0.5422	0

The matrix shows the Z-value obtained by Kruskal–Wallis multiple comparison test. The medians (see Fig. 4a and b) differ significantly in the Z-value are higher than 1.96 (bold letters). The upper right part of the matrix gives the data from females, the lower left part those of males.

within the same range. This may be caused by cross-reacting DHEA: though its cross-reaction with the antiserum was only 0.45%, its interference could not be neglected when taking into account its physiological concentrations in human blood (units or tens nmol/l) [23].

Finally, the method has been applied for determination of 7-oxo-DHEA in sera from healthy males and females. In general, large inter-individual variations were recorded in both sexes. The age dependence differed from that of its precursor DHEA and both 7-hydroxylated metabolites reported previously [23,24], characterized by an increase of their levels at the onset of puberty and reaching the maxima around 30 years of age, followed by a continuous decline with advanced age. The only similarity with DHEA and its 7-hydroxylated metabolites was in the very low levels in early childhood, followed by an increase during and after puberty. The age dependence was more distinct in males than in females. Rather surprising is the nadir of 7-oxo-DHEA in both sexes in the age group 36–50 years.

The development of a rapid method for determination 7-oxo-DHEA and its physiological values in human blood is the first step for further studies on its levels under various pathological conditions. At first we intend to measure it in patients with most frequent thyreopathies, known to have altered thermoregulation.

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Sex hormone-binding globulin in congenital adrenal hyperplasia

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Abstract

Background: Sex hormone-binding globulin biosynthesis is influenced by three hormonal systems: gonadal, insular and thyroid. Congenital adrenal hyperplasia is characterized by overproduction of adrenal androgens associated with impaired insulin sensitivity, hyperinsulinemia and often also with hypothyroidism. Only scarce data are available concerning congenital adrenal hyperplasia. The objective of this study was to determine the distribution of sex hormone-binding globulin and free testosterone levels in these patients and to what extent these values correlate with actual 17-hydroxyprogesterone and androstenedione levels, which are commonly used for monitoring of treatment effectiveness.

Materials and methods: A total of 300 retrospective laboratory records of 78 males and boys and 456 records of 162 girls and premenopausal women with diagnosis of congenital adrenal hyperplasia under common substitution treatment were evaluated statistically. The data were divided artificially into groups of low, normal and high levels, with respect to physiological concentrations for each sex and age. The percentages of the total in each group were calculated.

Results: Whereas an almost Gaussian distribution occurred for males, the data for females displayed a considerable shift to low sex hormone-binding globulin and accordingly high free testosterone levels. Sex hormone-binding globulin levels did not correlate with 17-hydroxyprogesterone.

Conclusion: Low sex hormone-binding globulin levels in congenital adrenal hyperplasia, at least in females, reflect their involvement in insular and eventually thyroid axes, rather than the effectiveness of substitution.

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Introduction

Congenital adrenal hyperplasia (CAH) due to deficiency of 21-hydroxylase or other enzymes of the corticosteroid biosynthetic pathway is often associated with impaired insulin sensitivity resulting in hyperinsulinemia (1–4) and also with hypothyroidism (5).

Sex hormone-binding globulin (SHBG) biosynthesis in the liver is influenced by three hormonal systems: gonadal, insular and thyroid. Androgens, irrespective of their origin (gonadal or adrenal), could suppress its biosynthesis (6–8). Decreased levels of SHBG in turn result in a higher portion of free androgens. Besides virilization, an exposure to high levels of free testosterone can be accompanied by growth disorders (9). Increased insulin levels occurring in patients with impaired insulin sensitivity and in obese subjects are also known to be associated with low SHBG resulting in further elevation of free testosterone (10, 11). Finally, lower than physiological levels of SHBG occur in hypothyroidism (12, 13).

When examining the retrospective laboratory data of patients with CAH under a common substitution regime, we noted considerable deviations of SHBG levels from physiological levels. The fact that CAH is often associated with insulin resistance and consequently with hyperinsulinemia might influence SHBG levels (2, 4). Because only scarce information is available on SHBG in patients with CAH, we analyzed a large set of retrospective data for SHBG and free testosterone in these patients. The following issues were addressed: 1. What is the distribution of SHBG and free testosterone levels in female and male patients with CAH. 2. Do these values correlate with actual 17-hydroxyprogesterone or androstenedione levels, considered to reflect effectiveness of substitution treatment.

Subjects and methods

Retrospective laboratory data collected in the Institute of Endocrinology, Prague during the years 2000–2007

Table 1 Physiological levels of 17-hydroxyprogesterone, testosterone, androstenedione, SHBG and free testosterone according to age and sex as reported in authors' laboratory.

Age group	17OH-P, nmol/L		Testosterone, nmol/L		Androstenedione, nmol/L		SHBG, nmol/L		Free testosterone, pmol/L	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
<1 month	0.50–10.0	0.50–10.0	0.40–16.2	0.30–1.70			42.7–108	28.3–69.5	6.08–137	5.83–18.6
1–3 months	0.40–12.2	0.50–10.0	0.40–16.2	0–0.60			76.0–137	68.0–149	4.04–110	0–3.49
3–4 months	0.50–10.0	0.50–10.0								
4–6 months	0.50–6.0	0.50–10.0								
6–10 months	0.50–4.70	0.50–4.70								
3 months–9 years	0.30–2.90	0.30–2.90	0.05–0.65	0–0.60	0.70–1.50	0.70–5.80	76.0–137	68.0–149	0.50–4.07	0–3.49
9–15 years	0.80–6.40	0.30–7.70			0.30–11.7	0.10–9.10				
<10 years			0.20–1.20	0.30–1.30			76.0–137	68.4–149	2.02–7.53	3.28–7.59
10–11 years			0.42–3.92	0.50–1.90			76.0–137	68.4–149	4.24–25.0	5.47–11.1
11–12 years			0.69–5.62	0.50–1.90			76.0–137	55.0–124	6.99–36.1	6.41–13.0
12–13 years			1.11–9.04	0.50–1.90			76.0–137	55.0–124	11.3–59.2	6.41–13.0
13–14 years			1.91–13.3	0.50–1.90			44.1–113	55.0–124	28.9–106	6.41–13.0
14–15 years			9.50–17.5	0.50–2.50			30.0–70.0	55.0–120	196–216	6.41–17.7
15–16 years			10.0–22.6	0.40–2.60			30.0–70.0	43.0–95.0	208–292	6.06–22.4
15–20 years					1.70–7.70	1.60–7.70				
15–30 years	1.30–3.40	1.10–8.40[a]								
16–25 years							23.0–50.0	43.0–95.0	239–630	6.06–25.9
>16 years			10.0–34.0	0.40–3.00						
>20 years					1.75–8.60	1.50–5.40				
25–40 years							23.0–55.0	43.0–95.0	239–587	6.06–25.9
>30 years	1.50–5.40	0.30–4.50[a]								
>40 years							31.0–66.0	43.0–95.0	204–507	6.06–25.9

Values represent the range of physiological levels for the age interval from within and above–below the respective age. [a]Up to 12.0 in luteal phase.

were processed and analyzed statistically. These included 300 records of 78 males (aged 1 month to 35 years, mean±SD 19.0±9.0, median 20 years) and 456 records of 162 premenopausal females and girls (aged 1 month to 39 years, mean±SD 17.2±9.1, median 16.0 years). All patients were diagnosed after birth as having congenital adrenal hyperplasia due to 21-hydroxylase deficiency or other enzymes in the corticosteroid biosynthetic pathway (E250–E259). Among them were 19 boys and 20 girls with salt loss. Diagnosis was based on clinical examination of external genitalia and laboratory findings of elevated 17-hydroxyprogesterone levels (for physiological levels reported in our laboratory see Table 1). All patients received substitution therapy of hydrocortisone and finally with fludrocortisone; the doses were continuously adjusted according to laboratory and clinical monitoring.

Hormone determination was done as follows: 17-hydroxyprogesterone (17OH-P), testosterone (Te), androstenedione (AD) and SHBG were measured in peripheral venous blood obtained after overnight fasting between 07.00 h and 08.00 h. Radioimmunoassay kits from Immunotech (Marseille, France) were used for determination of Te, 17OH-P and SHBG, whereas AD was determined by radioimmunoassays developed in the laboratory (14). The respective intra- and interassay coefficients of variation were 5.8, 5.2, 8.2 and 6.1, and 11.6, 6.2, 10.7 and 7.9 for AD, 17OH-P, Te and SHBG, respectively. Because physiological levels of these hormones strongly depend on sex and age, their physiological levels as determined in our laboratory are provided in Table 1. The values reflect physiological levels within and above–below the age and sex specific range. Free testosterone was calculated from total testosterone and SHBG levels, taking into account the association constants of testosterone with SHBG and albumin (15).

For statistical analysis, data were processed using Excel (Microsoft, ####, USA) and GraphPad Prism 4.0 (#####), and SPSS 11.5 software (#####) was used for Pearson's rank correlations.

Results

Table 2 shows the distribution of SHBG, total and free testosterone values for groups of low, normal and high levels, according to physiological levels for the respective age group as given in Table 1.

Concerning the amount of data in each group and their percentage of the total, it might be seen that whereas almost Gaussian distribution occurs for males, the data for females display a considerable shift to low SHBG and accordingly high free testosterone levels. In addition to the dependence on age, it should be emphasized that all patients were receiving substitution therapy.

The analogical distribution of 17-OH-P and AD levels, which are the most common parameters for laboratory monitoring of effectiveness of substitution, is shown in Table 3. In males more than three-quarters of 17OH-P levels and half of the records of AD levels fall into the range of high values, whereas in females the percentage of high values in both instances was lower than 50%.

To evaluate relationships among measured parameters, all laboratory data for male and female patients were mutually correlated. Particular focus was given to the relationship between SHBG and 17-OH-P. Pearson's correlation matrix is shown in Table 4. In this table, the section above and to the right of the diagonal shows the data for males, and the section below and to the left for females. Each cell from above represents the respective correlation coefficient (r), number of correlated pairs (n) and significance level (p). Correlations between free testosterone and total testosterone, and free testosterone and SHBG are not shown because these are inferred values. As expected, strong correlations were found

Table 2 SHBG, total testosterone (TTe) and free testosterone (FTe) levels in patients with CAH under common substitution regime, divided into groups of low, normal and high levels (see Table 1).

	Males			Females		
	Low	Normal	High	Low	Normal	High
SHBG						
n	86	135	79	171	196	89
% of total	28.7	45.0	26.3	37.5	43.0	19.5
TTe						
n	42	206	52	8	235	213
% of total	14.0	68.7	17.3	1.80	51.5	46.7
FTe						
n	90	131	79	20	160	276
% of total	30.0	43.7	26.3	4.4	35.1	60.5

Each cell shows the number of records (n) and their percentage from the total.

Table 3 17-Hydroxyprogesterone (17OH-P) and androstenedione (AD) levels in patients with CAH under common substitution regime, divided into groups of low, normal and high 17OH-P and AD levels (see Table 1).

	Males			Females		
	Low	Normal	High	Low	Normal	High
17OH-P						
n	14	51	212	14	205	212
% of total	5.1	18.4	76.5	3.2	47.6	49.2
AD						
n	7	130	137	7	239	184
% of total	2.6	47.4	50.0	1.6	55.6	42.8

Each cell shows the number of records (n) and their percentage from the total.

Table 4 Pearson's correlation matrix depicting mutual correlations among SHBG, total testosterone (TTe), free testosterone (FTe), 17-hydroxyprogesterone (17OH-P) and androstenedione (AD) levels in patients with CAH under common substitution regime.

	SHBG	TTe	FTe	17OH-P	AD
SHBG		–0.055 300 NS		–0.108 277 NS	–0.226 274 0.000
TTe				0.354 277 0.000	0.391 274 0.000
FTe				0.300 277 0.000	0.394 274 0.000
17OH-P	0.112 428 NS	**0.635** 428 **0.000**	**0.518** 428 **0.000**		**0.805** 258 **0.000**
AD	–0.090 429 NS	**0.783** 429 **0.000**	**0.739** 429 **0.000**	**0.622** 404 **0.000**	

Upper right section from the diagonal shows data for males, the lower left sections for females. Values in each cell are correlation coefficient (r), the number of correlated pairs (n) and statistical significance (p). Significant correlations ($p < 0.001$) with absolute r-values > 0.4 are in bold font. Because FTe is not an independent value, values of the respective correlated pairs are not given. NS denotes not significant.

among total as well as free Te, AD and 17OH-P, reflecting the fact that they represent steps in the steroid biosynthetic pathway. On the contrary, no correlation was revealed between SHBG and 17OH-P. In addition, no significant correlation was found between total Te and SHBG.

Discussion

Only a few reports deal with SHBG in patients with CAH, but in none of these was determination of SHBG the main outcome of these studies (9, 16). Thus, our study is the first report to specifically address this issue. The main finding was lower than normal SHBG levels and consequently higher levels of free testosterone for females (but not for males), irrespective of the substitution therapy.

An explanation could be that SHBG, as mentioned already, is involved in at least three endocrine axes: gonadal, insular and thyroid. SHBG has even been suggested to be an additional marker of hyperinsulinemia and insulin resistance (17). Hyperinsulinemia was also described in CAH (2, 4). In addition, insulin resistance with hyperinsulinemia and consequently a decrease of SHBG might also be induced by glucocorticoid treatment (18, 19). In females with CAH, in contrast to males, a considerable portion of androgens derive from the adrenals, which might also be the cause of the difference in testosterone and consequently SHBG between girls and boys. Because no correlation was revealed between SHBG and 17OH-P, this indicates that SHBG is a relatively independent variable. It could be speculated that whereas 17OH-P and AD reflect the adequacy of the substitution treatment of CAH, lower than normal SHBG levels are related to these patients and do not depend on their substitution treatment.

Insulin resistance and hyperandrogenemia, both associated with low SHBG, point to a similarity

between CAH and polycystic ovary syndrome, in spite of the fact that co-existence of both disorders is very rare (20, 21).

Finally, SHBG positively correlates with biologically active free thyroid hormones (13). CAH is often associated with congenital hypothyroidism, and in contrast to the association of PCOS with CAH, both diseases belong to the most frequent combination of congenital endocrine disorders (5). Unfortunately, data for thyroid hormone levels in our CAH patients were not available thus consideration of the effect of thyroid hormones on SHBG levels in CAH patients would be only speculative.

Based on data in this study as well as data in other studies, it can be concluded that low SHBG levels in CAH, at least in females, reflect its involvement in both insular and eventually thyroid axes, rather than the effectiveness of substitution.

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SHORT COMMUNICATION

The Content of Five Sex Steroids in Human Testis

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Summary

In order to assess whether intratesticular hormone content may be helpful for prediction of successful conception in men with fertility problems, five sex steroids, testosterone, dihydrotestosterone, androstenedione, estradiol and, for the first time epitestosterone, were measured in testicular tissue obtained by surgical retrieval from total 84 men. The group consisted of non-obstructive azoospermic men, aged 21-67 years who attended the centre for in vitro fertilization. Steroids after ether extraction and solvent partition were separated by high performance liquid chromatography and then measured by specific radioimmunoassays. The values varied considerably with means ± S.D. 2.43±2.47, 0.27±0.24, 0.080±0.13, 0.071± 0.089 and 0.31±0.27 for testosterone, dihydrotestosterone, androstenedione, estradiol and epitestosterone, respectively.

Key words

Androgens • Estradiol • Epitestosterone • Human testis • Biopsy

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Introduction

From the point of view of regulation, the testis represents to some extent an autonomous system characterized by an own transport mechanisms and feed-back loops. It is a site of formation of endocrine/ paracrine/autocrine acting molecules as growth factors, cytokines and last but not least steroids. Among their endocrine effects, steroids mediate inter-cell communications *via* their intratesticular receptors. Estradiol, for instance, besides its direct inhibitory action on testicular steroidogenesis, conducts paracrine action between Leydig and Sertoli cells (Levalle *et al.* 1994). Though also glucocorticoids may regulate testicular steroidogenesis by inhibiton of LH receptors expression through their own receptors in Leydig cells (Bambino and Hsueh 1981), sex steroids play the principal role in the testis. The importance of intratesticular between-cell communication is demonstrated among others by androgen-binding protein (ABP) mediating androgen transport from the site of their biosynthesis to target cells (Selva and Hammond 2006)

In spite of undoubted importance of steroids in the testis, there are not many reports on their intratesticular content. The first attempts of sex steroids determination in the human testicular tissue are dated from the seventies, for references see e.g. (Takahashi *et al.* 1982). They concerned young men with varicocele (McCoven *et al.* 1979), patients with prostate cancer (Leinonen 1980, Suescun *et al.* 1981, Kuber *et al.* 1991), various groups of infertile men with emphasis on the gonadotropin regulation of intratesticular sex steroid content (Takahashi *et al.* 1982, Levalle *et al.* 1994, Marie *et al.* 2001, Carreau *et al.* 2004), healthy male volunteers (Jarow and Zirkin 2005, Roth *et al.* 2010) and also *post mortem* tissues (Vermeulen and Deslypere 1986, Marie *et al.* 2001). The methods used for steroid determination differed in sample processing and not all included sufficient purification of the testicular extract. Meanwhile the methodology advanced enabling determination of minute amounts of steroids in various matrices including tissues. Here we present the data of four major sex steroids content in the whole human testicular tissue

obtained by surgical retrieval, namely testosterone, its precursor androstenedione, dihydrotestosterone and estradiol, after their separation by high performance liquid chromatography HPLC), in view to assess whether intratesticular hormone content may be helpful for prediction of successful conception in men with fertility problems. In addition, for the first time, epitestosterone concentration was determined, believed to act as an endogenous antiandrogen (Stárka 2003).

Materials and Methods

The whole testicular tissue was obtained from 84 non-obstructive azoospermic men aged 21-67 years (mean ± S.E.M.: 35.4±9.9) by surgical retrieval. Patients were evaluated by comprehensive history, physical examination, measurement of testicular size, at least two semen analysis and hormone parameters. The latter included serum follicle stimulating hormone (mean ± S.E.M.: 11.61±17.55 U/l), luteinizing hormone (mean ± S.E.M.: 6.01±5.06 U/l), prolactin (mean ± S.E.M.: 12.05±7.08 μg/l) and total testosterone (mean ± S.E.M.: 12.63±5.61 nmol/l) levels. All patients underwent screening for cystic fibrosis, Y chromosome microdeletion and karyotype analysis. Patients underwent microsurgical testicular sperm extraction (M-TESE) using optical x20-25 magnification (microscope OPMI Pico/S100, Carl Zeiss). In each case the informed written consent was obtained from the patients for working up the rests of tissues for scientific purposes. The material was immediately frozen in dry carbon dioxide and stored frozen at –70 °C until processed. The amounts of tissue varied from 2 to 65 mg. The tissues after thawing were homogenized in 1 ml of physiological solution, at first 1 min under increasing rate from 8000 up to 24 000 rpm, and the additional 1 min at 24 000 rpm using Ultraturrax T25 homogenizer. The homogenate was then extracted twice with 2 ml of diethyl ether, the ether extracts were combined and evaporated to dryness. The dry residues were partitioned between n-hexane (1 ml), methanol (2 ml) and water (0.5 ml). The upper hexane-methanolic phase containing the excessive fat was sucked off and the lower phase containing the steroids was evaporated again in the vacuum rotation evaporator. The dry residues were dissolved in 15 % acetonitrile (50 μl, v/v) and mixed properly to rinse the tube walls. The samples were centrifuged (2000 g, 3 min, 22 °C) and decanted solutions were transferred into chromatographic vials.

All the chemicals (analytical grade) and solvents (HPLC grade) were purchased from Merck (Darmstadt, Germany).

HPLC was used for separation of androstenedione (AD), testosterone (T), dihydrotestosterone (DHT), epitestosterone (ET) and estradiol (E2). The system (Dionex Softron, Germering, Germany) consisted of a HPLC pump P680 equipped with automatic flow rate control, an automated sample injector ASI-100, a thermostatic column compartment TCC-100, a photodiode array detector PDA-100 with wavelength range 190-600 nm and a fraction collector Foxy Jr. (Teledyne ISCO, Lincoln, NE). The separation was carried out on reverse phase EC 250/4 NUCLEOSIL® 100-5 C18 column (250 x 4 mm) with particle size of 5 μm (MACHEREY-NAGEL, Düren, Germany). To avoid possible column contamination the Phenomenex SecurityGuard system with cartridge C18 (4.0 x 3.0 mm) (Phenomenex, Torrance, CA) was used. For hormone separation the following protocol was used: The temperature in the column was maintained at 42 °C and the flow rate of the mobile phase was kept constant at 0.7 ml/min. The following gradient profile was used: 0.0-1.5 min constant mobile phase acetonitrile-water (10:90), 1.5-17.0 min linear gradient from methanol-acetonitrile-water (57: 4.8: 38.2) to methanol-acetonitrile-water (68: 4.8: 27.2), 17.0-21.0 min constant mobile phase acetonitrile-water (10:90). The column was equilibrated prior to sample injection. The standard solutions of AD, T, DHT, ET and E2 were used to set up the collecting windows. AD, T and ET were detected at 244 nm, DHT and E2 at 206 nm. The retention times of authentic AD, DHT, T, EpiT and E2 were 14.98, 15.45, 16.47, 17.77 and 15.38 min, respectively. The collected fractions were evaporated at 55 °C as above and the dry residues were analyzed by radioimmunoassay using the methods developed previously in the author´s laboratory,

Table 1. Intratesticular levels of five sex steroids from 84 azoospermic men, in nmol/g tissue.

Steroid	Mean	S.D.	Median	Lower quartile	Upper quartile
T	2.43	2.47	1.52	0.80	3.20
DHT	0.274	0.244	0.204	0.104	0.337
AD	0.080	0.128	0.043	0.017	0.106
E2	0.071	0.089	0036	0.015	0.096
ET	0.309	0.274	0223	0.114	0.390

Table 2. Survey of reported data on intratesticular steroids in humus.

Author, year	Sample (n)	Unit	T	DHT	AD	E2	ET	Method
McCowen *et al.* 1979	9 young males with varicocele after bilateral testicular biopsy	nmol/g tissue	5.16±0.32	0.14±071				RIA after extraction and thin layer chromatography
Pirke *et al.* 1979	45 males with azoospermia or oligozoospermia	nmol/g tissue	1.88					RIA after homogenization and extraction
Leinonen *et al.* 1980	25 males orchidectomized for prostate cancer	nmol/g tissue				0.0092±0.017		Extraction on Lipidex
Suescun *et al.* 1981	16 males with prostate cancer + 1 with carcinome of penis	nmol/g tissue	1.83±0.22	0.082±0.01				RIA after homogenization and extraction
Takahashi *et al.* 1982	40 infertile men after testicular biopsy	nmol/g tissue	4.58±2.95	0.067±0.040				RIA after extraction and separation on Sephadex LH 20
Vermeulen and Deslypere 1986	Cadaverous testes from 34 men	nmol/g tissue	1.55±0.22	0.064±0.013	0.058±0.045	0.019±0.002		RIA after homogenization, extraction and PC
Levalle *et al.* 1994	11 infertile men differing in FSH levels	nmol/g tissue	1.14-1.99			0.015-0.11		RIA after homogenization and extraction
Marie *et al.* 2001	10 infertile men after testicular biopsy	nmol/g tissue	6.14±1.33		0.62±0.27	0.64±0.41		RIA after homogenization and extraction
	Cadaverous testes from 20 healthy men		1.46±0.038		0.15±0.010	0.044±0.010		
Carreau *et al.* 2004	Three groups of aged men (total 37 men)	nmol/g tissue	1.68±0.26			0.047±0.015		Immunoassay after homogenization and extraction
This paper 2012	84 males with various fertility disorders	nmol/g tissue	2.43±2.47	0.27±0.24	0.080±0.128	0.071±0.089	0309±0.274	RIA after homogenization, extraction and HPLC
Jarrow and Zirkin 2005	9 male volunteers after bilatelar testicular aspiration	nmol/ml testicular volume	1.98±0.35	0.046±0.006		0.058±0.008		Tandem liquid chromatography-mass spectrometry
Roth *et al.* 2010	10 healthy male volunteers	nmol/ml testicular volume	1.49-3.11	0.004-0.016		0.0048-0.009		Tandem liquid chromatography-mass spectrometry

or by using commercial kits as follows: AD according to Putz *et al.* (1982), DHT by Hampl *et al.* (1990) and ET by Bílek *et al.* (1987). The corresponding intra-assay and inter-assay coefficients of variation (CV) in per cents were 5.8 and 11.6 (AD), 7.4 and 9.6 (ET), and 8.7 and 12.1 (DHT). Testosterone was measured by commercial RIA kit from Immunotech (Czech Republic division of Beckman Coulter, Marseille, France), estradiol by radioimmunoassay kit Spectria Estradiol RIA (Orion Diagnostica Oy, Espoo, Finland). The respective intra- and inter-assay CVs were 2.9 to 9.7 % (T) and 2.3 to 10.2 % (E2).

The losses during sample processing (extraction, solvent partition, HPLC) were estimated by spiking the pooled rests of testicular tissues (40 mg/sample) with radioactive tracers (50 000 dpm/sample) and by measuring the remaining radioactivity by liquid scintillation spectrometry (Beckman LS 6000 Liquid scintillation spectrometer). The radioactive tracers – [1,2,6,7-^{3}H] Testosterone, [1,2,6,7-^{3}H] Estradiol, [1,2,6,7-^{3}H] Androstenedione and [1,2,4,5,6,7-^{3}H] Dihydrotestosterone were purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA, USA). The recovery of tracers varied from 36.7 to 45.6 with mean 41.1 %. The results including those for ET were corrected for this value.

Results

The results of determination of five sex steroids in samples of testicular tissues from eighty four non-obstructive azoospermic men are shown in Table 1. The means, standard deviations, medians and upper and lower quartiles are provided. In the Table 2 the data are compared with those reported by other authors.

Discussion

The concentrations of testosterone are lower than those reported by early studies which did not use chromatographic separation of the ether extract but agree well with more recent ones. The values of estradiol and androstenedione are comparable with previous findings (Takahashi *et al.* 1982, Levalle *et al.* 1994). We have found higher intratesticular concentrations of dihydrotestosterone but the groups of subjects in other reports were much smaller. For the first time the values are provided for all steroids of interest in one sample, namely testosterone, its precursor androstenedione and 5alpha-reduced metabolite dihydrotestosterone, together with estradiol and epitestosterone. The number of samples and their heterogeneity do not allow statistical evaluation of the relation to the patient´s state, especially the sperm parameters, but the method is satisfactory for use in a larger number of samples.

Conflict of Interest

There is no conflict of interest.

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Review

Steroid metabolome in fetal and maternal body fluids in human late pregnancy

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ABSTRACT

Despite the extensive research during the last six decades the fundamental questions concerning the role of steroids in the initiation of human parturition and origin and function of some steroids in pregnancy were not definitely answered. Based on steroid metabolomic data found in the literature and our so far unpublished results, we attempted to bring new insights concerning the role of steroids in the sustaining and termination of human pregnancy, and predictive value of these substances for estimation of term. We also aimed to explain enigmas concerning the biosynthesis of progesterone and its bioactive catabolites considering the conjunctions between placental production of CRH, synthesis of bioactive steroids produced by fetal adrenal, localization of placental oxidoreductases and sustaining of human pregnancy. Evaluation of data available in the literature, including our recent findings as well as our new unpublished data indicates increasing progesterone synthesis and its concurrently increasing catabolism with approaching parturition, confirms declining production of pregnancy sustaining 5β-pregnane steroids providing uterine quiescence in late pregnancy, increased sulfation of further neuroinhibiting and pregnancy sustaining steroids. In contrast to the established concept considering LDL cholesterol as the primary substrate for progesterone synthesis in pregnancy, our data demonstrates the functioning of alternative mechanism for progesterone synthesis, which is based on the utilization of fetal pregnenolone sulfate for progesterone production in placenta. Close relationships were found between localization of

Abbreviations: 5α-DHT, 5α-dihydrotestosterone; 5β-DHP, 5β-dihydroprogesterone; ACTH, adrenocorticotropic hormone; AF, amniotic fluid; AKRs, aldo-keto reductases; AKR1C1, aldo-keto reductase family 1, member C1, 20α-hydroxysteroid dehydrogenase, hepatic dihydrodiol dehydrogenase; AKR1C2, aldo-keto reductase family 1, member C2, type III 3α-hydroxysteroid dehydrogenase; AKR1C3, aldo-keto reductase family 1, member C3, type II 3α-hydroxysteroid dehydrogenase; AKR1C4, aldo-keto reductase family 1, member C4, type I 3α-hydroxysteroid dehydrogenase; AKR1D1, 5β-reductase; ARSK, arylsulfatase K; CRH, corticotrophin releasing hormone; CRHBP, CRH binding protein; CNS, central nervous system; CYb5, cytochrome b5 enzyme; CYP11A1, cholesterol desmolase, cholesterol side chain cleavage enzyme; CYP11B1, 11β hydroxylase; CYP11B2, aldosterone synthase; CYP17A1, 17α-hydroxylase/17,20 lyase; CYP19A1, aromatase; CYP21A2, P450 21 hydroxylase; CYP3A4, glucocorticoid-inducible P450, taurochenodeoxycholate 6α-hydroxylase; CYP3A5, aryl hydrocarbon hydroxylase, xenobiotic monooxygenase; CYP3A7, aryl hydrocarbon hydroxylase, 16 α-hydroxylase; DHEA, dehydroepiandrosterone; DHEA16α, 16α-hydroxydehydroepiandrosterone; DHEA7α, 7α-hydroxydehydroepiandrosterone; DHEA7β, 7β-hydroxydehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; DZ, definitive zone of the fetal adrenal; FZ, fetal zone of the fetal adrenal; GA, gestational age; $GABA_A$-r, type-A γ-aminobutyric acid receptors; GH, growth hormone; GC–MS, gas chromatography–mass spectrometry; HSD, hydroxysteroid dehydrogenase; HSD11B, 11β-hydroxysteroid dehydrogenase HSD17B1-type 1 17β-hydroxysteroid dehydrogenase; HSD17B10, type 10 17β-hydroxysteroid dehydrogenase; HSD17B11, type 11 17β-hydroxysteroid dehydrogenase, 3(α → β)-hydroxysteroid epimerase; HSD17B12, type 12β-hydroxysteroid dehydrogenase; HSD17B2, type 2 17β-hydroxysteroid dehydrogenase; HSD17B6, type 6 17β-hydroxysteroid dehydrogenase; HSD17B7, type 7 17β-hydroxysteroid dehydrogenase; HSD3Bs, 3β-hydroxysteroid dehydrogenases/Δ(5 → 4)-isomerases; HSD3B1, type I 3β-hydroxysteroid dehydrogenase/Δ(5 → 4)-isomerase; HSD3B2, type II 3β-hydroxysteroid dehydrogenase/Δ(5 → 4)-isomerase; HSDs, hydroxysteroid dehydrogenases; IGF-1, insulin-like growth factor-1; K(m), Michaelis's constant; LC–MS, liquid chromatography–mass spectrometry; LDL, low-density lipoprotein; mRNA, messenger ribonucleic acid; MV, maternal cubital vein; MLN64, steroid acute regulatory protein-related lipid transfer (START) domain containing; NAS, neuroactive steroids; NMDA-r, N-methyl-D-aspartate receptors; OAT-4, organic anion transporter 4; POR, P450 (cytochrome) oxidoreductase; PregS, pregnenolone sulfate; Prog20α, 20α-dihydroprogesterone; SDRs, short-chain dehydrogenases/reductases; SRD5A1, type 1 5α-reductase; SRD5A2, type 2 5α-reductase; StAR, steroid acute regulatory protein; STAT5b, signal transducer and activator of transcription 5B; STS, steroid sulfatase; SULT2A1, human type 2A1 hydroxysteroid sulfotransferase; SULT1E1, estrogen preferring sulfotransferase; TZ, transitional zone of the fetal adrenal; UA, umbilical artery; UGT2B7, UDP glucuronosyltransferase 2B7; UV, umbilical vein.

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placental oxidoreductases and consistently higher levels of sex hormones, neuroactive steroids and their metabolites in the oxidized form in the fetus and in the reduced form in the maternal compartment.

Contents

1. Introduction

Despite the extensive research during the last six decades the questions concerning the role of steroids in the initiation of human parturition and origin and function of some steroids in pregnancy were not definitely answered. Human parturition is unique [1] and therefore the use of animal model is frequently inadequate. Therefore the information contained in steroid metabolome in human body fluids may be helpful for a better understanding the physiology of human pregnancy and parturition. Although the steroid metabolome in maternal circulation was extensively studied, the information is deficient concerning the metabolomic profiles in human fetal body fluids. In this study we attempted to review existing state of art in the steroid metabolomics focused on human late pregnancy. The data found in the literature will be reviewed as well as our so far unpublished GC–MS results that were obtained in the frame of our two recently published studies [2,3] in the group of women at labor from the 28th to 41st week of gestation. The latter data includes almost complete steroid metabolome in fetal umbilical arterial) and venous blood as well as in the maternal venous blood and amniotic fluid. The metabolomic profiles were recorded in 12 women giving birth after the 38th week of gestation who were without perinatological complications and the group of 38 preterm births being selected so that the reasons in preterm labors were independent of the steroid status (for details see [2,3]).

Some of the mechanisms explaining the hormonal control of pregnancy sustaining and onset of parturition involve progesterone withdrawal at concurrently increasing estradiol production before the onset of parturition [4,5]. However, progesterone levels in human maternal blood do not markedly change around parturition [6,7]. Regarding progesterone, the initiation of human delivery is rather connected to a changed expression of specific isoforms of progesterone receptors than to a change in progesterone levels. In addition to the increased circulating estradiol levels, the changing expression of specific isoforms of estradiol receptors probably also contributes to the onset of labor [8]. From the further steroids, cortisol may inhibit progesterone action in the regulation of 15-hydroxyprostaglandin dehydrogenase expression at term [9].

In pregnancy and parturition a role of the most abundant neuroinhibiting reduced progesterone metabolite allopregnanolone was suggested in rats [10]. Allopregnanolone, and probably also some other steroids, operate via positive modulation of the type-A γ-aminobutyric acid receptors ($GABA_A$-r) [11,12] on the membranes of hypothalamic oxytocin-producing cells. However, the role of allopregnanolone and further neuroactive steroids (NAS) in the timing of human parturition is still unclear.

The levels of pregnane NAS are excessively increased in pregnant women [13] in comparison with those in non-pregnant [14]. Besides $GABA_A$-r, the polar conjugates of the reduced 3α/β-hydroxy-5α/β-reduced pregnane steroids are also active on N-methyl-D-aspartate receptors (NMDA-r) showing positive and negative modulation for the 5α- and 5β-isomers, respectively [15]. Although CNS possesses independent steroid production [16], the peripherally produced NAS may pass the blood–brain barrier [17] and influence the steroid metabolome in the CNS. NAS may also operate at the peripheral level like allopregnanolone and progesterone, both attenuating myometrial contractions via the opening of voltage-dependent K^+-channels, contrary to estradiol, which is their antagonist [18,19]. The NAS may be also produced locally, exerting intracrine and paracrine effects. On the other hand, conjugated steroids may be easily transported by circulation in high amounts from more distant sources. The reduced progesterone

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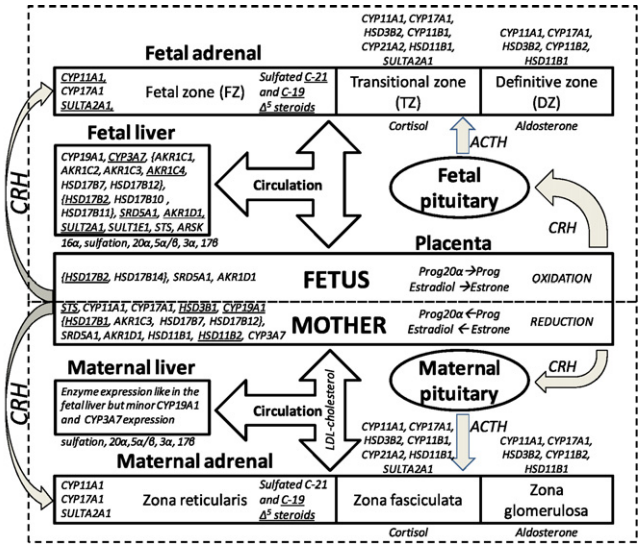

Fig. 1. Simplified scheme of steroidogenesis in human late pregnancy.

metabolites might also exert peripheral analgesic effects via blockade of T-type calcium channels, which are responsible for pain perception [20].

Besides non-genomic effects, reduced progesterone metabolites, which are synthesized in large quantities in pregnancy [13,21–27], may also bind on nuclear receptors such as progesterone receptors [28] providing uterine quiescence. Some studies including ours reported decreasing production of pregnancy sustaining 5β-pregnane steroids that provide uterine quiescence via binding to nuclear pregnane X-receptors [13,26,29–31].

The kinetics of irreversible catabolism of the bioactive steroids, oxidoreductive balances between active and inactive forms of steroids [32] and balances between free steroids and their conjugates [33] may be crucial for the regulation of their biological activity and consequently for the pregnancy sustaining.

Concerning the steroid metabolome in human body fluids, there are four key steroidogenic organs such as fetal and maternal adrenal, placenta, fetal and maternal liver (Fig. 1, our so far unpublished data). Considering the endocrine, autocrine and paracrine steroid effects, the uterus and fetal membranes might be of a great importance [34]. However, the contribution of the steroids produced in these tissues to steroid metabolome in fetal and maternal blood does not seem to be essential.

2. Steroid metabolism in fetal and maternal adrenal

2.1. The key role of placental CRH in the regulation of steroid biosynthesis in pregnancy

The paramount mechanism controlling overall production of the most of pregnancy steroids is based on placental production of CRH (Fig. 1, our so far unpublished data) [35]. CRH in non-pregnant subjects is a hypothalamic hormone controlling the pituitary secretion of ACTH and, in turn, the production of corticosteroids in an adult adrenal. The hypothalamic–pituitary–adrenal axis in these subjects is based on a negative feedback loop between the final active hormone, ACTH and CRH. The situation in pregnancy after luteo-placental shift is different. CRH is primarily expressed in human placenta and instead of the negative feedback loop cortisol–ACTH–CRH; there is a positive one between cortisol and CRH, while the ACTH production stagnates. CRH directly stimulates production of Δ^5 steroid sulfates in the fetal zone of the fetal adrenal (FZ) [36,37] and cortisol synthesis in the transitional zone of the fetal adrenal (TZ) [38] via binding to ACTH receptors [39]. ACTH receptor mRNA is localized in all cortical zones but its abundance is higher in DZ (definitive zone) than in FZ [40]. The fetal adrenal gland at term is almost the size of the fetal kidney and the FZ at term produces steroids more abundantly than normally secreting adrenal glands of the adult [35]. The C-19- and possibly also the C-21 Δ^5 steroids, originating in the FZ and being further processed in placenta and liver, represent the largest fraction of steroids in pregnancy [41–44]. However, progesterone is commonly considered to originate mainly in placenta from maternal LDL cholesterol [7,45].

After midgestation, the TZ cells may have the capacity to synthesize cortisol and be analogous to cells of the *zona fasciculata* of the adult adrenal. By the 30th week of gestation, the definitive zone of the fetal adrenal (DZ) and TZ begin to resemble the adult *zona glomerulosa* and *zona fasciculata*, respectively [46]. The FZ still producing conjugated C-19 Δ^5 steroids is similar to the adult *zona reticularis* but unlike the adult *zona reticularis*, the FZ produces excessive amounts of conjugated C-21 Δ^5 steroids, including sulfates of pregnenolone (PregS), 17-hydroxypregnenolone [35] and androstenediol (Fig. 2, our so far unpublished data). As generally accepted, the Δ^5 steroid sulfates (originating in the FZ) serve as precursors for the placental production of estradiol [36,37] and as suggested in our recent study [2], possibly also for progesterone synthesis.

The levels of CRH are extremely high in maternal and high in the fetal blood [47]. The rising levels of human placental CRH in maternal circulation in the last 4 weeks of pregnancy stimulate the production of conjugated C-19- [36] and probably also the C-21 Δ^5 steroids [2] in FZ in a dose-dependent manner. CRH is as effective as ACTH at stimulating sulfated dehydroepiandrosterone (DHEAS) production but is 70% less potent than ACTH at stimulating cortisol production. Although CRH increases the expression of cholesterol desmolase (CYP11A1, cholesterol side chain cleavage enzyme) it is not mitogenic for fetal adrenal cortical cells [36].

It should be outlined that the excessive production of placental CRH is specific for primates and the boost in CRH production in late pregnancy is specific only for human and great apes [48]. This should be considered when addressing the initiation of human parturition and this is the primary reason for which the animal models may not be optimal for investigation of human pregnancy. Only human beings and great apes produce a circulating binding protein for CRH (CRHBP), the levels of which fall at the end of pregnancy thus increasing the bioavailability of CRH [49,50].

Despite the substantial alterations in the placental CRH production in late pregnancy, the predictivity of the unstable CRH for an estimation of term is relatively poor [51]. Nevertheless, the CRH induced changes in the steroid metabolome may better predict the approaching parturition. When using the simultaneous quantification of the steroid metabolome in one sample by GC–MS or LC–MS and multivariate approach for evaluation of the results obtained, the cumulative effect of mutually strongly inter-correlated steroids substantially improves the predictivity. This algorithm appears to be less expensive and more informative. As demonstrated in our recent study [3], the predictivity of the primary products of the FZ for the onset of human parturition is high.

2.2. Steroid 17α-hydroxylase/17,20 lyase (CYP17A1)

Besides stimulation of CYP11A1, CRH also stimulates 17α-hydroxylase/17,20 lyase (CYP17A1) expression possessing both 17α-hydroxylase and 17,20-lyase activities [36]. CYP17A1 proteins and mRNAs were detected only in FZ and TZ, not in the DZ [52,53]. CYP17A1 also exhibits marked progesterone 16α-hydroxylase activity in human steroidogenic cells including those from the fetal adrenal [54]. CYP17A1 has extremely low C-17,20-

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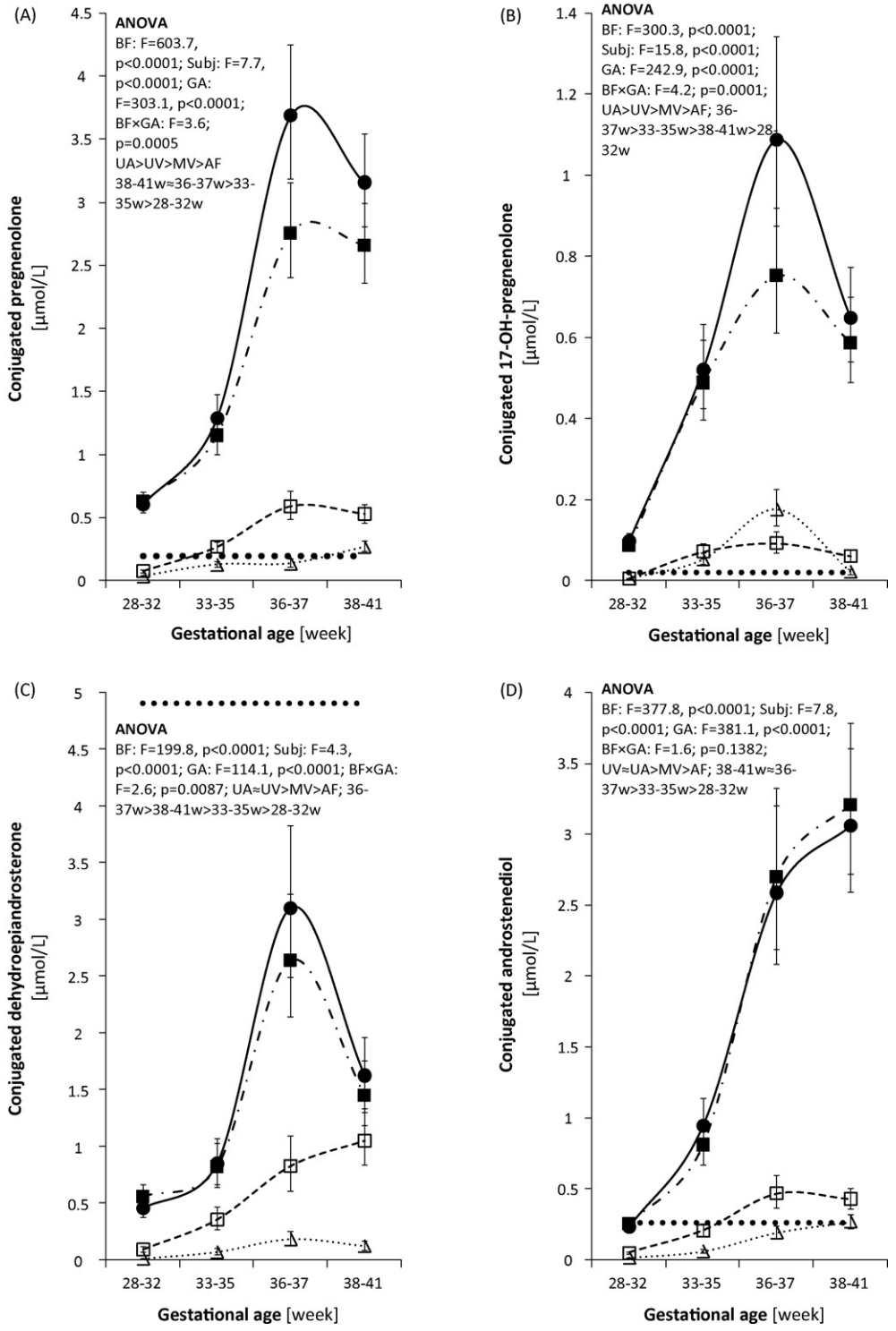

Fig. 2. Profiles of conjugated sulfated Δ^5 steroids in the plasma from the umbilical artery (UA), umbilical vein (UV) and maternal cubital vein (MV) and in amniotic fluid (AF) according to the gestational age. The repeated measures ANOVA model was used for the evaluation of the relationships between steroid levels, GA and the type of body fluid. The model consisted of within-subject factor body fluid (factor BF—four body fluids were investigated in each subject), subject factor (factor Subj), between-subject factor gestational age (factor GA—the subjects were separated into 4 groups according to the GA) and body fluid × GA interaction (BF × GA interaction). The symbol w denotes the week of gestation. Significant BF × GA interaction indicates that there is a significant difference between the dependences of the individual body fluids on GA. *F*-ratio represents the Fisher's statistic and *p* designates statistical significance for the factors and interaction. The symbols with error bars represent re-transformed means with their 95% confidence intervals for individual body fluids (full circles, UA; full squares, UV; empty squares, MV; empty triangles, AF. The significance testing in the form of the subgroup confidence intervals is for the interaction of body fluid (sample material) with GA. The 95% confidence intervals are computed using the least significant difference multiple comparisons ($p < 0.05$). The confidence intervals, which do not overlap each other, denote significant difference between the respective subgroup means. Further embedded table contains the multiple comparisons that are completed separately for the gestation week and for the sample material (body fluid). The symbol "≈" expresses insignificant difference, while the symbol ">" means "significantly higher than". The significance level was considered for $p < 0.05$. The horizontal line from the full circles represents the mean level of the steroid in the luteal phase of the menstrual cycle. These so far unpublished data were obtained in the frame of our recently published studies [2,3].

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lyase activity toward C-21 Δ^4 steroids and fails to convert these substances to corresponding C-19 steroids [54]. However, also the levels of sulfated C-21 Δ^5 steroids are elevated in the maternal blood [55] and excessively elevated in the fetal circulation in contrast to the situation in non-pregnant women (Fig. 2, our so far unpublished data), which indicates limited C-17,20-lyase activity in the FZ also for C-21 Δ^5 steroids.

2.3. Steroid sulfotransferases and sulfatases

The human type 2A1 hydroxysteroid sulfotransferase (SULT2A1) displaying reactivity towards 3α/β-hydroxysteroids, estrogens, and 17-hydroxyl group of androgens is highly expressed in the adrenal cortex [56]. TZ and FZ showed immunoreactivity for SULT2A1, but not the DZ [53]. SULT2A1 enzyme activities are independent of the gestational age (GA) [57]. In addition to the SULT2A1 expression, the estrogen preferring sulfotransferase (SULT1E1) activity [58] and relatively high steroid sulfatase (STS) immunoreactivity were also reported in the adult adrenal gland [59].

2.4. Activities of enzymes enrolled in the synthesis of corticoids

3β-Hydroxysteroid dehydrogenases/$\Delta(5\rightarrow4)$-isomerases (HSD3Bs) catalyzes the oxidative conversion of 3β-hydroxy-Δ^5 steroids. HSD3B immunoreactivity is not detected in the fetal adrenal prior to 22 weeks of gestation, but becomes discernible in the TZ and DZ after 23 weeks [52,53]. In late pregnancy, TZ and DZ provide the conversion of Δ^5 steroids to 3-oxo-Δ^4 precursors of corticosteroids expressing type 2 HSD3B (HSD3B2) [60]. Early in gestation, only the Δ^5 steroid production occurs in the TZ and FZ, which expresses CYP11A1 and CYP17A1 [52]. ACTH does not influence steroidogenesis in the FZ [61]. Like the HSD3B2, the enzymes CYP21A2 (P450 21 hydroxylase, or P450C-21), CYP11B1 (11β hydroxylase or P450c11) and CYP11B2 (aldosterone synthase) are necessary for corticoid synthesis. CYP21A2 immunoreactivity is minor in the DZ but is detectable in almost all cells in the TZ and FZ [53,62]. After 23 gestational weeks, the immunoreactivity for CYP21A2 is detected in all three zones [53]. TZ expressing CYP11A1, CYP17A1, HSD3B2, CYP21A2, CYP11B1 and type CYP11B2 has the capacity to synthesize cortisol after midgestation [53,62] while the DZ may synthesize mineralocorticoids, but not until near term [62]. CYP17A1, CYP11B1, and CYP11B2 immunoreactivities are present in the TZ and FZ but absent in the DZ but [62]. Later in gestation, the DZ produces mineralocorticoids, TZ produces glucocorticoids and the FZ continues to produce Δ^5 steroids [52].

Human adrenal glands also possesses 11β-hydroxysteroid dehydrogenase (HSD11B) activity catalyzing inactivation of glucocorticoids [63].

2.5. Adrenal C-3, C-17 and C-20 oxidoreductive conversions

HSDs, catalyzing reversible C-3, C-17 and C-20 oxidoreductive inter-conversions belong to either the short-chain dehydrogenases/reductases (SDRs) or the aldo-keto reductases (AKRs). Several SDRs are active in the adrenals.

The type 11 17β-HSD (HSD17B11) $3(\alpha\rightarrow\beta)$-hydroxysteroid epimerase prefers the oxidative conversion converting 5α-androstan-3α,17β-diol to androsterone [64,65].

Type 6 17β-HSD (HSD17B6) possessing both oxidoreductase and $3(\alpha\rightarrow\beta)$-hydroxysteroid epimerase activities acts on both C-19 and C-21 3α-hydroxysteroids. Because bioactive steroids commonly exert their effect in a stereo-specific manner, epimerase activity may be of biological importance [66].

Type 7 17β-HSD (HSD17B7) preferably operates as reductase and catalyzes the reduction of the oxo-group in either 17- or 3-position of the substrate to the corresponding 17β- or 3α-hydroxy-counterparts, respectively. HSD17B7 exhibits also minor 3βHSD-like activity towards progesterone and 20α-dihydroprogesterone (Prog20α) [67]. Like the HSD17B7 the type 12 17β-HSD (HSD17B12) also prefers the reductive direction catalyzing the conversion of estrone into estradiol and was also detected in the adrenal [68].

2.6. 16α-Hydroxylation

16α-Hydroxylation being primarily provided by cytochrome P450 CYP3A7 enzyme probably regulates the levels of precursors for the synthesis of hormonally active steroids. The CYP3A7 is also active in the fetal adrenal but the levels of the CYP3A7 isozyme in fetal adrenals are only 33% of that in fetal livers [69,70].

2.7. Differences in enzyme expression between fetal and adult adrenal

While the expression of some enzymes like CYP17A1, 21-hydroxylase, 11β hydroxylase, and CYP11B2 do not significantly differ between the fetal adrenal in late pregnancy and adult adrenal, others show pronounced differences. CYP11A1, cytochrome b5 enzyme (CYb5) and P450 cytochrome oxidoreductase (POR) mRNA expression is nearly twice higher in fetal than in adult adrenal, and SULT2A1 transcript shows even 13-fold higher levels in the fetal adrenal. Alternatively, HSD3B2 mRNA expression in midgestation is 127-fold lower than that in the adult adrenal. It is evident that increased expression of CYP11A1 in fetal adrenal reflects high cholesterol utilization for steroidogenesis. CYb5 and POR cofactors may stimulate CYP17A1 activity and thus the production of sulfated Δ^5 steroids in the fetal adrenal [71]. Markedly higher expression of SULT2A1 reflect high claim for steroid sulfation enabling a production of sufficiently soluble precursors, which can be easily transported in excessive amounts by circulation for the placental synthesis of sex hormones. Alternatively, the lack of HSD3B2 in the FZ provides preferential synthesis of the Δ^5 C-21 steroids over cortisol production.

3. Steroid metabolism in fetal and maternal liver

The activities of CYP11A1 and HSD3Bs in the fetal liver are negligible or even absent in human pregnancy [72]. However, other steroidogenic enzymes in the maternal and particularly in the fetal liver may substantially influence the steroid metabolome in both fetal and maternal circulation.

3.1. Liver 16α-hydroxylation and estrogen formation

16α-Hydroxylation is provided by cytochrome P450 CYP3A7 enzyme that is pronouncedly expressed in the microsomal fraction from fetal liver [70,73] although this activity in the adult liver is negligible [43]. While CYP3A4 and CYP3A5 enzymes are responsible for the production of 7α-hydroxy-DHEA (DHEA7α), 7β-hydroxy-DHEA (DHEA7β), and 16α-hydroxy-DHEA (DHEA16α) in the adult liver microsomes, the fetal/neonatal CYP3A7 produces DHEA16α and DHEA7β [74].

The fetal liver is the primary source of 16α-hydroxy-metabolites of Δ^5 steroids, as also documented by consistently higher levels of 16α-hydroxy-metabolites of the substances in fetal circulation when compared with the maternal compartment [75,76], confirmed also by our unpublished results (Fig. 3). However, some authors [54,75] suggested 16α-hydroxy-progesterone (Prog16α) synthesis from progesterone catalyzed by CYP17A1 localized in the placenta.

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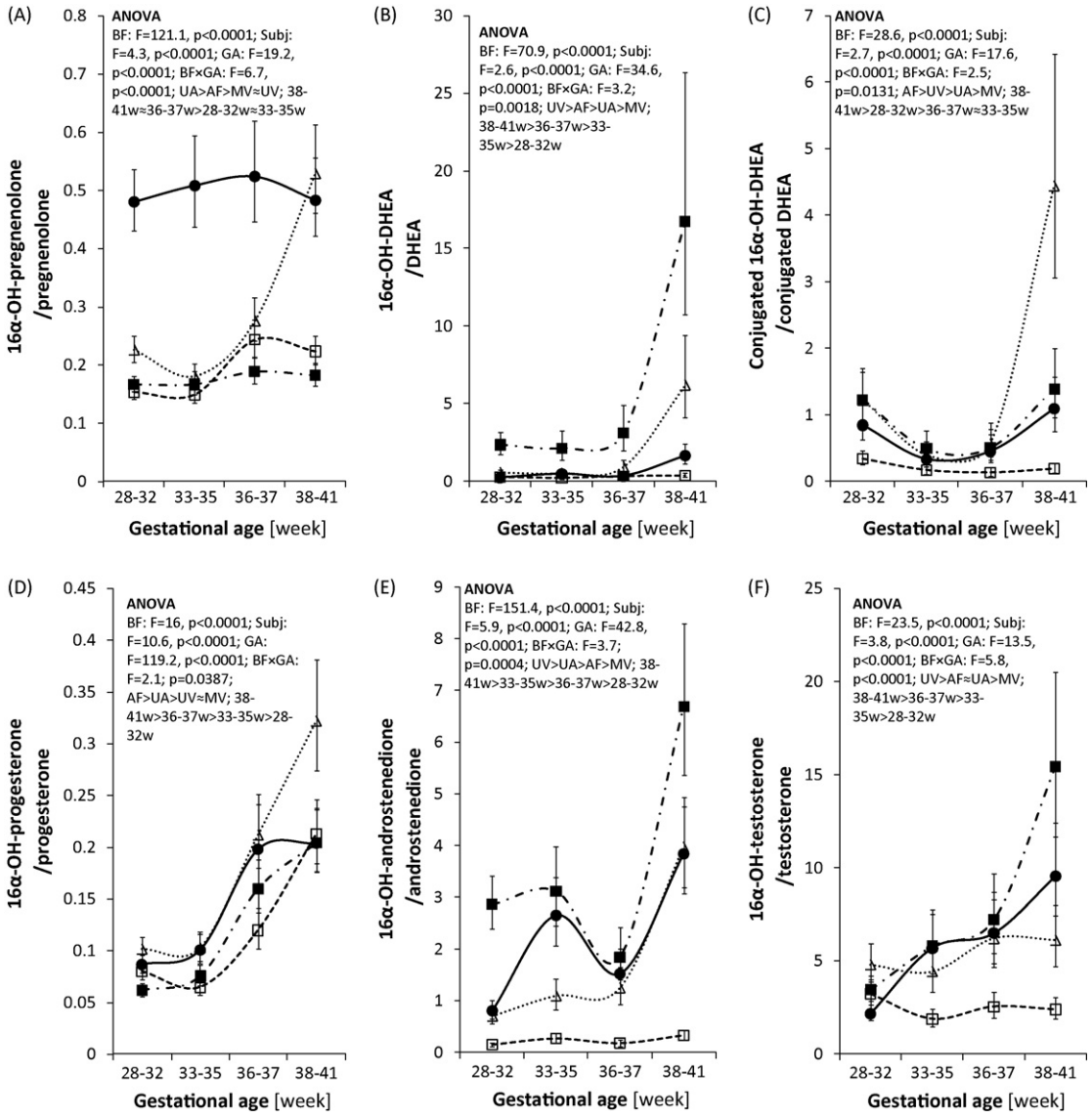

Fig. 3. Profiles of the ratios of 16α-hydroxysteroids to the corresponding 16-deoxy-steroids in the plasma from the umbilical artery (UA), umbilical vein (UV) and maternal cubital vein (MV) and in amniotic fluid (AF) in preterm and normal labor. The drawings and symbols are the same as for Fig. 2. These so far unpublished data were obtained in the frame of our recently published studies [2,3].

The levels of 16α-hydroxysteroids in the fetal blood increase from the second to the third trimester [61,75,76] and rise considerably at delivery [77]. In addition, the ratios of 16α-hydroxy-metabolites to 16-deoxy-steroids significantly increased after 30th week of gestation indicating increasing catabolism of the sex hormone precursors in the fetal liver [78] (Fig. 3, our so far unpublished data).

According to the Diczfalusy's concept [79], the DHEAS from the fetal adrenals is hydroxylated at the 16α-position in the fetal liver and then aromatized to estriol in the placenta and most of this huge amount of estriol exits the placenta into the uterine vasculature and maternal circulation.

The inhibitory effect of sulfated DHEA16α on estrogen production is minimal at low DHEAS concentrations (favoring the secretion of estrone and estradiol) and is greatly enhanced at concentrations of DHEAS that induced maximum estrone and estradiol secretions. In trophoblastic cells, the metabolism of DHEAS can modulate estriol secretion, and the metabolism of sulfated DHEA16α can modulate the secretion of estrone and estradiol [80]. However, whilst each substrate appeared to inhibit the aromatization of the other, the 16-deoxy-C-19 steroids are more potent inhibitors [81]. 16α-Hydroxy-metabolites of testosterone and androstenedione are only poor substrates for the placental aromatase (CYP19A1) in contrast to the corresponding 16-deoxy-steroids [82] and the initial rates of estrogen formation are higher for the 16-deoxy-C-19 steroids [81].

Lee et al. [83] reported that at a physiologically relevant low substrate concentration (10 nmol/L), CYP3A7 had a strong catalytic activity for the 16α-hydroxylation of estrone, and the ratio of its 16α-hydroxylation to 2-hydroxylation was 107%. However, when estradiol was the substrate, CYP3A7 had only very weak catalytic activity for 16α-hydroxylation, and the ratio of its 16α-hydroxylation to 2-hydroxylation was 10–33%. Moreover, the maximum velocity/K(m) ratio was more than 100 times higher for the 16α-hydroxylation of estrone than for estradiol. This prompts that estrone originating in placenta from androstenedione is transported by circulation into the fetal liver, where may be further conjugated by sulfatases and glucoronidases, converted to estradiol by reductive and SDRs and AKR1Cs. Estradiol of the placental

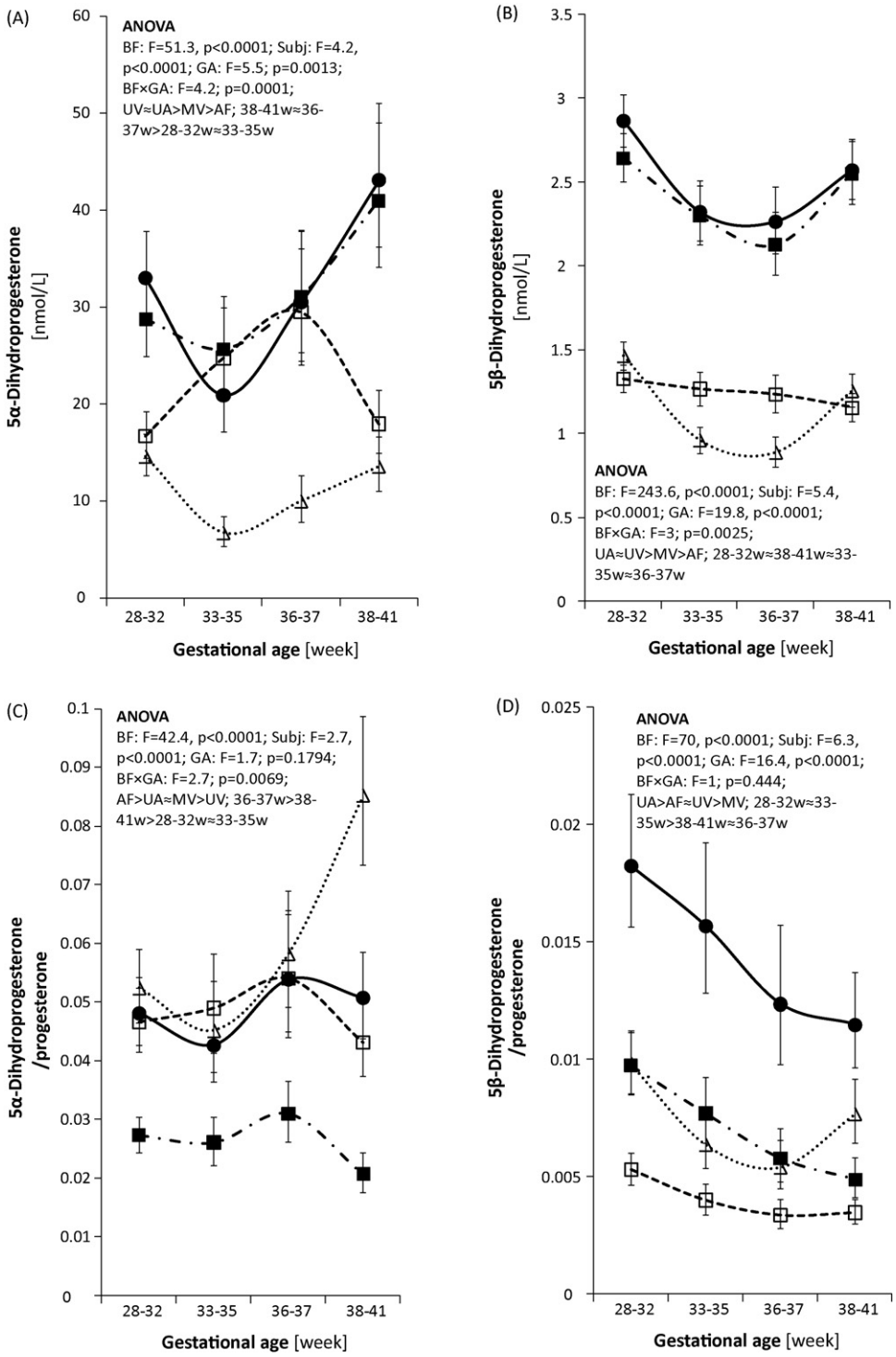

Fig. 4. Profiles of 5α-dihydroprogesterone and 5β-dihydroprogesterone and their ratios to progesterone in the plasma from the umbilical artery (UA), umbilical vein (UV) and maternal cubital vein (MV) and in amniotic fluid (AF) in preterm and normal labor. The drawings and symbols are the same as for Fig. 2. These so far unpublished data were obtained in the frame of our two recently published studies [2,3].

and liver origin may be also sulfated, then the free and conjugated estrone may be 16α-hydroxylated by CYP3A7 (the most potent AK1C4 is independent of the substrate sulfation status) and finally, liver SDRs and AKR1Cs may catalyze further conversion of the free and conjugated (in C-3 position) 16α-hydroxy-estrone to free and conjugated estriol.

Like in the case of CYP3A7, the adult liver exhibits little CYP19A1 activities but the fetal liver is capable to extensively aromatize various C-19 steroids to estrogens [84,85] and the CYP19A1 activity in the fetal hepatocytes appears to be up regulated by glucocorticoids [86].

3.2. C-3, C-17 and C-20 oxidoreductive conversions

Because active hydroxysteroids generally exert their effect in a stereo-specific manner, epimerase activity may potentially play an important role in regulating the biological activities of various steroids.

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Human liver contains all isoforms (AKR1C1–AKR1C4) of dihydrodiol dehydrogenase with 20α-, 17β-, 3α- or 3β-hydroxysteroid dehydrogenase-like activity [87–89]. Activities of AKR1Cs could control occupancy of the androgen- and $GABA_A$-r [90]. *In vivo*, all AKR1Cs preferentially work as reductases [91] and are capable to reduce estrone and progesterone to estradiol and Prog20α, respectively. On the other hand, AKR1Cs may decrease the neurosteroid concentrations by inactivating allopregnanolone and eliminating the precursors like progesterone from the synthetic pathways via reduction of the 20-oxo-steroid group [32,92]. The AKR1C2 preferring 3α-reduction over the 3β-reduction may catalyze 3α-, 17β- and 20α-HSD reactions [32,89,92,93]. AKR1C3 catalyze the reduction of 5α-dihydrotestosterone (5α-DHT), androstenedione, estrone and progesterone to produce 5α-androstan-3α,17β-diol, testosterone, estradiol and Prog20α, respectively [88]. AKR1C4, the expression of which is limited to the liver [32,94,95], catalyzes the transformation of the 5α-DHT into 5α-androstan-3α,17β-diol. Liver specific AKR1C4 shows superior catalytic efficiency versus the other isoforms. This efficiency exceeded those obtained with the other isoforms by 10–30-fold. In contrast to the other isoforms, the catalytic efficiency for AKR1C4 is unaffected by steroid conjugation [89].

Two liver SDRs, HSD17B7 and HSD17B12, also preferentially work as reductases. HSD17B7 preferring the reduction of the oxo-group in 20-, 17- or 3-position to the corresponding 20α-hydroxy-, 17β-hydroxy- or 3α-hydroxy-counterparts is also significantly expressed in the liver [67,96] as well as HSD17B12 catalyzing the transformation of estrone into estradiol [68].

HSD17B2, HSD17B10 and HSD17B11, which are also highly expressed in the liver, prefer the oxidative direction. HSD17B2 may contribute to formation of 20-oxo- and 17-oxo-steroids from their 20α- and 17β-counterparts [42]. HSD17B6 prefers oxidoreductase and 3(α → β)-hydroxysteroid epimerase activities and acts on both C-19 and C-21 3α-hydroxysteroids [66]. Type 10 17β-HSD (HSD17B10) being abundantly expressed in the liver, is capable of catalyzing the oxidation of steroid modulators of $GABA_A$-r [97]. HSD17B10 catalyzes the oxidation of 5α-androstan-3α,17β-diol to 5α-DHT [98] and conversion of allopregnanolone and allotetrahydrodeoxycorticosterone (3α,5α-THDOC) to the corresponding inactive 3-oxo-steroids. The catalysis of HSD17B10 appears to be essential for maintaining normal functions of GABA-ergic neurons [99]. Finally, the HSD17B11 [64] can convert 5α-androstan-3α,17β-diol to androsterone [64,65].

Commonly, 20α-hydroxysteroids are considered as inactive catabolites. However, 20α-dihydropregnenolone relax the tonic contractions induced by KCl in a concentration-dependent way [100].

3.3. 5α/β-Reductases

5α- and 5β-Reductions are important for the biosynthesis of NAS. Conjugation of the androgens occurs extensively in the liver which has high activity of 5α- and 5β-reductases [101,102].

There are two isoforms of 5α-reductase, with a limited degree of homology, different biochemical properties and distinct tissue distribution. Type 1 5α-reductase (SRD5A1) is widely distributed in the body, with the highest levels in the liver. SRD5A1 converts testosterone into 5α-dihydrotestosterone and progesterone or corticosterone into their corresponding 5α-3-oxo-steroids. In the androgen-dependent structures, 5α-DHT is almost exclusively formed by 5α-reductase type 2 (SRD5A2) [103]. In the peripheral tissues, including the liver, SRD5A1 and reductive 3α-HSD isoforms work consecutively to eliminate the androgens and protect against the hormone excess [104].

5β-Reductase (AKR1D1) belonging to AKRs, efficiently catalyzes the reduction of both C-19 and C-21 3-oxo-Δ^4 steroids to the corresponding 5β-reduced metabolites. 11β-Hydroxy-group in corticoids hinders the transformation [102].

The higher levels of several 5β-reduced progesterone metabolites in the fetus than in maternal compartment (Figs. 4B, D and 5C, D) (our unpublished data) indicate higher placental expression of AKR1D1 towards the fetal compartment and/or higher expression of AKR1D1 in the fetal liver. The latter possibility appears to be more likely because both 5β-pregnanolone isomers display lower levels in the blood from umbilical vein (UV) than in blood from the umbilical artery (UA) (Fig. 5C and D, our so far unpublished data). In addition, the ratio of 5β-dihydroprogesterone (5β-DHP) to progesterone is significantly higher in UA than in UV (Fig. 4D, our so far unpublished data).

3.4. Balance between polar conjugates and unconjugated steroids

The balance between the sulfated and unsulfated NAS may be decisively influenced by the activities of liver sulfatases, sulfotransferases and perhaps also the glucuronosyltransferases. The pregnane and androstane 5α/β-reduced metabolites being frequently neuroactive are readily sulfated in the liver. As already mentioned, the balances between free steroids and their conjugates [33] may be crucial for the regulation of their biological activity and consequently for the sustaining of pregnancy. The 5α/β-reduced metabolites with a hydroxyl in the 3α-position positively modulate $GABA_A$-r. Their sulfates operate in the opposite way, though on different binding sites. Sulfation may also decrease the concentration of unconjugated NAS, the polarity of which is more favorable for crossing the blood–brain barrier. The modulation efficiencies of the conjugated neurosteroids on $GABA_A$-r may reach about 1/10 of those for the corresponding unconjugated substances [33]. Nonetheless, in maternal circulation the concentrations of conjugated pregnane steroids are about two orders of magnitude higher when compared with their unconjugated analogues. Conjugation is a prerequisite for the activity of 3α/β-hydroxy-5α/β-reduced pregnane steroids on N-methyl-D-aspartate receptors (NMDA-r) showing positive and negative modulation for the 5α- and 5β-isomers, which are neuroactivating and neuroinhibiting substances, respectively [15]. Finally, the sulfation might influence the activity and/or availability of the peripherally active pregnancy sustaining steroids like the 5α/β-reduced pregnane and androstane steroids but may also facilitate their transport by circulation. However, even in these cases, the sulfation rather shift the biological activity towards induction of labor, catabolizing the 5β-reduced steroids that provide uterine quiescence via pregnane X-type receptors [29] and allopregnanolone that relaxes myometrium through voltage-dependent K^+ channels [105].

Our previous [13] and current data consistently show rising sulfation of all pregnanolone isomers including neuroinhibiting GABA-ergic substances in late pregnancy (Fig. 5E–H, our so far unpublished data).

The sulfotransferase SULT2A1 is highly expressed in human liver [56,106–108]. In the fetal liver, SULT2A1 activity exhibits remarkable inter-individual variability, which may be the cause for an absent correlation with the GA [57]. Liver UDP glucuronosyltransferase 2B7 (UGT2B7) catalyzes the glucuronidation of bile acid substrates but also the 3α-hydroxylated androgenic steroids, and 17β-estrogens at very high rates [109].

The sulfotransferase enzyme SULT1E1 has the lowest K(m) values for estrogens and catecholestrogens of the known human SULT isoforms [110]. SULT1E1 is responsible for the sulfation and inactivation of estradiol at physiological concentrations. The enhanced SULT1E1 activity may have a role in inhibiting GH-stimulated STAT5b phosphorylation and IGF-1 synthesis via the sulfation and inactivation of estradiol [111]. SULT1E1 may also play an impor-

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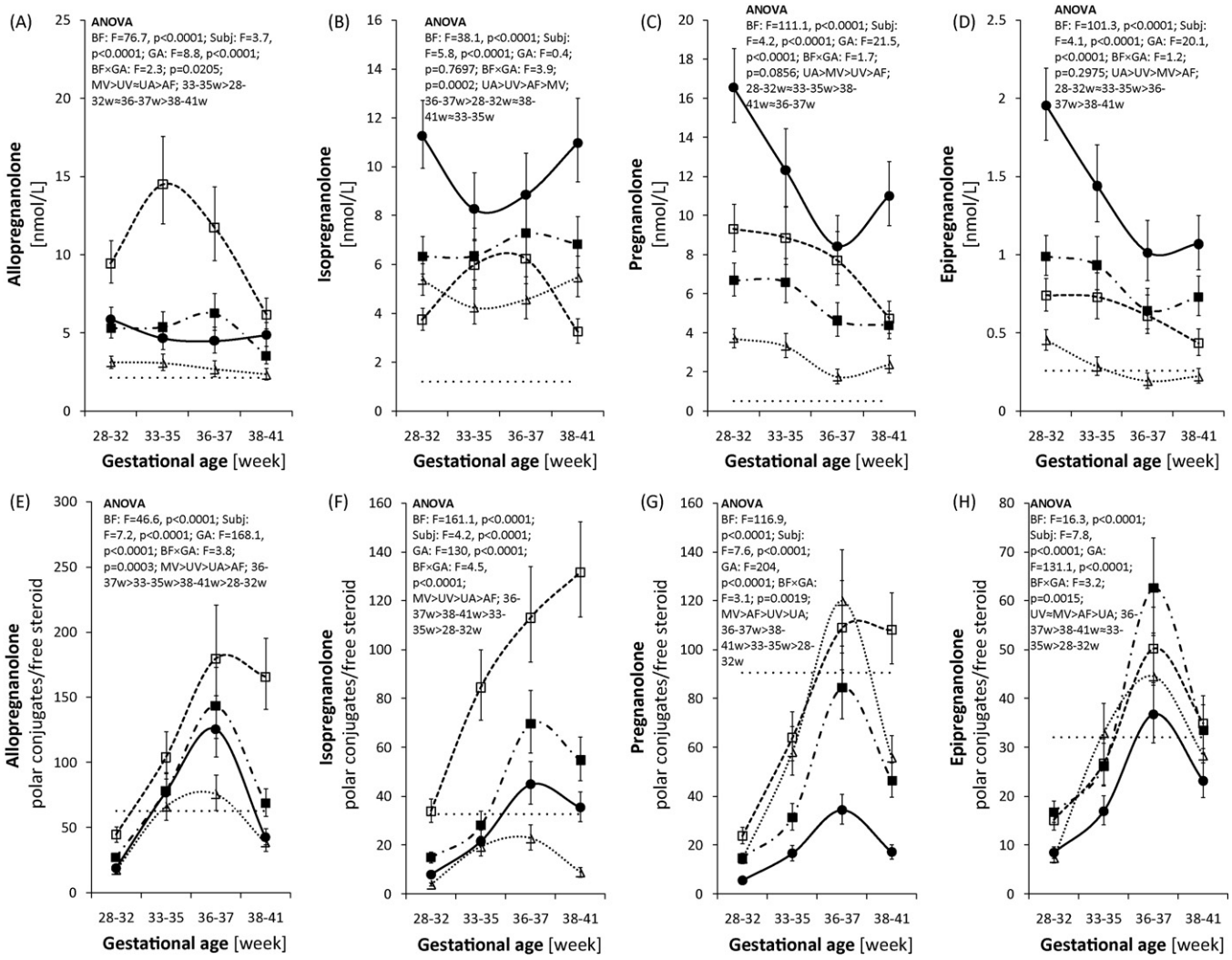

Fig. 5. Profiles of unconjugated pregnanolone isomers and ratios of conjugated pregnanolone isomers to corresponding unconjugated steroids in the plasma from the umbilical artery (UA), umbilical vein (UV) and maternal cubital vein (MV) and in amniotic fluid (AF) in preterm and normal labor. The drawings and symbols are the same as for Fig. 2. These so far unpublished data were obtained in the frame of our recently published studies [2,3].

tant role in protecting peripheral tissues from possible excessive estrogenic effects [58].

Besides placenta, also the liver shows strong expression of STS [112]. Although Warren and French [113] reported about four times less activity for DHEAS hydrolysis in human liver when compared with placenta, Selcer et al. reported comparable STS immunoreactivity in these tissues [112]. In contrast to placenta where the arylsulfatase K (ARSK) is not expressed, this enzyme might contribute to the hydrolysis of steroid sulfates in the liver [114].

3.5. Inactive steroid catabolites and prediction of term

Taking into account the simple availability of maternal blood in contrast to the fetal blood and amniotic fluid, the GA-predicting steroids in maternal plasma are of greatest interest. The inactive catabolites of sulfated Δ^5 steroids produced by the FZ or placental estrogens frequently exhibit even better predictivity for an estimation of GA that the parent steroids. These catabolites appear to be the end products of the steroid metabolism, the biosynthesis of which is readily catalyzed by the liver enzymes. For instance, the excellent predictivity was recorded for conjugated 16α-hydroxy-metabolites of Δ^5 steroids and estrogens (Fig. 6, our so far unpublished data), polar conjugates of 5α/β-reduced C-19 steroids (Fig. 7A–C, our so far unpublished data) and some 5-androstene-3β,7α/β,17β/oxo-steroids (Fig. 7D–F, our so far unpublished data) [3]. Moreover, an acceleration of 16α- and possibly also 7β-hydroxylation was reported with approaching term [61,75,76].

4. Transport of steroid sulfates into the placenta

The transport of steroid sulfates from the fetal circulation into the placental cells (where they are further metabolized) appears to be mediated by an organic anion transporter OAT-4, which is localized in the cytotrophoblast membranes and at the basal surface of the syncytiotrophoblast [115] (Fig. 8, our so far unpublished data). The data indicates the transport of steroid conjugates between the fetal and maternal compartment without preceding hydrolysis.

5. Steroid metabolism in placenta

Sex hormones produced by the placenta play a key role in the endocrine control of pregnancy and parturition. Placental CRH stimulates the production of estradiol in a time- and dose-dependent manner and also the mRNA levels of the key enzymes for estrogen synthesis such as CYP19A1, type 1 17β-HSD (HSD17B1)

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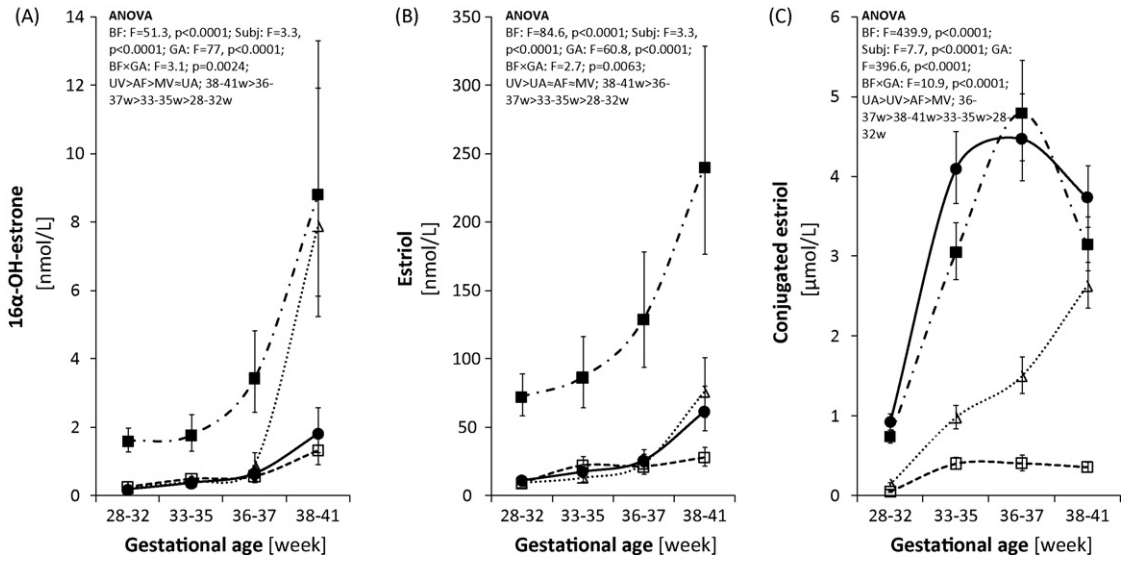

Fig. 6. Profiles of 16α-hydroxy-estrogens in the plasma from the umbilical artery (UA), umbilical vein (UV) and maternal cubital vein (MV) and in amniotic fluid (AF) in preterm and normal labor. The drawings and symbols are the same as for Fig. 2. These so far unpublished data were obtained in the frame of our recently published studies [2,3].

[116] as well as enzymes involved in progesterone synthesis like type 1 3β-HSD (HSD3B1) and CYP11A1 [117].

5.1. Cholesterol desmolase in placenta

In contrast to other tissues producing cholesterol, placenta lacks short-term modulation of steroid synthesis. In this tissue, the electron supply to CYP11A1 limits the conversion rate permitting pregnenolone synthesis to proceed at only 16% maximum velocity. Thus, the mitochondria have a near-saturating cholesterol concentration for CYP11A1, likely provided by the StAR-like protein MLN64. Cholesterol translocation to the CYP11A1 is not critical for placental progesterone synthesis and the subsequent pregnenolone conversion to progesterone [118].

5.2. Steroid sulfatases and placental production of sex hormones

The principal metabolic step preceding further placental metabolism of sulfated Δ^5 steroids originating in FZ is their desulfation being provided by the placental STS, which is localized in the endoplasmic reticulum [119]. The placental STS expression in pregnancy explicitly outweighs the production in other tissues [58]. STS allows access of DHEA to the HSD3B1 and CYP19A1 within the syncytiotrophoblast layer and conversion to estrogens [120]. Placental STS is independent of substrate concentration [121] and of GA [122–124].

5.3. 3β-Hydroxysteroid dehydrogenase activity

HSD3B1 is necessary for the placental synthesis of progesterone and C-19 3-oxo-4-ene steroids. The latter substances are further metabolized to estrogens [60,125]. HSD3B1 placental activity is predominantly located in the syncytiotrophoblast and intermediate trophoblast cells [126,127]. The specific activities of HSD3Bs for C-21 steroids in mitochondrial and microsomal preparations from human term placenta are about two times higher than for the C-19 steroids [128]. Like in the case of sulfatase activity, placental HSD3B1 activities are constant throughout the human gestation [129,122,123] and around parturition [124,130]. Progesterone and DHEAS may cause marked HSD3Bs inhibition in physiological conditions [121,131].

5.4. Estrogen formation

Placenta is the primary site of estrogen formation in pregnancy. CYP19A1 catalyzing the last steps of estrogen biosynthesis from Δ^4 C-19 steroids is abundantly expressed in syncytiotrophoblast [132]. Estrogens regulate their own synthesis by the product inhibition. The substrate inhibition is more apparent for 16-deoxy-estrogens than for their 16α-hydroxy-metabolites [133]. 16-Deoxy- and 16-hydroxy-C-19 substrates bind at separate, but interactive sites and each substrate on binding inhibits the aromatization of the other [134,135].

CYP19A1 activity strongly depends on GA. The increase in estradiol levels in maternal blood from the 2nd to the 3rd trimester is greater than that of the placental weight and there is significantly higher placental CYP19A1 activity in the 3rd trimester than in the 2nd trimester [122]. The aforementioned results as well as our recent data [3] indicate high predictivity of parturition onset for some estrogens (Fig. 9, our so far unpublished data).

5.5. 16α-Hydroxylation

Although the CYP3A7 is primarily expressed in the fetal liver, its activity was also found in the placenta. The amounts of placental and endometrial CYP3A7 mRNA and protein substantially increase from the first to the second trimester of pregnancy [73].

5.6. 5α/β-Reductases

The pioneer studies on placental 5α-reductase [129] reported *in vitro* synthesis of 5α-reduced pregnanes [3H]5α-pregnane-3,20-dione and [3H]3β-hydroxy-5α-pregnan-20-one from [3H]progesterone by placental tissue. 5α-Reduced steroids, including allopregnanolone, suppress neuronal activity and may have neuroprotective effects in the fetus. Placental expression of both isoenzymes increased with advancing gestation. Placental 5α-reductases may provide precursors for allopregnanolone synthesis in fetal brain [136].

AKR1D1 is primarily expressed in the liver but its activity was also detected in other tissues including placenta [30]. The progesterone metabolite 5β-dihydroprogesterone (5β-DHP) is a potent tocolytic. Acute *in vitro* treatment with 5β-DHP causes rapid uter-

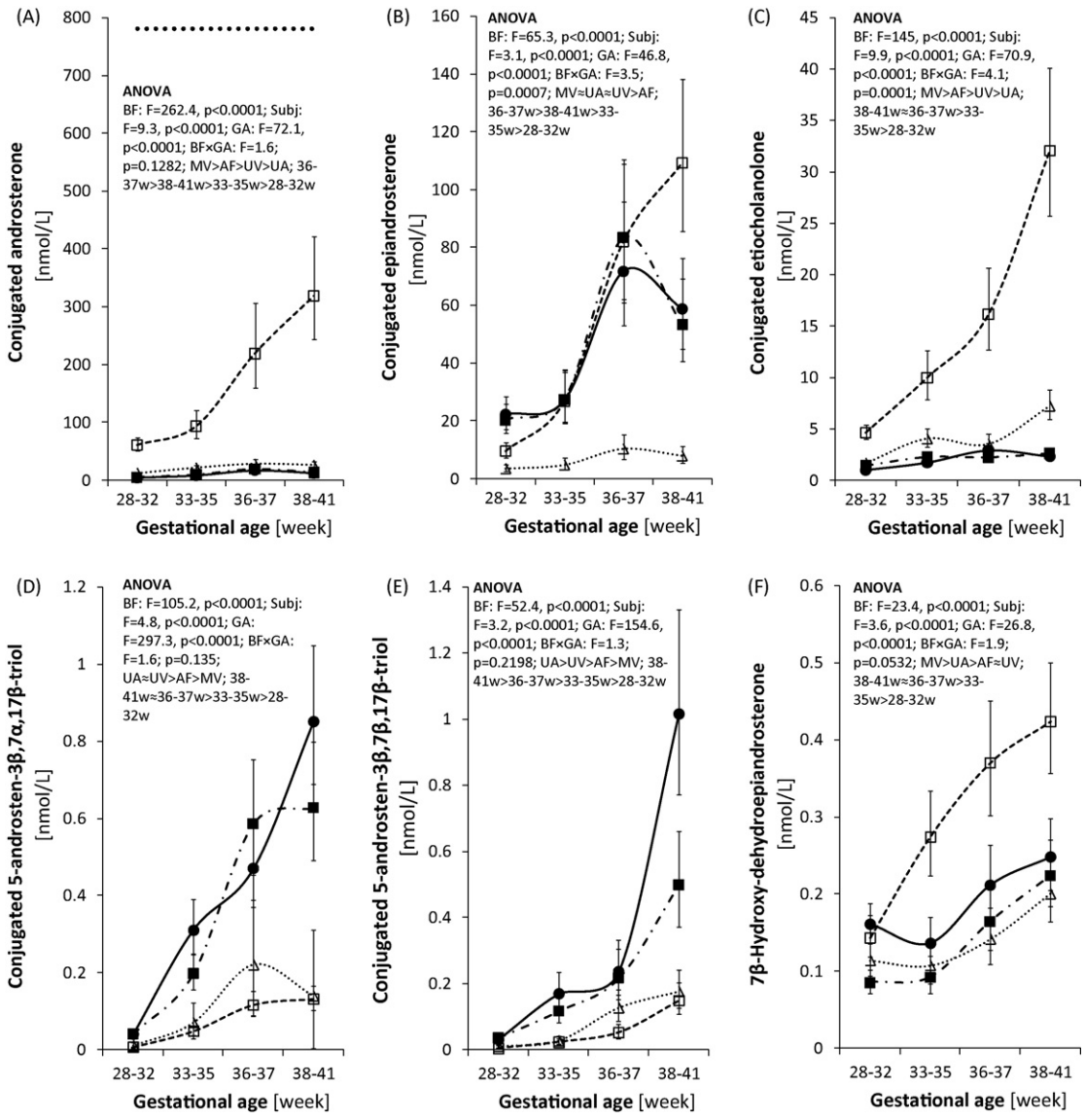

Fig. 7. Profiles of three conjugated 3α/β-hydroxy-5α/β-androstane-17-ones and some 5-androstene-3β, 7α/β,17β/17-oxo-steroids in the plasma from the umbilical artery (UA), umbilical vein (UV) and maternal cubital vein (MV) and in amniotic fluid (AF) in preterm and normal labor. The drawings and symbols are the same as for Fig. 2. These so far unpublished data were obtained in the frame of our recently published studies [2,3].

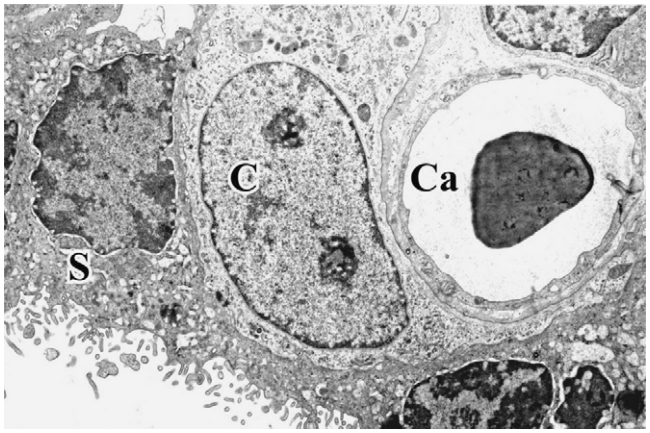

Fig. 8. Transmission electron micrograph of the human placenta; S, syncytiotrophoblast; C, cytotrophoblast; Ca, fetal villous capillary; bar, 2 μm (our so far unpublished data).

ine relaxation that is not mediated by pregnane X-type receptors (PXR) but the 5β-reduced metabolites of progesterone may also act chronically in pregnancy through a PXR-mediated mechanism [29]. In the placenta and myometrium, relative expression of AKR1D1 decreases in association with labor by about 2-fold and 10-fold, respectively [30]. In contrast to the turnover of progesterone to 5α-DHP reflecting 5α-reductase activity which remains stable (Fig. 4C, our so far unpublished data), the conversion of progesterone to 5β-DHP reflecting 5β-reductase activity decreases later in pregnancy [13,26,30] (Fig. 4D, our so far unpublished data). This data is consistent with a possible role for 5β-DHP in the onset of spontaneous human parturition. The placental expression of 5β-reductase mRNA is about two orders of magnitude higher than in myometrium and about three orders of magnitude higher than in chorion and amnion [30].

5.7. Steroid sulfotransferase

SULT2B1 catalyzing sulfation of DHEA but not estradiol is present in syncytiotrophoblast [107,108,137,138] while SULT1E1

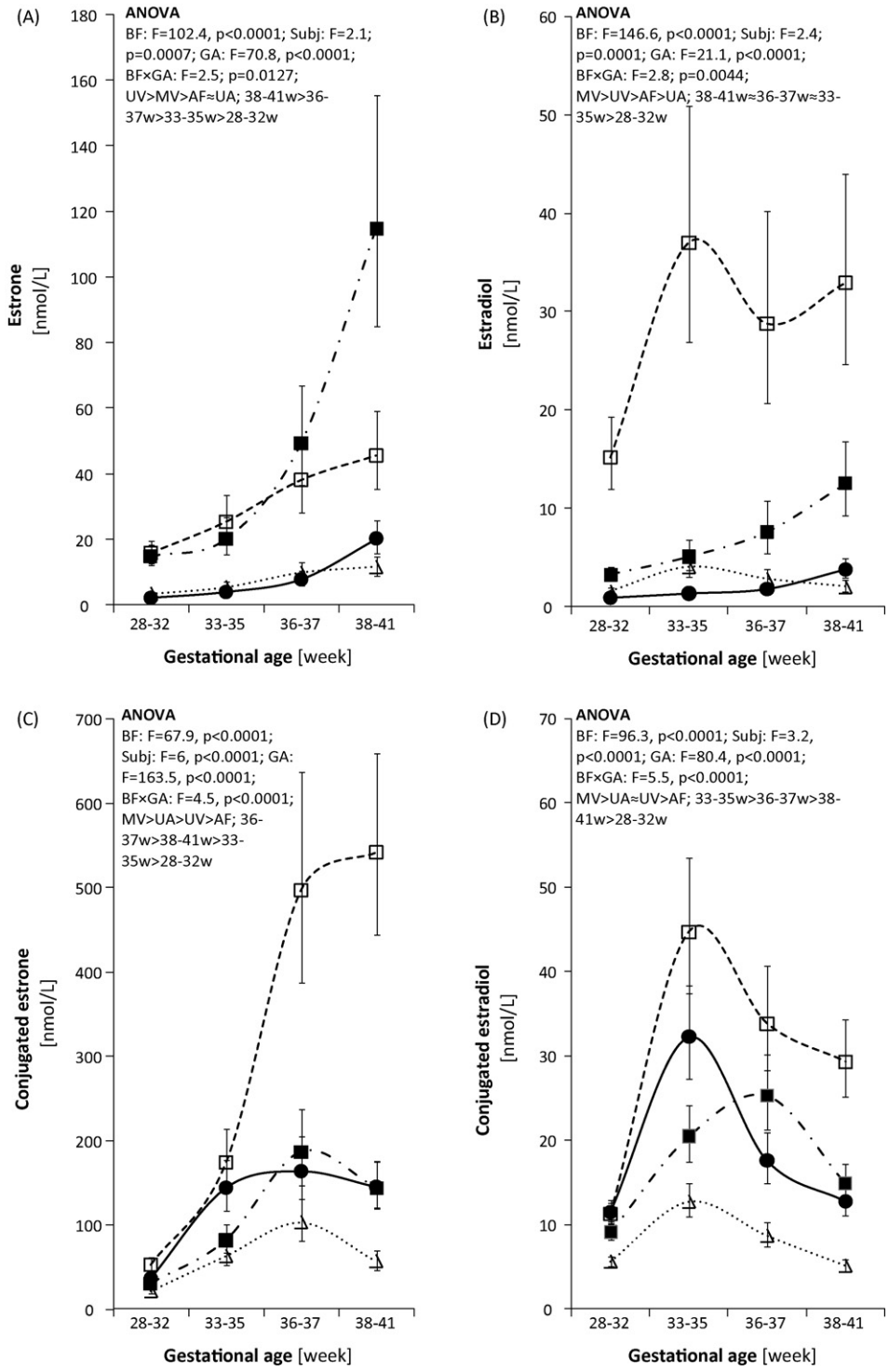

Fig. 9. Profiles of free and conjugated estrogens in the plasma from the umbilical artery (UA), umbilical vein (UV) and maternal cubital vein (MV) and in amniotic fluid (AF) in preterm and normal labor. The drawings and symbols are the same as for Fig. 2. These so far unpublished data were obtained in the frame of our two recently published studies [2,3]. These so far unpublished data were obtained in the frame of our recently published studies [2,3].

and SULT2A1 show negligible functional activity in placental tissues [139], which means that estrogens are sulfated in an extra placental way, most probably in the liver.

5.8. Reversible C-3, C-11, C-17 and C-20 oxidoreductive inter-conversions in placenta

Placenta expresses various dehydrogenases belonging to SDRs and AKRs. From the SDRs, the cytoplasmic HSD17B1 is highly expressed in syncytiotrophoblast [42]. Besides catalyzing the conversion of estrone and progesterone to estradiol and Prog20α, respectively, HSD17B1 may also catalyze the formation of 5-androstene-3β,17β-diol from DHEA [140,141]. Syncytiotrophoblast, coming directly into contact with maternal blood, converts estrone to estradiol. In contrast to type HSD17B1 mRNA, type HSD17B2 mRNA is not detectable in cell cultures of human cytotrophoblast or syncytiotrophoblast [142]. Besides HSD17B1, the AKR1 member C3 enzyme (AKR1C3), HSD17B7 and HSD17B12

may also catalyze progesterone deactivation to Prog20α and conversion of inactive estrone to bioactive estradiol [68,88,132,141].

AKR1C3 is pluripotent widely distributed enzyme catalyzing the conversion of aldehydes and ketones to alcohols [143,144]. AKR1C3 functions as a bi-directional 3α-, 17β- and 20α-HSD and can interconvert active androgens, estrogens and progestins with their cognate inactive metabolites, however, like other AKR1Cs *in vivo*, AKR1C3 preferentially works as a reductase [88,91,143].

Regarding 3α-pregnanolone isomers positively modulating $GABA_A$-r, the oxidoreductive conversion of 20-oxo- to 20α-hydroxy-group or a modification of the C17,20 side chain results in a selective (subtype dependent) reduction of positive allosteric modulation of $GABA_A$-r (about 6-fold) [145]. In addition, the reversible oxidoreductive interconversion of 3α-hydroxy/3-oxo/3β-hydroxy-5α/β-reduced pregnane and androstane steroids may influence the ratio of neuroinhibiting 3α-hydroxy-5α/β-reduced metabolites, which are allosteric positive modulators of $GABA_A$-r, to the corresponding 3-oxo-metabolites and 3β-hydroxy-metabolites. The latter ones are biologically inactive but compete with the 3α-hydroxy-isomers for the active sites on the receptors [146].

In contrast to the aforementioned enzymes, the HSD17B2 prefers the oxidative direction catalyzing the progesterone biosynthesis from inactive Prog20α as well as the conversion of bioactive estradiol to biologically inactive estrone [42]. The site of expression of HSD17B2 was identified in two studies, either in endothelial cells of fetal capillaries and some stem villous vessels [42] or in endothelial cells of villous arteries and arterioles [147]. Moghrabi et al. suggested a protective role of the HSD17B2 from the excess of bioactive estrogens and androgens in the fetus [42]. Besides HSD17B2, the type 14 17β-HSD (HSD17B14) a member of SDRs may also convert estradiol to estrone and 5-androstene-3β,17β-diol to DHEA [147].

The metabolism of placental sex steroids in the reductive direction increases as pregnancy advances and significantly rises during human parturition [129,148]. This phenomenon may be of an importance in the mechanism of initiation and continuation of labor and might indicate a mechanism of progesterone withdrawal in association with the onset of human parturition.

HSD11B1 expression is abundant in syncytiotrophoblast microvillus membranes juxta the maternal circulation whereas HSD11B2 expression is extensive throughout the remainder of the syncytiotrophoblast, including the basal cell membrane and epithelial basal lamina [149]. HSD11B1 expression is constant, but the expression of HSD11B2 in the placenta increases significantly with GA. The adaptation of HSD11B2 activity prevents the increasing maternal cortisol concentrations from transplacental passage [150].

Distribution of placental oxidoreductases and sources of progesterone, estrogens and neuroactive steroids in pregnancy.

As indicated by growing progesterone levels in UV (Fig. 10, our so far unpublished data), placental production of progesterone probably increases shortly before termination of pregnancy but its levels in UA, maternal cubital vein (MV), and amniotic fluid (AF) remain constant. This means that there should be concurrently increasing progesterone catabolism in this period.

Paradoxically, although progesterone is the most important steroid in human pregnancy there are a lot of peculiarities and contradictions regarding its biosynthesis. As already mentioned, the FZ is analogous to adult *zona reticularis*. However, while both FZ and *zona reticularis* produce large amounts of DHEAS, the extensive production of PregS is specific for FZ. This substantial dissimilarity between FZ and *zona reticularis* remains unexplained. Although, DHEAS from the FZ is generally accepted as the substrate for placental estrogen synthesis, the physiological role of PregS in human pregnancy is unknown.

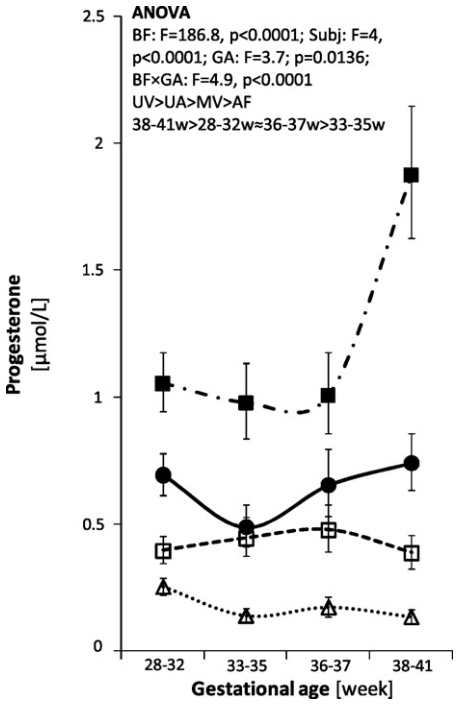

Fig. 10. Profile of progesterone in the plasma from the umbilical artery (UA), umbilical vein (UV) and maternal cubital vein (MV) and in amniotic fluid (AF) in preterm and normal labor. The drawings and symbols are the same as for Fig. 2. These so far unpublished data were obtained in the frame of our recently published studies [2,3].

On the other hand, the maternal LDL cholesterol is considered to be a single substrate for placental progesterone synthesis [7,45], although the conversion of cholesterol/sulfate to pregnenolone/sulfate is the rate limiting step but not the cholesterol transport to active sites like in extra placental tissues. It is generally accepted that the activities of STS and HSD3B1 are enormous in comparison with other human tissues and being independent of GA are capable to readily convert DHEAS to estrogens. Inconsistently, the fate of PregS was not considered although its concentrations in late pregnancy are at least the same as DHEAS levels. Whereas DHEAS easily penetrates to the active sites in placenta being desulfated and converted to androstenedione and testosterone, there is no reason for PregS to act differently. Neither STS nor HSD3B1 activities are rate limiting for placental progesterone synthesis [118]. Therefore, it may be more expediential to utilize fetal PregS instead of the necessity to synthesize total maternal progesterone *de novo* from maternal LDL cholesterol.

The progesterone is vital for pregnancy sustaining and so there may be independent sources for its production. Whereas the conversion of maternal LDL cholesterol may be the first of them, there is no reason for placenta to reject processing of pregnenolone sulfate in the same way as DHEAS, obviously, except for the final step, i.e. estrogen synthesis. The former source provides stability of progesterone production in the cases where the steroid production in the FZ fails, however, the latter may substantially contribute to the progesterone production. As our data indicates, the rise in production of Δ^5 steroids with approaching labor is linked to rise of progesterone levels in UV but not in other body fluids. This data indicates that primarily placental and perhaps also the liver oxidoreductases may readily convert progesterone to its metabolite Prog20α and *vice versa* and that the different location of the reductase- or oxidoreductase-preferring isoforms in placental tissues may be decisive for the reductive or oxidative status of steroid metabolome in mother and fetus. Not only the progesterone levels,

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Table 1
Correlations (after power transformation to Gaussian distribution and constant variance) between progesterone (Prog), 20α-dihydroprogesterone (Prog20α), and 20α-dihydroprogesterone polar conjugates (Prog20αC) in umbilical venous blood (UV) and maternal venous blood (MV); Pearson's and partial correlations (with adjustment of all variables in the correlation matrix to constant except the pair under investigation) are above and below the diagonal, respectively.

		UV			MV			
		Prog	Prog20α	Prog20αC	Prog	Prog20α	Prog20αC	PEARSON'S CORRELATIONS
UV	Prog		0.277 46 0.063	0.196 46 0.192	0.083 46 0.582	0.103 46 0.497	0.188 46 0.210	
	Prog20α	0.241 46 0.124		**0.569** **46** **0.000**	**0.326** **46** **0.027**	**0.632** **46** **0.000**	**0.527** **46** **0.000**	
	Prog20αC	0.030 46 0.852	0.254 46 0.105		0.202 46 0.178	**0.515** **46** **0.000**	**0.747** **46** **0.000**	
MV	Prog	0.090 46 0.569	-0.124 46 0.433	-0.135 46 0.395		**0.680** **46** **0.000**	**0.310** **46** **0.036**	
	Prog20α	-0.156 46 0.323	**0.432** **46** **0.004**	0.071 46 0.655	**0.647** **46** **0.000**		**0.602** **46** **0.000**	
	Prog20αC	0.072 46 0.651	-0.016 46 0.922	**0.597** **46** **0.000**	-0.032 46 0.842	0.283 46 0.070		
		PARTIAL CORRELATIONS						

but also the concentration of estrogens, NAS and other substances which influence pregnancy sustaining like 5α/β reduced pregnane and androstane metabolites in fetal and maternal circulations are in all probability controlled by the distribution of placental oxidoreductases.

The reason why the placental production was considered to be independent of the fetal PregS might be the absence of correlations between maternal and fetal progesterone although the levels of estradiol (synthesized from the fetal DHEAS) also do not correlate between mother and fetus. However, as reported in our recent study [2], there are significant partial correlations for both free and conjugated Prog20α between UV and MV and the correlation between Prog20α and progesterone in MV (Table 1). In addition, there are also significant partial correlations between estrone polar conjugates in UV and unconjugated estradiol in MV and a correlation between estrone and estradiol in MV (Table 2) (our unpublished data).

Assuming that the distribution of placental oxidoreductase isoforms controls the reductive and oxidative status of steroid inter-conversions in maternal and fetal compartment, respectively, the difference between oxidative fetal and reductive maternal steroid metabolomic status should be the most apparent when comparing blood from UV, containing placental steroids before their further metabolism in other fetal tissues (mainly liver), and MV. In accordance with the aforementioned assumption, the blood from UV contains higher proportions of 20-oxo-steroids like progesterone, 17-oxo-steroids (e.g. estrone and DHEA), 3-oxo-steroids like 5α/β-DHP and 3β-hydroxysteroids (isopregnanolone and epipregnanolone), while maternal venous blood contains higher proportions of 20α-hydroxysteroids like 20α-dihydroprogesterone, 17β-hydroxysteroids such as estradiol and

Table 2
Correlations (after power transformation to Gaussian distribution and constant variance) between estrone (E1), estrone polar conjugates (E1C), estradiol (E2), and estradiol polar conjugates (E2C), in umbilical venous blood (UV) and maternal venous blood (MV); Pearson's and partial correlations (with adjustment of all variables in the correlation matrix to constant except the pair under investigation) are above and below the diagonal, respectively.

		UV				MV				
		E1	E1C	E2	E2C	E1	E1C	E2	E2C	PEARSON'S CORRELATIONS
UV	E1		**0.540** **45** **0.000**	**0.702** **45** **0.000**	**0.337** **46** **0.022**	**0.427** **47** **0.003**	**0.615** **46** **0.000**	**0.355** **47** **0.014**	**0.366** **47** **0.011**	
	E1C	-0.015 45 0.930		**0.596** **46** **0.000**	**0.557** **48** **0.000**	**0.335** **48** **0.020**	**0.853** **48** **0.000**	**0.396** **48** **0.005**	**0.629** **48** **0.000**	
	E2	**0.623** **45** **0.000**	0.109 46 0.503		**0.691** **47** **0.000**	**0.343** **47** **0.018**	**0.610** **46** **0.000**	0.278 47 0.059	**0.421** **47** **0.003**	
	E2C	**-0.395** **46** **0.012**	0.024 48 0.881	**0.698** **47** **0.000**		0.226 49 0.119	**0.507** **48** **0.000**	**0.430** **49** **0.002**	**0.683** **49** **0.000**	
MV	E1	0.001 47 0.998	-0.189 48 0.231	0.185 47 0.248	-0.207 49 0.182		**0.458** **49** **0.001**	**0.614** **50** **0.000**	**0.406** **50** **0.004**	
	E1C	0.201 46 0.215	**0.691** **48** **0.000**	0.042 46 0.796	-0.035 48 0.824	0.279 49 0.070		**0.397** **49** **0.005**	**0.612** **49** **0.000**	
	E2	0.194 47 0.224	0.075 48 0.636	-0.195 47 0.223	0.152 49 0.332	**0.526** **50** **0.000**	-0.180 49 0.249		**0.658** **50** **0.000**	
	E2C	0.072 47 0.656	0.149 48 0.347	-0.280 47 0.076	**0.503** **49** **0.001**	0.002 50 0.988	0.191 49 0.220	**0.399** **50** **0.007**		
		PARTIAL CORRELATIONS								

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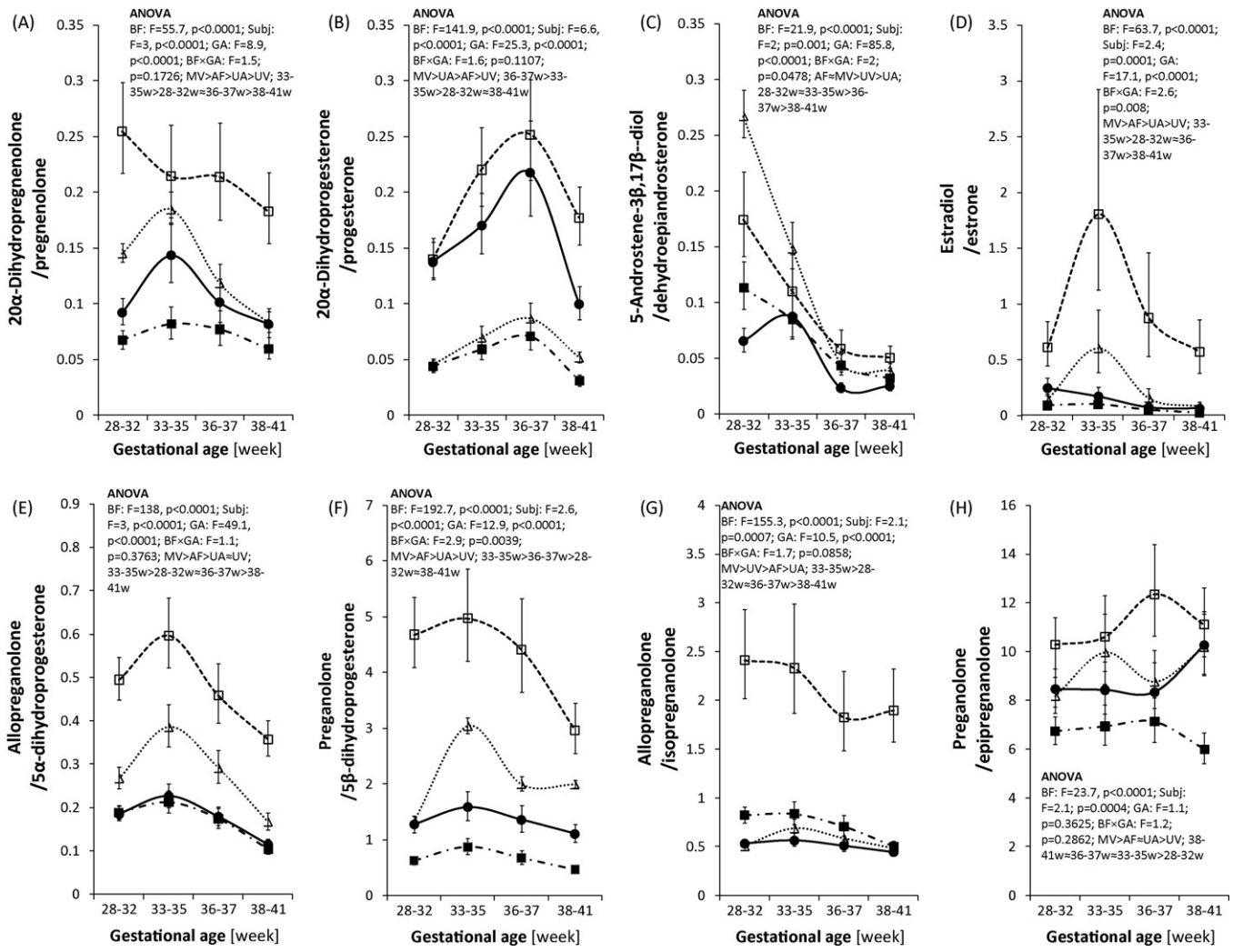

Fig. 11. Profiles of the ratios of steroids in reduced forms to the corresponding oxidized forms in the plasma from the umbilical artery (UA), umbilical vein (UV) and maternal cubital vein (MV) and in amniotic fluid (AF) in preterm and normal labor. The drawings and symbols are the same as for Fig. 2. These so far unpublished data were obtained in the frame of our recently published studies [2,3].

androstenediol and 3α-hydroxysteroids like neuroinhibiting allopregnanolone and pregnanolone (Fig. 11, our so far unpublished data). Furthermore, the levels of conjugated 3α-hydroxy-5α/β-reduced C-19 steroids in MV are pronouncedly higher (Fig. 7A–C, our so far unpublished data) than in the fetal circulation and amniotic fluid, while the 3β-isomer conjugated epiandrosterone do not significantly differ between mother and fetus (Fig. 7B, our so far unpublished data).

Besides the neuroinhibiting effects in the CNS (which are probably counterbalanced by changed phosphorylation status of the $GABA_A$-r [10]) the higher levels of the 3α-hydroxysteroids in MV might be useful for pregnancy sustaining by reducing myometrial activity via the voltage-gated K^+ channels [105].

Gilbert Evans and colleagues reported 3α-hydroxysteroid oxidoreductase-mediated turnover of 5α- and 5β-DHP to their metabolites allopregnanolone and pregnanolone. In the maternal circulation, between the 28th and 38th week of gestation, a decrease of allopregnanolone and increase of pregnanolone occurred [26]. On the contrary, our data showed a consistent decrease in both allopregnanolone/5α-DHP and pregnanolone/5β-DHP ratios between the 28th and 41st week of gestation (Fig. 11E and F, our so far unpublished data). In addition, we have recorded a slight but significant decrease even in the allopregnanolone/isopregnanolone ratio (Fig. 11G, our so far unpublished data). Considering the enzyme distribution in placenta, these results indicate increasing activity of placental and perhaps also the liver HSD17B7 in late pregnancy. The data also points to decreasing synthesis of neuroinhibiting GABA-ergic steroids with advancing gestation.

6. Conclusions

The data available in the literature including our recent findings and new unpublished data indicate increasing progesterone synthesis that is accompanied by increasing catabolism with approaching parturition. The data also confirms declining production of pregnancy sustaining 5β-pregnane steroids. These substances provide uterine quiescence in late pregnancy. There is also an increasing sulfation of neuroinhibiting and pregnancy sustaining steroids with approaching term. In contrast to the established concept considering LDL cholesterol as the primary substrate for progesterone synthesis in pregnancy, our data demonstrates the functioning of alternative mechanism for progesterone synthesis, which is based on the utilization of fetal pregnenolone sulfate for progesterone production in placenta. Close relationships were found between localization of placental oxidoreductases and consistently higher levels of sex hormones, neuroactive steroids and their metabolites in the oxidized form

in the fetus and in the reduced form in the maternal compartment.

Acknowledgement

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