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Steroid metabolome in plasma from the umbilical artery, umbilical vein, maternal cubital vein and in amniotic fluid in normal and preterm labor☆

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ABSTRACT

The boost in placental production of CRH in late pregnancy is specific for human. CRH receptors are expressed in the fetal zone of the fetal adrenal (FZFA). Hence, we evaluated the associations between the steroid metabolome and gestational age (GA). The levels of 69 steroids and steroid polar conjugates such as 3β-hydroxy-5-ene steroids (3βOH5S), 3-oxo-4-ene steroids (3O4S), progesterone 5α/β-reduced metabolites, 20α-hydroxy-metabolites of C21 steroids, C19 5α/β-reduced metabolites, 7α/β-hydroxy-metabolites of 3βOH5S, estrogens and 16α-hydroxy-metabolites of 3βOH5S and 3O4S, were measured by GC–MS in plasma from the umbilical artery (UA), umbilical vein (UV), and maternal cubital vein (MV) and in amniotic fluid (AF) in 12 women at normal labor and 38 women at preterm labor due to pathologies unrelated to steroid status. Using multivariate regression, prediction models for GA were completed for the individual body fluids. The conjugated 3βOH5S (the key products of the FZFA), estrogens, some polar conjugates of progesterone 5α/β-reduced metabolites and some steroid 7α/β- and 16α-hydroxy-metabolites showed strong positive correlations with the GA. The predictivity decreased in the following sequence UV (R = 0.950), UA (R = 0.945), MV (R = 0.895), and AF (R = 0.891). Although the predictivity of steroids in maternal blood was slightly less effective when compared with the UV and UA, it was the best solution for further practice.

1. Introduction

Although numerous authors including us [1–43] have analyzed steroids in the body fluids of pregnant women and fetuses, none of them carried out quantification of all key steroids related to pregnancy. This includes free and conjugated precursors and catabolites of active hormones and other active substances like immunomodulatory and neuroactive steroids.

The mechanism of mammalian pregnancy is multi-factorial in nature and distinctively species-specific [44]. In contrast to the common effects of sex hormones [45] and corticosteroids [46,47], there is limited information on the actions of progesterone metabolites [48–52] and immunomodulatory 7α/β-hydroxy-derivatives of 3β-hydroxy-5-ene steroids (3βOH5S) [53] during pregnancy and before labor. Catabolism of the active hormones to their hydroxy-metabolites and/or the formation of polar steroid conjugates may be crucial for the regulation of their biological activity and consequently for the uterine contractions and the onset of labor [54–56].

Several authors reported a boost in the placental production of CRH in late pregnancy (which is specific for human and great apes). The levels of CRH are exceedingly elevated in the maternal circulation and relatively less pronouncedly in the fetal blood. It might be of an importance that CRH receptors are expressed in the fetal zone of the fetal adrenal (FZFA). The data indicates that placental CRH may directly stimulate the activity of the FZFA. Despite the substantial alterations in the placental CRH production in late pregnancy, the predictivity of CRH for an estimation of term is relatively poor. To sidetrack the expensive determination

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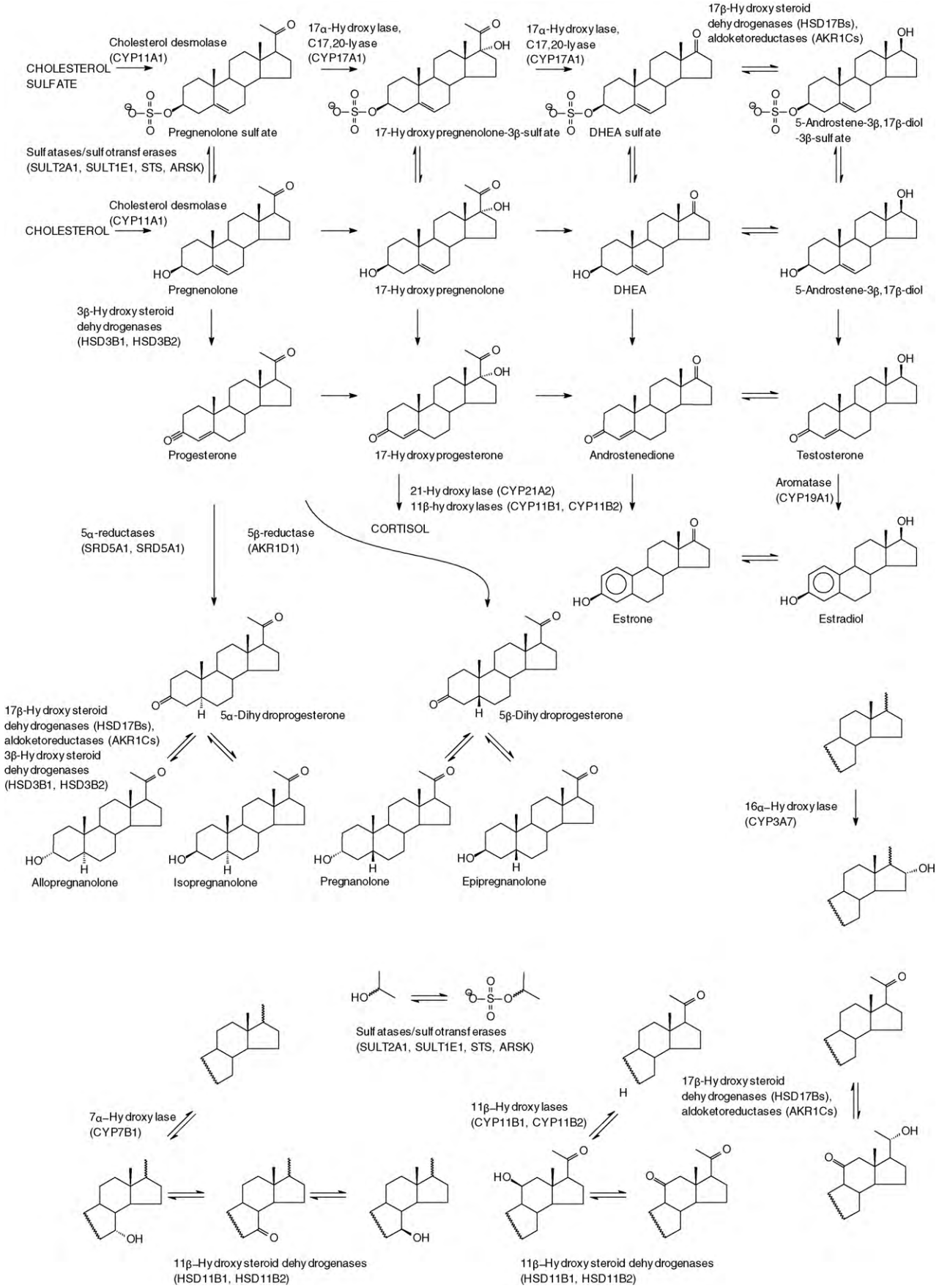

Fig. 1. Simplified scheme of steroid metabolism.

Table 1
Characteristics from analysis of steroid metabolome in the plasma from umbilical artery, umbilical vein and maternal cubital vein and in amniotic fluid at labor from 28th to 41st week of pregnancy.

Gradient	Steroid	Retention time [min]			*m/z* [Da]
		Peak 1	Peak 2	σ[a]	
G1	5-Androstene-3β,7α,17β-triol	8.41[b]	–	0.016	208, 327, 432
G1	Androstenediol	9.04	–	0.015	254, 344
G1	Androsterone	9.54	–	0.013	270, 360
G1	Etiocholanolone	9.65	–	0.012	270, 360
G1	5-Androstene-3β,7β,17β-triol	9.72	–	0.012	208, 327, 432
G1	EpiE2 (IS)	9.97	–	0.011	285, 416
G1	17-Hydroxy-pregnenolone	10.15	–	0.011	213, 231, 270, 304
G1	Epiandrosterone	10.29	–	0.011	270, 360
G1	DHEA	10.30	–	0.011	268, 358
G1	Testosterone	10.71	10.87	0.010	268, 358, 389
G1	Pregnenolone	11.23	–	0.009	288, 312, 386
G1	16α-Hydroxy-pregnenolone	11.45	–	0.009	384, 474
G1	Androstenedione	11.60	11.73	0.009	313, 344
G1	16α-Hydroxy-androstenedione	11.67	11.75	0.009	302, 311, 401, 432
G1	5β-Dihydroprogesterone	11.88	11.90	0.009	275, 288, 343
G1	5α-Dihydroprogesterone	12.15	12.17	0.008	275, 288, 343
G1	17-Hydroxy-progesterone	13.08	13.17	0.010	197, 284, 357
G2	EpiE2 (IS)	11.85	–	0.031	231, 285, 416
G2	Epipregnanolone	13.03	–	0.012	241, 298, 388
G2	Allopregnanolone	13.18	–	0.010	241, 298, 388
G2	Pregnanolone	13.28	–	0.009	241, 298, 388
G2	Isopregnanolone	13.68	–	0.008	241, 298, 388
G2	Progesterone	14.51	14.58	0.007	273, 286, 372, 341
G3	5β-Pregnane-3β,20α-diol	9.88	–	0.012	269, 284, 347, 449
G3	EpiE2 (IS)	9.97	–	0.011	285, 416
G3	5β-Pregnane-3α,20α-diol	10.08	–	0.010	269, 284, 347, 449
G3	5α-Pregnane-3α,20α-diol	10.55	–	0.011	269, 284, 347, 449
G3	5α-Pregnane-3β,20α-diol	10.63	–	0.011	269, 284, 347, 449
G3	20α-Dihydropregnenolone	10.66	–	0.011	267, 282, 372
G3	20α-Hydroxy-5β-pregnane-3-one	11.34	11.36	0.008	288, 303
G3	20α-Hydroxy-5α-pregnane-3-one	11.64	11.66	0.008	288, 303
G3	20α-Dihydroprogesterone	11.85	–	0.008	153, 296, 301
G4	EpiE2 (IS)	9.97	–	0.011	231, 270, 285, 416
G4	Estradiol	10.27	–	0.010	231, 270, 285, 416
G4	Estrone	11.17	–	0.010	231, 340, 371
G4	Estriol	11.22	–	0.009	231, 311, 345, 414, 504
G4	16α-Hydroxy-estrone	11.38	11.44	0.008	231, 285, 311, 345, 428
G5	5β-Androstane-3β,17β-diol	13.81	–	0.016	241, 331
G5	5α-Androstane-3α,17β-diol	14.03	–	0.013	331, 346
G5	5β-Androstane-3α,17β-diol	14.14	–	0.012	346, 421
G5	5α-Androstane-3β,17β-diol	14.72	–	0.009	241, 421
G6	7α-Hydroxy-DHEA	7.96	–	0.012	266, 356, 387
G6	EpiE2 (IS)	7.99	–	0.012	285, 416
G6	16α-Hydroxy-DHEA	8.59	8.71	0.012	266, 356, 446
G6	7β-Hydroxy-DHEA	8.82	–	0.012	266, 356, 387
G6	7α-Hydroxy-pregnenolone	9.10	–	0.011	384, 415
G6	16α-Hydroxy-testosterone	9.74	9.83	0.009	266, 356, 387, 446
G6	7β-Hydroxy-pregnenolone	9.76	–	0.008	384, 415
G6	16α-Hydroxy-progesterone	10.47	10.53	0.006	156, 188, 429

[a] Standard deviation of retention time for quantitation peak.
[b] Peaks used for quantification are underlined.

of unstable CRH with insufficient predictability, it may be expedient to exploit the potential association between the CRH boost and rising activity of FZFA (which may manifest by rising levels of some steroids and steroid conjugates being associated with the FZFA function). Therefore, the quantification of the steroid metabolome (Fig. 1) in one sample may be much more informative concerning the estimation of term than the measurement of CRH. Moreover, the cumulative effect of mutually strongly intercorrelated steroids on the predictivity may be expected when using an appropriate multivariate approach. Taking into account the aforementioned information, it is likely that the steroid levels may be suitable for estimation of fetal maturation and prediction of the onset of labor. Additionally, there are various disturbances associated with human pregnancy like postpartum blues [50,51], some hereditary diseases like steroid sulfatase deficiency [57], Down's syndrome [58], intrahepatic cholestasis [59] or various forms of congenital adrenal hyperplasia [60,61] for the detection of which a suitable multi-component method evaluating the steroid metabolomic profile may be helpful, however, the diagnostics of these pathologies is not addressed in the present study.

Therefore, the first goal of this study was to develop a multi-component method for quantification of steroid metabolome from the umbilical artery (UA), umbilical vein (UV), maternal cubital vein (MV) and in amniotic fluid (AF). Although there are several methods including LC–MS/MS available for rapid quantification of

steroids in human body fluids, human steroids include a number of isomers having almost identical fragmentation pattern. Biological activities commonly differ between the isomers. Therefore, these substances should be perfectly separated before the detection. The separation by GC is commonly more efficient than in the case of LC and thus, it is advantageous to use the GC–MS in this study.

The second aim of the study was to suggest a model for the estimation of gestational age (GA) and prediction of term, which is based on the assessment of steroid metabolome, and to find which steroids are appropriate for a construction of this model. The parameters of the model should indicate whether there is a positive correlation between the activity of FZFA and GA in late pregnancy as expected.

Respecting the trouble-free availability of maternal plasma in contrast to fetal plasma and amniotic fluid, the third goal was to evaluate whether the steroids in MV may possess comparable predictivity for an estimation of the term like those in UV, UA and AF.

2. Materials and methods

2.1. Subjects

The study group consisted of 50 women (21–41 years) at labor from the 28th to 41st week of gestation. Twelve (24%) women giving birth after the 38th week of gestation were without perinatological complications. From the 38 (76%) labors coming on within the 28th and 37th weeks of gestation, 29 (76.3%) pregnancies were terminated by caesarean section due to health risks to mother or fetus and 9 (23.7%) were vaginal deliveries with spontaneous uterine activity. In these women the reason for premature uterine activity was the infection in mother as documented by the high CRP levels, leucocytosis, and fewer. In contrast to the group of healthy women after the 38th week of gestation, all preterm births with spontaneous uterine activity were induced by sudden unexpected complications in which the association with gradual changes in steroid metabolome was less probable. The dependence of preterm labors conducted by caesarean section on steroids status

Table 2
Analytical criteria for multi-component analysis of steroid metabolome in the sera from umbilical artery, umbilical vein and maternal cubital vein and in amniotic fluid at labors from 28th to 41st week of pregnancy.

Gradient	Steroid	Limit of detection[a]		R^2[b] (linearity of a test procedure)	Intra-assay CV [%] ($n=6$)			Inter-assay CV [%] ($n=6$)
		[pmol/L]	[fg]		1000 pg	100 pg	10 pg	
G1	Pregnenolone	2.13	33.6	0.9995	6.0	3.3	5.2	7.8
G1	17-Hydroxy-pregnenolone	28.2	467	0.9989	5.5	4.1	10.5	28.2
G1	DHEA	2.2	31.7	0.9986	2.2	2.3	5.5	3.5
G5	Androstenediol	0.41	6	0.9999	0.7	0.5	10.1	4.4
G2	Progesterone	7.7	121	>0.9999	2.5	4.5	5.5	7.7
G1	17-Hydroxy-progesterone	10.6	175	0.9985	6.4	3.3	6.0	12
G1	Androstenedione	11.3	162	0.9996	4.6	5.8	25.3	8.2
G1	Testosterone	4.77	68.7	0.9992	6.1	3.6	14.1	9.2
G4	Estrone	5.86	79.2	0.9996	2.8	3.1	26.1	8.5
G4	Estradiol	0.97	13.2	0.9991	2.0	1.3	24.6	3.5
G4	Estriol	2.52	36.2	0.9996	1.3	3.0	26.5	7.7
G4	16α-Hydroxy-estrone	0.59	8.5	0.9999	2.5	3.7	25.1	3.4
G1	5α-Dihydroprogesterone	4.39	69.3	0.9999	5.0	8.5	28.2	7.4
G2	Allopregnanolone	1.53	24.4	0.9999	3.0	2.2	2.4	3.1
G2	Isopregnanolone	1.17	18.6	>0.9999	2.3	1.7	2.3	3.7
G1	5β-Dihydroprogesterone	6.31	99.7	>0.9999	5.0	7.4	13.8	13
G2	Pregnanolone	1.52	24.2	0.9998	3.7	1.9	1.6	5.7
G2	Epipregnanolone	2.35	37.3	0.9998	6.4	3.3	6.0	2.2
G3	20α-Dihydropregnenolone	2.23	35.5	0.9999	1.7	1.1	9.4	5.8
G3	20α-Dihydroprogesterone	17.1	271	0.9975	2.6	3.3	–	6
G3	20α-Hydroxy-5α-pregnane-3-one	4.5	71.6	0.9994	3.1	4.4	–	6.8
G3	5α-Pregnane-3α,20α-diol	2.55	40.8	>0.9999	1.8	0.8	3.2	3.5
G3	5α-Pregnane-3β,20α-diol	3.84	61.5	0.9998	1.9	1.4	3.6	4.4
G3	20α-Hydroxy-5β-pregnane-3-one	1.57	25	0.9997	2.2	2.8	38.5	4.1
G3	5β-Pregnane-3α,20α-diol	3.74	59.8	>0.9999	1.5	0.6	1.7	2.2
G3	5β-Pregnane-3β,20α-diol	1.56	25	0.9977	1.5	0.7	2.9	3.6
G1	Androsterone	0.39	5.7	0.9997	4.7	4.1	4.6	8.3
G1	Etiocholanolone	0.3	4.3	>0.9999	4.4	4.2	3.4	6.4
G1	Epiandrosterone	2.24	32.5	0.9999	4.2	2.8	3.9	5.4
G5	5α-Androstane-3α,17β-diol	3.72	54.3	0.9999	1.5	2.1	9.2	7.2
G5	5α-Androstane-3β,17β-diol	2.46	35.9	0.9998	0.7	1.5	8.9	6.1
G5	5β-Androstane-3α,17β-diol	31.1	454	0.9999	1.6	1.4	10.5	11.6
G5	5β-Androstane-3β,17β-diol	9.65	141	0.9994	2.8	3.6	12.1	13.1
G6	7α-Hydroxy-pregnenolone	0.4	6.7	0.9996	12.7	0.9	28.8	10
G6	7β-Hydroxy-pregnenolone	0.45	7.4	0.9999	13.0	2.1	29.9	11.7
G6	7α-Hydroxy-DHEA	0.58	8.8	0.9999	10.4	1.3	29.2	3.5
G6	7β-Hydroxy-DHEA	0.43	6.6	0.9996	10.3	1.0	29.0	11.8
G1	5-Androstene-3β,7α,17β-triol	0.06	0.9	0.9999	6.4	3.3	6.0	2.8
G1	5-Androstene-3β,7β,17β-triol	0.04	0.6	0.9988	4.3	5.1	4.0	2.2
G1	16α-Hydroxy-pregnenolone	0.15	2.6	0.9999	9.2	2.6	6.6	12.1
G6	16α-Hydroxy-DHEA	0.39	5.9	0.9997	9.5	1.2	30.6	9.2
G6	16α-Hydroxy-progesterone	2.05	33.8	0.9999	9.4	1.0	28.5	18.5
G1	16α-Hydroxy-androstenedione	2.94	44.5	0.9999	8.7	2.1	27.0	5
G6	16α-Hydroxy-testosterone	8.72	132	0.9983	9.3	1.9	30.5	14.1

[a] Limits of detection for steroid polar conjugates were 10 times higher due to different sample dilution.
[b] Correlations coefficients of linear regression (R) were computed for concentration range covering the range for all body fluids tested.

Table 3
Levels of 69 steroids in the serum from human maternal cubital vein at premature and normal labor from the 28th to 41st week of gestation.

Steroid	Our results [nmol/L]				Results of other authors [nmol/L]
	Umbilical artery	Umbilical vein	Maternal cubital vein	Amniotic fluid	
Preg	25.8 (23.5, 31.2)	37 (35.7, 44.9)	6.59 (6.09, 12.64)	7.15 (6.71, 10.4)	(RIA, VD: UA 81.6, UV 108 [1]); (RIA, VD: UA 4.68, UV 4.24 [2]); (RIA, w40: MV 23.7 [3]); (RIA, VD: UA 57.0, UV 69.0, 35.8 [4]); (RIA, GC–MS (sulfates), SC w37-41: UA 98.1, UV 93.0 MV 47.2 [5]); (GC–MS, VD: MV 946 [6]); (HPLC-RIA, w36–38: MV 57.3 [7]); (RIA, VD: MV 11.1 [8]); (GC–MS, w36–38 LP: MV 10 [9])
PregC	3214 (3081, 3999)	2691 (2500, 3517)	514 (506, 645)	258 (244, 396)	(RIA, VD: UA 6079, UV 4987 [1]); (RIA, w40: MV 1329 [3]); (RIA, VD: UA 5949, UV 4494, MV 1582 [4]); (RIA, GC–MS (sulfates), SC w37–41: UA 1994, UV 1845, MV 886 [5]); (HPLC/GC–MS, VD w37–42: UA 2120, UV 1789, MV 382 [10]); (RIA, VD: MV 1610 [8])
Preg17	26.9 (23.6, 57.1)	8.9 (7.2, 15.3)	16.4 (13.2, 22.6)	4.12 (3.72, 4.73)	(RIA, VD: MV 9.64, AF 2.41 [11]); (RIA, w40: MV 6.33 [3]); (RIA, VD: UA 104, UV 31.0, MV 56.0 [4]); (RIA, VD: U 27.1–80.5 [12]); (RIA, VD: MV 9.61 [8])
Preg17C	941 (878, 1462)	699 (659, 1214)	83.2 (64.8, 129)	25.5 (24.9, 72.1)	(RIA, VD: MV 12.7, AF 19.3 [11]); (RIA, VD: U 1560-5030 [12]); (HPLC/GC-MS, VD w37–42: UA 820, UV 860, MV 89 [10])
DHEA	8.1 (7.5, 11.7)	2.19 (2.1, 2.72)	14.1 (13.9, 16.1)	1.58 (1.44, 2.07)	(RIA, VD: UA 46, UV 13.2 [1]); (RIA, VD: UA 5.24, UV 4.27 [2]); (RIA, w40: MV 12.2 [13]); (RIA, VD: UA 16.0, UV 7.05, MV 25.7 [4]); (RIA, GC–MS (sulfates), SC w37-41: UA 25.0, UV 17.3, MV 15.1 [5]); (RIA, w30–34: MV 6.9 [14]); (RIA, VD: MV 20.6 [8]); (RIA, VD: MV 15.7, U 18.4 [15]); (LC/MS/MS, w32 LP: MV 7.32 [16])
DHEAC	1698 (1291, 2516)	1625 (1525, 2289)	1288 (1178, 1883)	97.4 (73.8, 220)	(RIA, VD: U 2465 [17]); (RIA, VD: UA 8674, UV 7153 [1]); (RIA, w40: MV 3300 [13]); (RIA, VD: UA 8090, UV 5694, MV 3507 [4]); (RIA, GC–MS (sulfates), SC w37–41: UA 3142, UV 3010, MV 3297 [5]); (RIA, w30–34: MV 1700 [14]); (GC–MS, VD: MV 5285 [18]); (RIA, VD: UA 1.36, UV 1.16, MV 1.92 [19]); (RIA, w40: MV 2300–5800 [20]); (RIA, w41: U 2600 [21]); (HPLC/GC–MS, VD w37–42: UA 2641, UV 2756, MV 620 [10]); (RIA, VD: MV 4360 [8]); (RIA, VD: U 6493, MV 4167 [15]); (LC/MS/MS, w32 LP: MV 850 [16])
Adiol	0.226 (0.175, 0.297)	0.081 (0.068, 0.121)	0.642 (0.592, 0.859)	0.058 (0.052, 0.113)	(RIA, w40: MV 2.2 [13]); (RIA, w30–34: MV 1.4 [14]); (RIA, VD: UA 22.7, UV 21.8, MV 10.4 [22]); (EIA, 3th trimester: MV 6.87 ± 1.95 [23])
AdiolC	3406 (3058, 4061)	3680 (3432, 4303)	388 (323, 597)	244.5 (235.8, 395.5)	(EIA, 3th trimester: MV 254 ± 19 [23])
Prog	745 (599, 1249)	1440 (1411, 2177)	386 (369, 497)	150 (145, 169)	(RIA, w40: MV 478 [24]); (RIA, w40: MV 94.1 [25]); (GLC, 3th trimester: MV 239–427 [26]); (GC–MS, w40–42: MV 535 [27]); (GLC, RIA, VD: RIA: UA 162 ± 62, UV 388 ± 121 MV 56 ± 26 GLC: UA 324 ± 94, UV 704 ± 227 MV 129 ± 49 [28]); (RIA, w34–40: MV 106–522 [29]); (RIA, VD: UA 828, UV 1082 [2]); (RIA, w40: MV 475 [3]); (RIA, VD: UA 1592, UV 3248, MV 541 [4]); (RIA, GC–MS (sulfates), SC w37–41: UA 382, UV 640, MV 287 [5]); (RIA, VD: UA 722 ± 63, UV 585 ± 131, MV 140 ± 28 [30]); (GC–MS, VD: MV 439, 226 pre-pain, delivery [6]); (RIA, w40: MV 49–584 [20]); (RIA, w41: U 822, MV 783 [21]); (LC–RIA, VD: MV 478 [31]); (HPLC-RIA, w36–38: MV 659 [7]); (LC/MS/MS, w32 LP: MV 224 [16]); (GC–MS, w36–38 LP: MV 520 [9])
Prog17	30 (25.9, 38)	60.1 (55, 71.4)	19.1 (18, 66.1)	6.78 (6.65, 10.8)	(RIA, w34–40: MV 60.6–75.2 [29]); (RIA, w40: MV 25.8 [3]); (RIA, VD: UA 43.6, UV 52.7, MV 15.4 [4]); (RIA, VD: U 53.3-304 [12]); (RIA, w40: MV 12.2–36 [20]); (LC/MS/MS, w32 LP: MV 10.53 [16])

A2	3.42 (3.19, 4.21)	2.22 (2.01, 2.61)	9.9 (9.8, 11.9)	3.26 (2.96, 4.41)	(RIA, w40: MV 9.8 [3]); (RIA, VD: UA 3.53, UV 2.52, MV 11.7 [4]); (RIA, w40: MV 8.1-10.6 [20]); (RIA, VD: U 12.0, MV 12.4 [15]); (LC/MS/MS, w32 LP: MV 2.65 [16])
T	1.25 (1.13, 1.76)	1.04 (0.97, 1.99)	3.6 (3.44, 4.37)	1.06 (1.00, 1.70)	(RIA, w40: MV 3.5 [13]); (RIA, VD: U m: 1.24, f: 0.896, MV m:3.99, f: 3.95 [32]); (RIA, VD: UA 1.15, UV 1.22, MV 3.23 [4]); (RIA, w40: MV 3.3–5.7 [20]); (RIA, VD: U 0.729, MV 5.20 [15])
E1	17.4 (15.3, 40.7)	113 (112, 166)	50.3 (45.6, 65.4)	12.3 (11.5, 16.5)	(RIA, w40: MV 9.26–74.1 [33]); (GC–MS, LP: MV 37.0 [34]); (RIA, w38–40 LP: MV 8.44–39.1 [35]); (RIA, w34–40: MV 39.6–78.9 [29]); (RIA, VD: U 114, MV 28.1 [15])
E1C	157 (135, 232)	146 (133, 232)	623 (579, 728)	54.0 (50.1, 83.8)	(RIA, w40: MV 11.9–119 [33]); (GC–MS, LP: MV 157 [34])
E2	3.50 (3.27, 7.36)	13.7 (13, 16.6)	36.2 (33.6, 45.8)	2.10 (1.90, 3.22)	(RIA, VD: MV 15 [36]); (RIA, w40: MV 23.2–220 [33]); (GC–MS, LP: MV 34.56 [34]); (RIA, w38–40 LP: MV 67.7–91.9 [35]); (RIA, w34–40: MV 50.4–84.9 [29]); (RIA, w40: MV 95.6 [13]); (RIA, VD: U m: 0.335, f: 0.243, MV m:0.75, f:0.496 [32]); (RIA, VD: UA 9.82, UV 18.4, MV 50.7 [4]); (RIA, GC–MS (sulfates), SC w37–41: UA 4.49, UV 16.9, MV 59.2 [5]); (RIA, VD: UA 13.4 ± 3.9, UV 46.4 ± 11.4, MV 19.5 ± 5.2 [30]); (RIA, w40: MV 1.64–11.13 [20]); (RIA, VD: U 34.9, MV 83.8 [15]); (LC/MS/MS, w32 LP: MV 0.0227 [16])
E2C	16.0 (15.4, 16.9)	17.6 (17.1, 18.4)	32.5 (31.3, 38.2)	5.63 (5.13, 6.74)	(RIA, w40: MV 4.04–63.2 [33]); (GC–MS, LP: MV 18.4 [34])
E3	53 (52.1, 94.0)	256 (252, 389)	31.2 (28.5, 36.6)	90.5 (66.5, 204)	(RIA, VD: MV 11.1 [36]); (RIA, VD: MV 4895 [17]); (RIA, w40: MV 14.9–85.8 [33]); (GC–MS, LP: MV 27.8 [34]); (RIA, w38–40 LP: MV 29.7–34.0 [35]); (RIA, w34–40: MV 1.74–50.3 [29]); (RIA, VD: UA 8.30, UV 8.16 [2]); (RIA, w40: MV 43.4 [13]); (GC–MS, VD: MV 377 [18]); (RIA, VD: UA 1132, UV 1639, MV 255 [19]); (RIA, VD: UA 163 ± 39, UV 56.8 ± 15.5, MV 18.5 ± 4.3 [30]); (RIA, VD: U 774.3, MV 62.5 [15])
E3C	3382 (3237, 4630)	2950 (2899, 3805)	349 (338, 499)	2959 (2597, 3479)	(RIA, w40: AF 3472 [37]); (RIA, w40: MV 41.0–694 [33]); (GC–MS, LP: MV 365 [34]); (RIA, GC–MS (sulfates), SC w37–41: UA 4167, UV 4132, MV 260.4 [5]); (GC–MS, VD: MV 551 [38])
E116α	1.53 (1.52, 2.45)	9.21 (8.76, 11.3)	1.38 (1.29, 1.41)	9.37 (8.46, 11.4)	(GC–MS, LP: MV 8.74 [34])
P5α	38.3 (34.6, 53.4)	36.9 (32.9, 54.0)	17.6 (16.0, 25.8)	12.9 (12.4, 13.6)	(GC–MS, w40–42: MV 180 [27]); (HPLC-RIA, w36–38: MV 31 [7]); (GC–MS, w36–38 LP: MV 222 [9]); (GC–MS, w40 LP: MV 75.6 [39])
P3α5α	4.60 (4.40, 5.60)	3.63 (3.51, 4.47)	6.40 (6.10, 8.20)	2.22 (2.15, 4.91)	(GC–MS, LP: MV 13.5 [40]); (GC–MS, w38–41 LP: MV 28.8, U 27.0 [41]); (LC-RIA, VD: MV 157 [31]); (GC–MS, w36–38 LP: MV 40.91 [9]); (GC–MS, w40 LP: MV 44 [39])
P3α5αC	210 (200, 257)	239 (238, 311)	1087 (1037, 1373)	73.0 (69.0, 114)	(GC–MS, LP: MV 2500 [40]); (GC–MS, w38–41 LP: MV 358, 141 [41]); (GC–MS, w40 LP: MV 766 [39])
P3β5α	9.50 (9.10, 12.9)	5.96 (5.63, 8.77)	2.87 (2.81, 3.93)	5.99 (5.42, 7.01)	(GC–MS, LP: MV 5.01 [40]); (GC–MS, w38–41 LP: U 19.9, MV 8.31 [41]); (GC–MS, w36–38 LP: MV 20.3 [9]); (GC–MS, w40 LP: MV 14 [39])
P3β5αC	356 (356, 433)	318 (301, 480)	436 (410, 569)	48.9 (45.7, 75.2)	(GC–MS, w38–41 LP: U 146, MV 169 [41]); (GC–MS, w40 LP: MV 330 [39])
P5β	9.80 (8.90, 16.2)	9.80 (7.80, 21.2)	1.31 (1.27, 1.78)	1.54 (1.33, 2.38)	(HPLC-RIA, w36–38: MV 2.3 [7]); (HPLC-RIA, VD: MV 178 [42]); (GC–MS, w36–38 LP: MV 3.54 [9]); (GC–MS, w40 LP: MV 4.45 [39])
P3α5β	12.8 (12.1, 15.1)	4.42 (3.92, 6.33)	4.40 (4.20, 6.20)	2.87 (2.45, 3.94)	(GC–MS, w38–41 LP: MV 18.9, U 32.8 [41]); (GC–MS, w36–38 LP: MV 18.51 [9]); (GC–MS, w40 LP: MV 19.7 [39])

Table 3 (*Continued*).

Steroid	Our results [nmol/L]				Results of other authors [nmol/L]
	Umbilical artery	Umbilical vein	Maternal cubital vein	Amniotic fluid	
P3α5βC	184 (177, 253)	193 (175, 229)	475 (444, 573)	108 (101, 208)	(GC–MS, LP: MV 650 [40]); (GC–MS, w40 LP: MV 435 [39])
P3β5β	1.04 (0.89, 1.38)	0.66 (0.60, 1.03)	0.36 (0.33, 0.66)	0.28 (0.17, 0.42)	(GC–MS, w38–41 LP: U 5.92, MV 2.32 [41]); (HPLC-RIA, w36–38: MV 2.21 [7]); (GC–MS, w40 LP: MV 2.04 [39])
P3β5βC	51.5 (47.5, 81.1)	64.6 (59.7, 81.2)	42.9 (40.3, 51.8)	18.4 (16.8, 25.4)	(GC–MS, w38–41 LP: U 14.7, MV 15.8 [41]); (GC–MS, w40 LP: MV 37.6 [39])
Preg20α	2.47 (2.41, 2.76)	1.68 (1.67, 3.09)	1.82 (1.74, 2.03)	0.48 (0.46, 1.80)	
Preg20αC	1924 (1843, 2463)	2028 (1770, 2798)	996 (848, 1208)	262 (207, 344)	(GC–MS, VD: MV 1770 [6])
Prog20α	76 (73, 98)	50.2 (45.1, 73.3)	64.4 (62.1, 93.7)	7.0 (6.8, 9.1)	(GLC, RIA, VD: RIA: UA 28 ± 10, UV 33 ± 15 MV 16 ± 8 GLC: UA 17 ± 5, UV 17 ± 3 MV 15 ± 15 [28]); (RIA, w40: MV 79.1 [3]); (GC–MS, VD: MV 330, 186 pre-pain, delivery [6])
Prog20αC	77.9 (77.8, 104.1)	94.9 (92.8, 117)	35.2 (32.9, 52.9)	23.1 (16.9, 35.2)	
P5α20α	56.6 (49.1, 80.2)	40.3 (39.0, 54.2)	28.9 (26.5, 36.6)	11.1 (10.0, 21.1)	
P5α20αC	109 (100, 176)	90 (88, 148)	52.3 (44, 69)	69.6 (57.1, 129)	
P3α5α20α	0.382 (0.361, 0.516)	0.233 (0.215, 0.346)	0.423 (0.392, 0.496)	0.276 (0.234, 0.509)	
P3α5α20αC	126 (112, 137)	122 (83, 352)	17.5 (14.2, 27.4)	13.6 (11.5, 36.1)	(GC–MS, LP: MV 8900 [40])
P3β5α20α	2.14 (1.84, 2.65)	1.59 (1.48, 2.35)	1.65 (1.49, 1.95)	0.69 (0.59, 2.14)	
P3β5α20αC	1500 (1387, 1947)	1615 (1576, 1750)	4008 (3805, 5412)	530 (365, 956)	(GC–MS, LP: MV 6100 [40])
P5β20α	13.9 (12.9, 26.7)	13.0 (10.6, 22.3)	1.65 (1.59, 2.74)	1.06 (0.99, 1.22)	
P5β20αC	54.6 (51.1, 92.1)	59.2 (54.5, 91.7)	11.6 (11.4, 13.9)	13.3 (11.8, 25.2)	
P3α5β20α	12.1 (11.6, 15.4)	2.85 (2.57, 3.80)	5.28 (4.34, 8.04)	3.12 (1.98, 7.81)	
P3α5β20αC	1657 (1462, 2386)	1362 (1250, 1719)	1806 (1760, 2967)	1090 (916, 1449)	(GC–MS, LP: MV 8100 [40])
P3β5β20α	0.424 (0.393, 0.569)	0.187 (0.168, 0.268)	0.235 (0.221, 0.335)	0.115 (0.1, 0.274)	
P3β5β20αC	311 (254, 392)	307 (267, 389)	587 (489, 867)	65.6 (49.8, 231)	
A3α5α	0.170 (0.160, 0.198)	0.057 (0.052, 0.081)	0.302 (0.271, 0.420)	0.065 (0.061, 0.128)	
A3α5αC	11 (11, 19)	13.1 (11, 18)	384 (361, 428)	31 (28, 45)	
A3β5αC	58.3 (46.2, 93.7)	58.4 (56, 87.8)	94.9 (88.6, 174.9)	7.7 (6.9, 13.3)	
A3α5β	0.050 (0.042, 0.0664)	0.056 (0.054, 0.0687)	0.148 (0.141, 0.168)	0.062 (0.052, 0.138)	
A3α5βC	2.58 (2.47, 3.03)	2.55 (2.47, 3.08)	30.9 (29.7, 46.3)	8.76 (8.49, 11.3)	
A3α5α17βC	13.5 (12.6, 18.7)	13.13 (12.7, 20.4)	25.3 (21.8, 38.5)	4.38 (3.9, 11.7)	
A3β5α17βC	4.33 (3.66, 6.90)	6.13 (5.58, 7.98)	18.7 (17.2, 22.2)	1.93 (1.86, 5.61)	
A3α5β17βC	3.22 (3.20, 3.87)	3.88 (3.67, 4.84)	5.58 (5.38, 9.77)	0.77 (0.68, 5.03)	
A3β5β17βC	1.08 (1.05, 1.66)	1.53 (1.52, 1.71)	0.52 (0.39, 1.05)	0.92 (0.82, 1.71)	
Preg7α	0.346 (0.325, 0.496)	0.509 (0.500, 0.673)	0.215 (0.198, 0.253)	0.089 (0.086, 0.115)	
Preg7β	0.189 (0.184, 0.342)	0.423 (0.422, 0.439)	0.300 (0.286, 0.441)	0.082 (0.077, 0.153)	

DHEA7α	1.79 (1.72, 2.17)	1.83 (1.70, 2.23)	1.67 (1.64, 2.01)	0.78 (0.70, 1.04)	
DHEA7β	0.226 (0.222, 0.265)	0.234 (0.230, 0.265)	0.392 (0.378, 0.489)	0.168 (0.164, 0.239)	
AT7α	0.023 (0.019, 0.038)	0.006 (0.005, 0.010)	0.068 (0.061, 0.171)	0.009 (0.009, 0.011)	
AT7αC	0.729 (0.664, 1.44)	0.485 (0.454, 0.586)	0.114 (0.104, 0.250)	0.152 (0.142, 0.177)	
AT7β	0.010 (0.009, 0.018)	0.006 (0.005, 0.009)	0.058 (0.056, 0.073)	0.006 (0.006, 0.008)	
AT7βC	0.779 (0.603, 1.45)	0.523 (0.494, 0.750)	0.123 (0.115, 0.247)	0.201 (0.163, 0.303)	
Preg16α	9.30 (9.20, 17.3)	5.08 (4.36, 6.34)	1.23 (1.11, 1.71)	3.49 (2.92, 12.5)	(GC–MS, VD: MV 1196 [6])
Preg16αC	10.8 (10.8, 15.9)	12.3 (11.69, 26.6)	2.09 (1.65, 3.52)	15.9 (15.8, 27.9)	(HPLC/GC–MS, VD w37–42: UA 1595, UV 1841, MV n.d. [10])
DHEA16α	18.2 (16.7, 21.5)	52.5 (48.9, 65.7)	6.27 (5.95, 9.05)	18.0(16.3, 37.0)	(RIA, VD: U 5987 [17]); (RIA, VD: UA 25.5, UV 36.5 [1]); (RIA, VD: UA 7467, UV 5987 [2]); (GC–MS, VD: MV 531 [18])
DHEA16αC	1368 (1129, 4221)	1645 (1325, 4831)	219 (211, 425)	521 (290, 843)	(RIA, GC–MS (sulfates), SC w37–41: UA 8684, UV 7171, MV 441 [5]); (GC–MS, VD: MV 762 [38]); (HPLC/GC–MS, VD w37–42: UA 5660, UV 4270, MV 160 [10])
Prog16α	180 (171, 261)	393 (377, 694)	80.1 (77.7, 123)	44.1 (36.6, 115)	(RIA, w40: MV 72.3 [3])
A216α	15.9 (15.5, 19.6)	14.4 (14.0, 21.0)	3.17 (2.91, 4.54)	15.0 (14.0, 24.8)	(HPLC-RIA, VD: UA 12.4, UV 17.8, MV15.7 [43])
T16α	11.8 (10.2, 20.3)	17.0 (14.4, 21.9)	7.8 (7.2, 9.9)	8.12 (7.92, 13.0)	

UA, umbilical artery; UV, umbilical vein; MV, maternal cubital vein; U, mixed umbilical blood; AF, amniotic fluid; RIA, radioimmunoassay. Levels of 69 steroids in the serum from human maternal cubital vein at premature and normal labor from the 28th to 41st week of gestation.

was even less likely. Although we had no tools to verify our anticipation that the reasons in preterm labors with spontaneous uterine activity were independent of the steroid status, our exertion was to select the women to provide maximum conformity of steroid metabolome with the actual GA in both preterm and in-time labors.

The local Ethical Committee approved the study. After signing written informed consent, the women underwent the sample collection.

2.2. Sample collection

Samples of umbilical arterial and venous blood and samples of blood from maternal cubital vein were withdrawn immediately after the separation of the newborn from the umbilical cord. The amniotic fluid was collected at the second stage of labor. Second stage of labor is the period from the full dilatation of the cervix until the baby is completely out of the birth canal. The amniotic fluid in our study was collected after the delivery of the baby's head. Each sample was collected into a cooled plastic tube containing 100 μl of 5% EDTA. The plasma was obtained after centrifugation for 5 min at 2000 × *g* at 4 °C. The samples of plasma and amniotic fluid were stored at −20 °C until analyzed.

2.3. Chemicals and reagents

The steroids were purchased from Steraloids (Newport, RI, USA), the Sylon B from Supelco (Bellefonte, PA, USA), the methoxylamine hydrochloride from Sigma (St. Louis, MO, USA) and the solvents from Merck (Darmstadt, Germany).

2.4. Instruments

The GCMS-QP2010 Plus system from Shimadzu (Kyoto, Japan) consisted of a gas chromatograph equipped with automatic flow control, AOC-20s autosampler and a single quadrupole detector with an adjustable electron voltage of 10–195 V. A capillary column with a medium polarity RESTEK Rxi (diameter 0.25 mm, length 15 m, film thickness 0.1 μm) was used for analyses. Electron-impact ionization with electron voltage fixed at 70 V and emission current set to 160 μA was used for the measurements. The temperature of the injection port, ion source and interface was maintained at 220, 300, and 310 °C, respectively. Analyses were carried out in the splitless mode with a constant linear velocity of the carrier gas (He) which was maintained at 60 cm/s. The septum purge flow was set up to 3 mL/min. The samples were injected using the high-pressure mode which was applied at 200 kPa and this pressure was maintained for 1 min. The detector voltage was set to 1.4 kV.

2.5. Steroids measured

The levels of 40 unconjugated steroids and 29 steroid polar conjugates (the letter C at the end of the abbreviations of the unconjugated steroids symbolizes the corresponding steroid polar conjugates) were measured in the maternal and fetal body fluids using GC–MS. We have quantified the 3β-hydroxy-5-ene steroids (3βOH5S) – pregnenolone (Preg), 17-hydroxy-pregnenolone (Preg17), dehydroepiandrosterone (DHEA) and 5-androstene-3β,17β-diol (Adiol) including the corresponding polar conjugates, 3-oxo-4-ene steroids (3O4S) – progesterone (Prog), 17-hydroxy-progesterone (Prog17), androstenedione (A2), testosterone (T), estrogens – estrone (E1), estradiol (E2), estriol (E3), 16α-hydroxy-estrone (E116α), including the corresponding polar conjugates except of the E116αC, 5α/β-reduced-20-oxo-metabolites of progesterone – 5α-dihydroprogesterone (P5α), allopregnanolone (P3α5α), isopregnanolone (P3β5α), 5β-dihydroprogesterone (P5β), pregnanolone (P3α5β), epipregnanolone (P3β5β), 20α-hydroxy-metabolites of Prog and Preg – 20α-hydroxy-pregnenolone (Preg20α) and 20α-dihydroprogesterone (Prog20α), including the corresponding polar conjugates, 5α/β-reduced-20α-hydroxy-metabolites of progesterone – 20α-hydroxy-5α-pregnane-3-one (P5α20α), 5α-pregnane-3α,20α-diol (P3α5α20α), 5α-pregnane-3β,20α-diol (P3β5α20α), 20α-hydroxy-5β-pregnane-3-one (P5β20α), 5β-pregnane-3α,20α-diol (P3α5β20α), 5β-pregnane-3β,20α-diol (P3β5β20α), including the corresponding polar conjugates, C19 5α/β-reduced-17-oxo-steroids – androsterone (A3α5α), etiocholanolone (A3α5β) and also the corresponding polar conjugates, and the polar conjugates of epiandrosterone (A3β5αC), polar conjugates of C19 5α/β-reduced-17β-hydroxy-steroids – 5α-androstane-3α,17β-diol (A3α5α17βC), 5α-androstane-3β,17β-diol (A3β5α17βC), 5β-androstane-3C,17β-diol (A3α5β17βC), and 5β-androstane-3β,17β-diol (A3β5β17βC), 7α/β-hydroxy-metabolites of Preg and DHEA – 7α-hydroxy-pregnenolone (Preg7α), and 7β-hydroxy-pregnenolone (Preg7β), 7α-hydroxy-DHEA (DHEA7α), 7β-hydroxy-DHEA (DHEA7β), 7α/β-hydroxy-metabolites of Adiol – 5-androstene-3β,7α,17β-triol (AT7α), 5-androstene-3β,7β,17β-triol (AT7β) as well as the corresponding polar conjugates, 16α-hydroxy-metabolites of 3βOH5S – 16α-hydroxy-pregnenolone (Preg16α), 16α-hydroxy-DHEA (DHEA16α) and also the corresponding polar conjugates, and 16α-hydroxy-metabolites of 3O4S – 16α-hydroxy-progesterone (Prog16α), 16α-hydroxy-androstenedione (A216α), and 16α-hydroxy-testosterone (T16α).

2.6. Sample pre-treatment

The unconjugated steroids were extracted from 1 mL of plasma or amniotic fluid with diethyl-ether (3 mL). The diethyl-ether extract was dried in the block heater at 37 °C. The lipids in the dry residue of the diethyl-ether extract were separated by partitioning between a mixture of methanol–water 4:1 (1 mL) and pentane (1 mL). The pentane phase was discarded and the polar phase was dried in the vacuum centrifuge at 60 °C (2 h). The dry residue from the polar phase was derivatized first with methoxylamine hydrochloride solution in pyridine (2%) on oxo-groups (60 °C, 1 h). The mixture after the first derivatization was dried in the flow of nitrogen and the dry residue was treated with the reagent Sylon B (99% of bis(trimethylsilyl)-trifluoroacetamide and 1% of trimethylchlorosilane) forming trimethylsilyl derivatives on hydroxy-groups (TMS-MOX derivatives) (90 °C, 1 h). Finally, the mixture after the second derivatization step was dried in the flow of nitrogen, the dry residue was dissolved in 20 μL of isooctane and 1 μL of the solution was used for GC–MS analysis.

Steroid conjugates remaining in the polar residues after diethyl-ether extraction were analyzed as follows: the polar residues were dried in the vacuum centrifuge at 37 °C (5 h) and the dry residues were hydrolyzed as described elsewhere [62]. The hydrolyzed samples were again dried in the vacuum centrifuge at 37 °C (5 h). The dried residues were reconstituted with 1 mL of chromatographic water and then and further processed in the same way as the free steroids. In contrast to the sample preparation of free steroids, the dry residue after the second derivatization step was dissolved in 200 μL of isooctane instead of the 20 μL of isooctane. Prior to further processing, the original samples and the polar phases after diethyl-ether extraction, which were used for the quantification of the steroid conjugates, were spiked with 17α-estradiol (as an internal standard) to attain a concentration of 1 and 10 ng/mL, respectively. The internal standard was recorded at effective masses m/z = 231, 285 and 416. The addition of internal standard to body fluid before sample preparation (free steroids) and to polar phase after diethyl-ether extraction

(conjugated steroids) assured that the losses during the sample processing were not critical for steroid quantification. Nevertheless, we have also tested the extraction efficiency of diethyl-ether extraction as well as the efficiency of partitioning of dried diethyl-ether extract between a mixture of methanol–water and pentane. In the former case, the extraction efficiency ranged from 75.6 to 96.2% being higher for the less polar steroids. For partitioning of dried diethyl-ether extract between a mixture of methanol–water and pentane the extraction efficiency was lowest for relatively non-polar pregnanolone isomers such as pregnanolone (76.3%) and reached almost 100% for steroids with higher polarity than that of progesterone showing 99.6% extraction efficiency in this step.

Although the addition of an appropriate steroid conjugate as the internal standard into the original sample would be more correct than the addition of unconjugated standard into the polar residue after diethyl-ether extraction of free steroids, we have found no appropriate and commercially available steroid conjugate having negligible levels in the human circulation. Nevertheless, the efficiency of the hydrolysis step for several commercially available steroid conjugates was verified comparing the known quantities of unconjugated steroids (1 ng) after diethyl-ether extraction and the equimolar amount of the corresponding sulfates after hydrolysis and diethyl-ether extraction of the released steroids. We have tested the efficiency of the hydrolysis in 10 parallel measurements and we have found the values 98.4 ± 1.8%, 89.8 ± 1.3%, 82 ± 1.5%, 93.7 ± 1.7%, 91.8 ± 1.9%, 77.4 ± 1.5%, 79.6 ± 2.3%, 92.5 ± 2.8%, 84 ± 2.2%, 66.8 ± 2%, and 72.4 ± 2% (mean ± SEM) for the sulfates of androsterone, etiocholanolone, epiandrosterone, 17-hydroxy-pregnenolone, dehydroepiandrosterone, estrone, pregnenolone, epipregnanolone, allopregnanolone, pregnanolone, and isopregnanolone, respectively. These values indicated that the estimation of the steroid conjugates using the hydrolysis method suggested by Dehennin et al. mostly well reflects the actual circulating levels of the conjugated steroids. Although it would be appropriate to use the obtained values for a correction of our results for the steroid polar conjugates, the sulfates were commercially available only for minority of the investigated steroids. Therefore, we have not applied these correction factors. The losses of steroids, which were independent of the corresponding hydrolysis step, were covered by an addition of internal standard into the polar residue after the diethyl-ether extraction of unconjugated steroids containing the steroid conjugates and before hydrolysis and further processing of the polar residue.

2.7. Temperature and pressure gradients for the GC–MS analysis of trimethylsilyl derivatives and the retention times of the steroids

To effectively utilize the biological material, the individual samples were applied in independent courses, in each case employing a part of the steroids under investigation. The choices of the steroids measured within the individual courses, the temperature and pressure gradients, and the effective masses used for the measurement in selected ion monitoring (SIM) mode were all optimized to attain minimum limit of detection (LOD) at sufficient selectivity. All temperature gradients used for steroid analysis were conducted at constant linear velocity 60 cm/s.

The temperature gradient used for the 1st, 3rd and 5th runs (G1, G3 and G4) was as follows: 1 min delay at 80 °C, increase to 190 °C (40 °C/min) increase to 210 °C (4 °C/min) increase to 300 °C (20 °C/min), 5 min delay at 300 °C, initial pressure 34 kPa, injector temperature 220 °C, analysis duration 18.25 min.

The gradient used for the 2nd run (G2) was: increase from 80 to 190 °C (40 °C/min), increase to 205 °C (1.6 °C/min) increase to 300 °C (40 °C/min), 5 min delay at 300 °C, initial pressure 34 kPa, injector temperature 240 °C, analysis duration 19.5 min.

The gradient used for the 5th run (G5) was: 1 min delay at 80 °C, increase to 170 °C (40 °C/min) increase to 180 °C (1 °C/min) increase to 300 °C (40 °C/min), 5 min delay at 300 °C, initial pressure 34 kPa, injector temperature 220 °C, analysis duration 21.5 min.

The gradient used for the 6nd run (G6) was: 1 min delay at 80 °C, increase to 200 °C (40 °C/min) increase to 240 °C (8 °C/min) increase to 300 °C (40 °C/min), 5 min delay at 300 °C, initial pressure 34 kPa, injector temperature 220 °C, analysis duration 15.5 min.

The effective masses, retention times of chromatographic peaks, sequence number of injection for steroid groups and gradients that were used for quantification of individual steroids are shown in Table 1 (see also Fig. 1).

2.8. Validation procedure

The linearity was demonstrated at least over the ranges found for individual steroids within all analyzed body fluids (nominal analyte concentration ±20%) using serial dilution from the highest concentration. The correlation coefficients (r) ranged from 0.9936 to 0.9999 for individual steroids. Intra-assay variability was tested using 10 replicates obtained from the same pooled maternal plasma. Inter-assay variability was checked using a sample of pooled maternal plasma in six independent runs. Limit of detection for individual steroids was calculated using the triple of the respective signal-to-noise ratio. The recovery was based on the relationship between the amounts of the added steroid and the amounts detected by the GC–MS assay. This validation step was done in quadruplicate. The individual pooled body fluids (UA, UV, MV, and AF) were spiked with the steroid measured in amounts about the triple of median values found for the individual steroids and the recovery values were expressed as percentage of assayed concentration relative to the calculated concentration (recovery). The recoveries ranged from 70.9 to 102%. The determination of recoveries was completed for unconjugated steroids (most of the polar conjugates are not available).

In all cases, the mixtures of authentic standards and internal standard were processed in the same way as samples. The mixtures were specific for each of the independent courses as mentioned above. The standards were injected in duplicates in three different amounts for each steroid (10, 100 and 1000 pg). Respecting the excellent linearity for all substances investigated (Table 2); the calibration line was used for data processing.

2.9. Statistical data analysis

To eliminate skewed data distribution and heteroscedasticity, the original data was transformed to a Gaussian distribution before further processing by a power transformation using the statistical software Statgraphics Centurion, version XV from Statpoint Inc. (Herndon, Virginia, USA). The transformed data underwent multivariate regression using the method of orthogonal projections to latent structure (OPLS) [63]. This method is effective in coping with the problem of severe multicollinearity within the matrix of independent variables. In our model, the GA was chosen as the single dependent variable while the steroid levels represented the independent variables. The variability was separated into two independent components. The first component contained the variability in the steroid levels, which was shared with GA (the predictive component) while the second component contained the variability shared within the steroid levels (the orthogonal component). The OPLS enabled us to find the best predictors as well as the best combination of predictors for estimation of GA from steroid levels. Using this approach, we were also able to compare the predictivity of the steroids in different biological fluids. After standardization

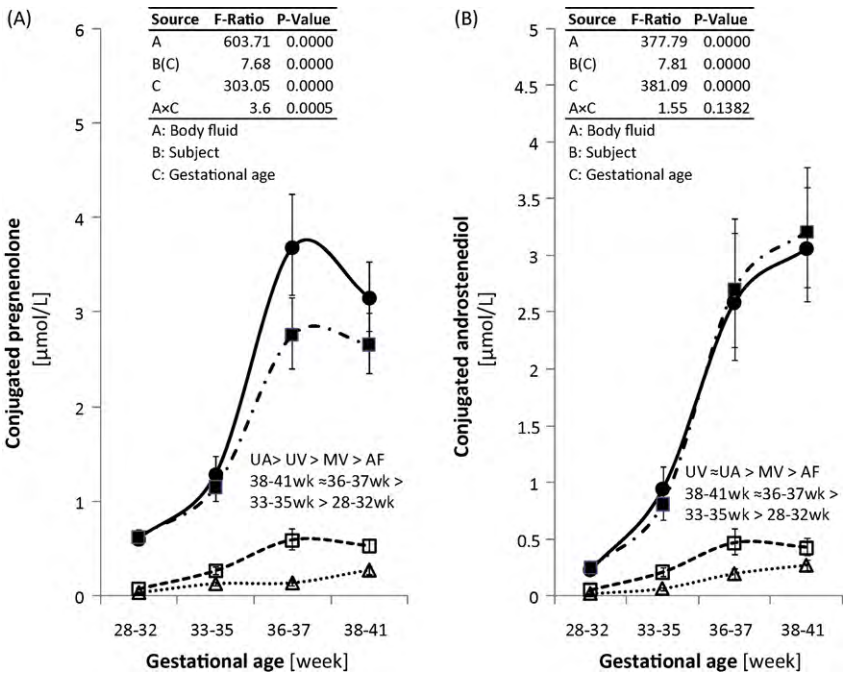

Fig. 2. Profiles of conjugated pregnenolone (panel A) and conjugated androstenediol (panel B) in the plasma from the umbilical artery (UA), umbilical vein (UV) and maternal cubital vein (MV) and in amniotic fluid (AF) according to the gestational age (GA). The repeated measures ANOVA model was used for 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 A – four body fluids were investigated in each subject), subject factor (factor B), between-subject factor GA (factor C – the subjects were separated into four groups according to the GA) and body fluid × GA interaction (A × C interaction). Significant A × C interaction indicates that there is a significant difference between the dependences of the individual body fluids on GA. *F*-ratio represents 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, UA). 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 $p < 0.05$.

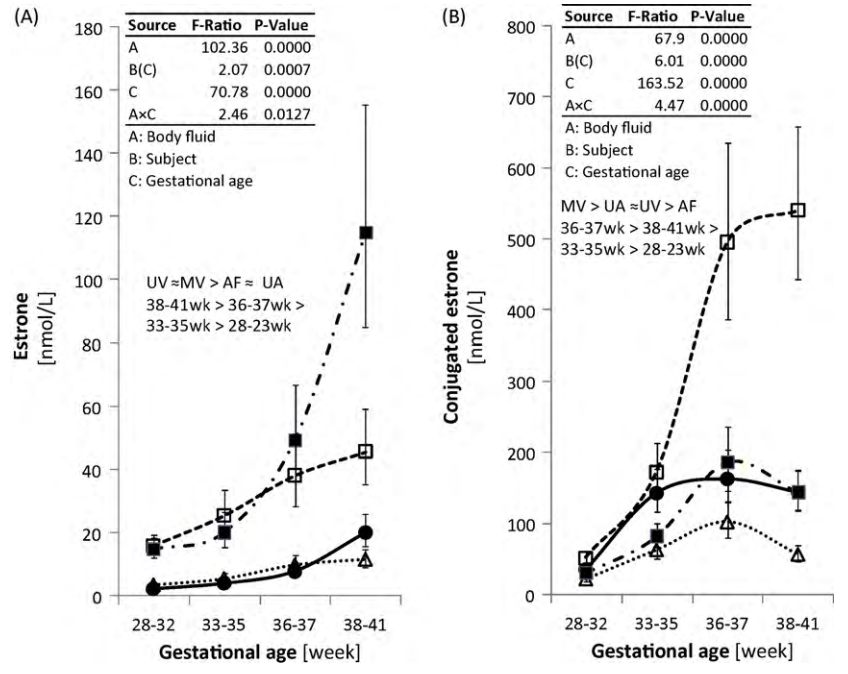

Fig. 3. Profiles of estrone (panel A) and conjugated estrone (panel B) in the plasma from the umbilical artery (UA), umbilical vein (UV) and maternal cubital vein (MV) and in amniotic fluid (AF) in preterm and normal labor. The drawings and symbols are the same as for Fig. 2.

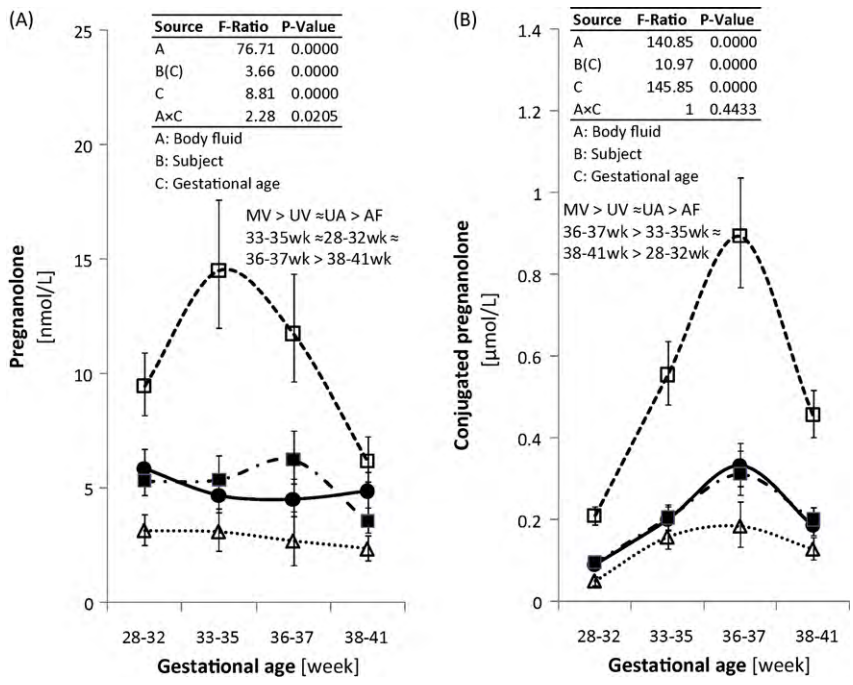

Fig. 4. Profiles of pregnanolone (panel A) and conjugated pregnanolone (panel B) in the plasma from the umbilical artery (UA), umbilical vein (UV) and maternal cubital vein (MV) and in amniotic fluid (AF) in preterm and normal labor. The drawings and symbols are the same as for Fig. 2.

of the variables, the OPLS model can be expressed as follows:

$$\mathbf{X} = \mathbf{T}_p\mathbf{P}_p^t + \mathbf{T}_0\mathbf{P}_0^t + \mathbf{E}$$

$$\mathbf{Y} = \mathbf{T}_p\mathbf{P}_p^t + \mathbf{F}$$

where **X** is the matrix with l independent variables and i subjects, **Y** is the matrix of m dependent variables and i subjects. In our data $\mathbf{T}_p$ represent the vector of component scores from the single predictive component and i subjects extracted from **Y**, $\mathbf{T}_0$ is the vector of component scores from the single orthogonal component and i subjects extracted from **X**, $\mathbf{P}_p$ represents the vector of component loadings for the predictive component extracted from **Y**. $\mathbf{P}_0$ represents the vector of component loadings for the orthogonal component extracted from **X** and l independent variables, while **E** and **F** are the error terms.

The statistical software SIMCA-P v.11.5 from Umetrics AB (Umeå, Sweden), used for data analysis enabled to find the number

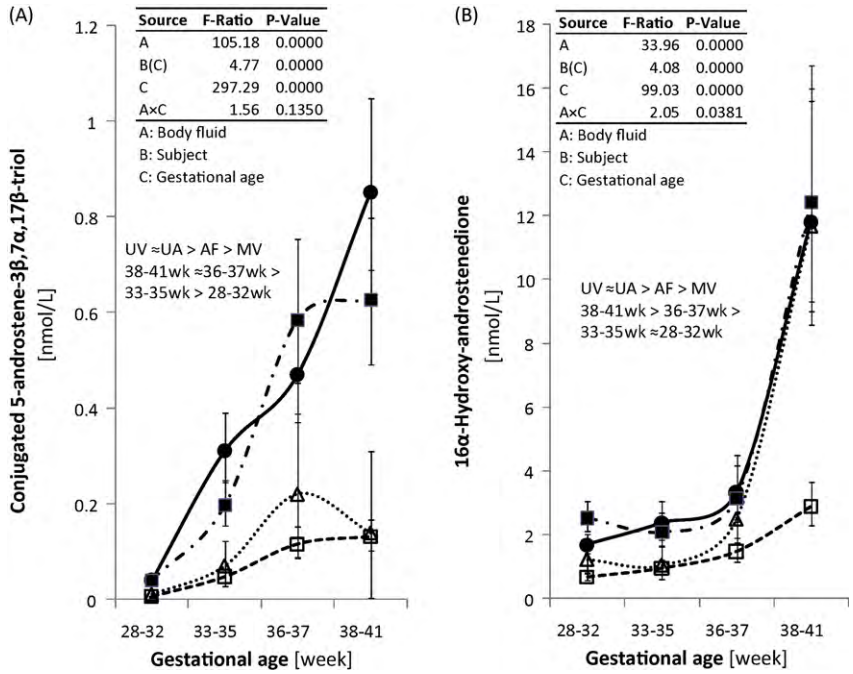

Fig. 5. Profiles of conjugated 5-androstene-3β,7α,17β-triol (panel A) and conjugated 16α-hydroxy-androstenedione (panel B) in the plasma from the umbilical artery (UA), umbilical vein (UV) and maternal cubital vein (MV) and in amniotic fluid (AF) in preterm and normal labor. The drawings and symbols are the same as for Fig. 2.

of the relevant components using the prediction error sum of squares and also allowed the detection of multivariate non-homogeneities and testing the multivariate normal distribution and homoscedasticity (constant variance).

3. Results and discussion

3.1. Method development

The selectivity of the method was provided by the use of optimum gradients in independent runs. This approach enabled complete separation of the steroid isomers from each other. The separation of steroid isomers with almost identical fragmentation is most critical for providing sufficient selectivity. We also considered fragmentation patterns and physiological concentrations of steroids and sterols, which can be co-eluted with the measured substances and we have recorded no critical interference. The sufficient separation of the steroids is demonstrated in Table 1. The steroids were well detectable as documented by the comparison of median levels in individual body fluids with the respective LODs (Table 2). The correct identification of the substances was ensured by congruence of fragmentation pattern and retention time with the standard (at least 2 fragments + retention time(s)). In addition, the correlations were checked between precursors and products considering the known steroid metabolic pathways (data not shown). Further, the steroids containing oxo-group mostly produced two-peak response, thus in this case we also checked the ratios of peak 1 to peak 2 for individual fragments. The analytical criteria concerning selectivity, linearity of a test procedure, repeatability and LOD for each steroid is shown in Tables 1 and 2. The recovery values ranged from 70.9 to 102%. The linearity was satisfactory for all steroids. The correlation coefficients of two-parameter linear regression ranged from 0.9971 to 0.9999 (Table 2).

The levels of free and conjugated steroids are shown in Table 3. A number of reduced androstane steroids, 7-hydroxy-metabolites of 3β-hydroxy-5-ene-steroids, several 20α-hydroxy-C21 steroids, their conjugates and 16α-hydroxy-testosterone, were measured in maternal and fetal body fluids for the first time. Comparison of the results with the data reported from other studies is provided ibidem. The steroid levels are generally in accordance with the data reported elsewhere. Surprisingly, the levels of reduced 20-oxo progesterone metabolites were mostly lower when compared with the data from other reports, including our

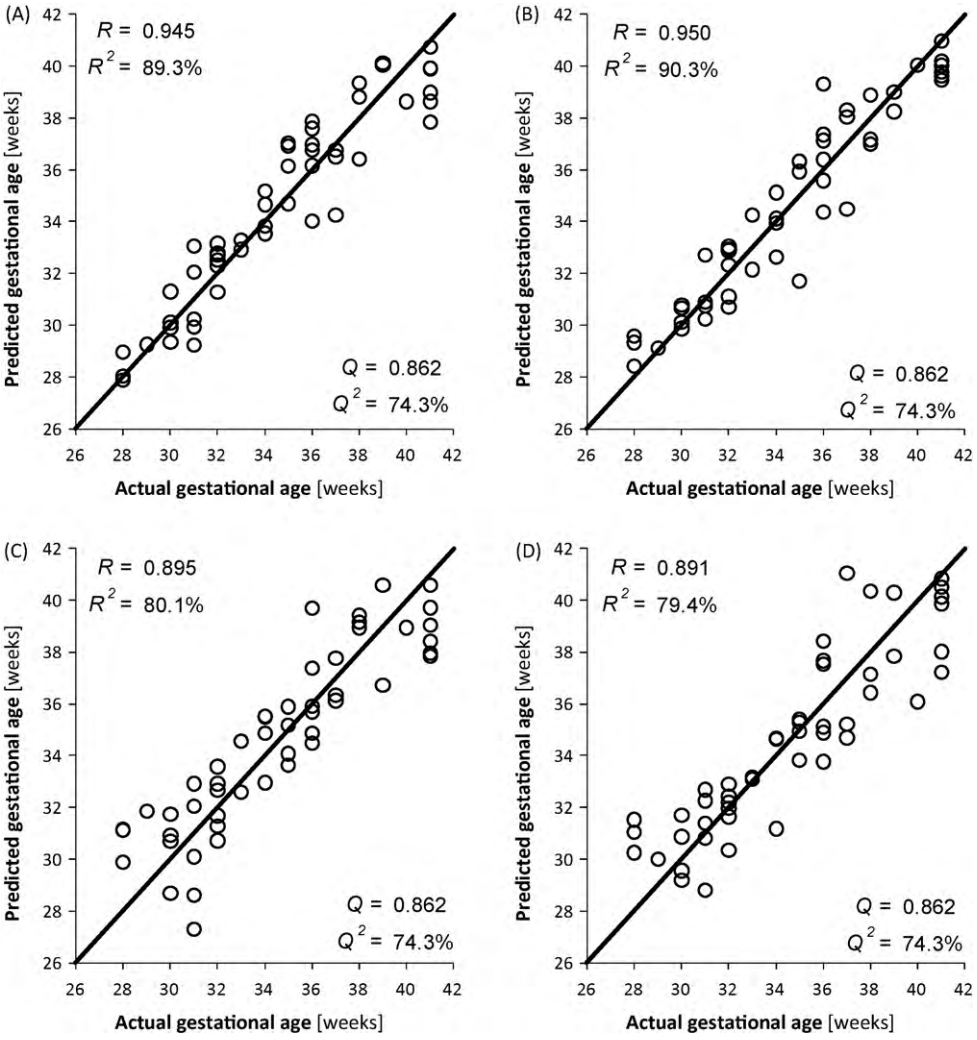

Fig. 6. Comparison of predictivity of OPLS models in predicting gestational age from steroid levels in the plasma from the umbilical artery (UA), umbilical vein (UV) and maternal cubital vein (MV) and in amniotic fluid (AF); R^2 is the percent of variation of the matrix of the dependent variable **Y** (gestational age) explained by the OPLS model. R^2 or R is a measure of fit, i.e. how well the model fits the data. A large R^2 (close to 1) is a necessary condition for a good model; Q^2 is the percent of variation of the matrix of the dependent variable **Y** explained by the OPLS, predicted by the model according to cross validation. Q^2 indicates how well the model predicts new data. A large Q^2 ($Q^2 > 0.5$) indicates good predictivity.

own past reports, however, the levels of the respective steroid conjugates exhibited better compatibility. This may be ascribed to sample collection in a different period of the parturition. As reported in our previous study, the levels of unconjugated 20-oxo progesterone metabolites rapidly change after labor while the concentrations of the respective conjugates exhibit higher stability [64].

3.2. Model of gestational age and prediction of the onset of labor

Most of the steroids under investigation significantly depended on GA at labor (Figs. 2–5). As regards the practical use of our results, there is a question as to whether the aforementioned relationships can be used for prediction of the onset of labor in pregnant women. In women, who were enrolled in this study, the causes of preterm

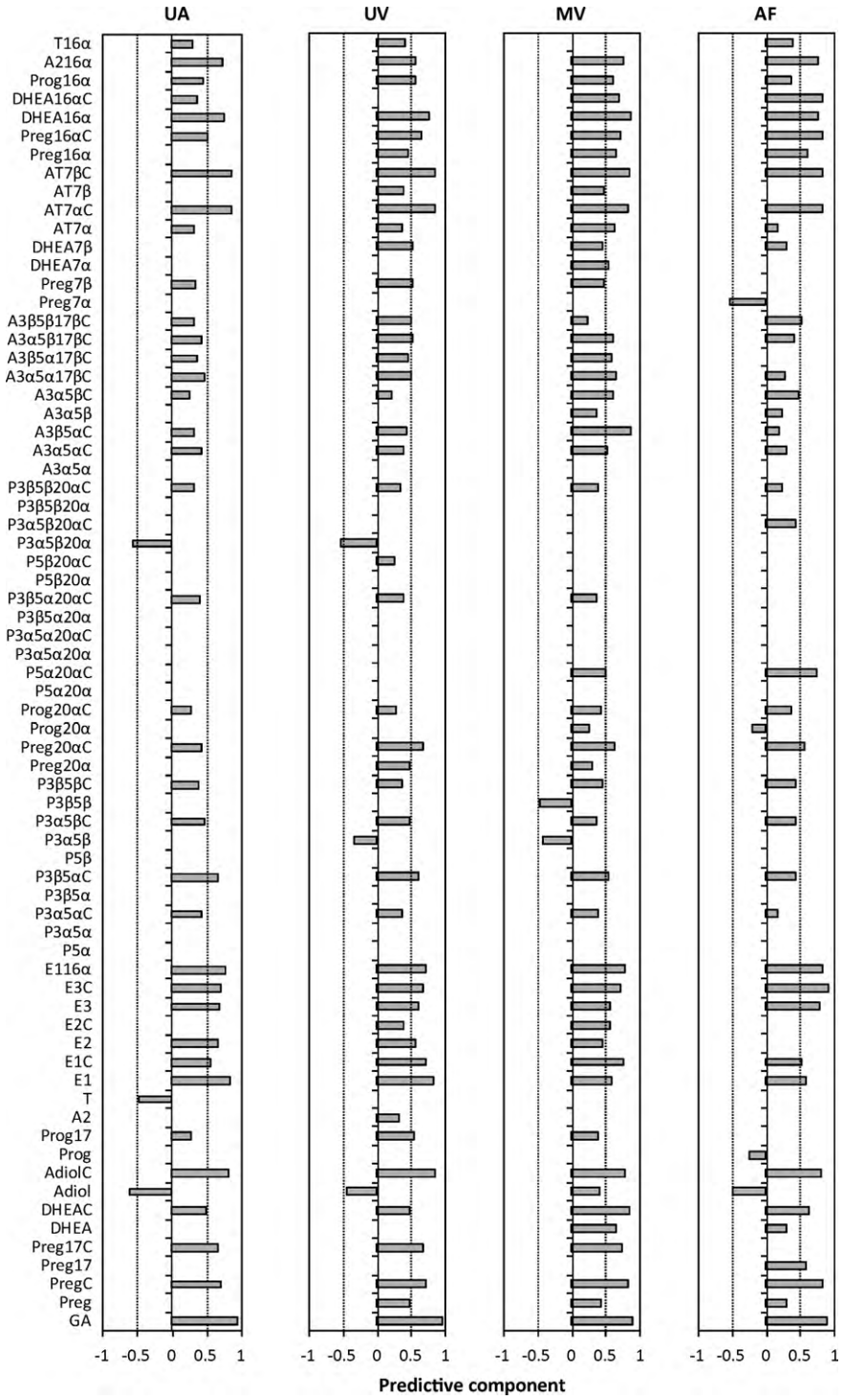

Fig. 7. The correlations of the steroids and GA with the common predictive component. The final OPLS model (orthogonal projections to latent structures) is illustrated as a bar graph displaying significant component loadings in the form of correlation coefficients with a common predictive component for GA and steroid levels. The missing bars in this figure symbolize insignificance of the corresponding steroids in the initial model, which includes all investigated steroids ($n = 69$) and the absence of the insignificant steroids in the final model, which includes only the steroids being significant ($p < 0.05$) in the initial model. The loadings are computed to reach maximum correlations between GA and steroid levels.

Table 4
Levels of relevant steroids in the OPLS model evaluating the relationships between steroids in the plasma from maternal cubital vein and gestational age at premature and physiological labor from the 28th to 41st week of gestation.

Steroid	Group A 28th–32nd week, $n = 19$	Group B 33rd–35th week, $n = 10$	Group C 36th–37th week, $n = 9$	Group D 38th–41st week, $n = 12$	Differences between groups K–W[a] test[b], K–W multiple comparisons ($p < 0.05$)
Preg	5.1 (4.2, 7.6)	7.9 (6.6, 9.4)	8.9 (7, 11)	6.6 (5.8, 13)	$p = 0.0057$, AB, AC, AD
PregC	70 (60, 102)	274 (221, 354)	540 (444, 957)	514 (407, 645)	$p < 0.0001$, AB, AC, AD, BC, BD
Preg17C	3.5 (1.8, 19)	81 (62, 113)	93 (48, 158)	83 (36, 129)	$p < 0.0001$, AB, AC, AD
DHEA	2 (0.92, 4.2)	5.8 (4.5, 6.6)	5.6 (4.4, 12)	14 (8, 16)	$p < 0.0001$, AC, AC, AD, BD,
DHEAC	92 (45, 153)	382 (202, 548)	1220 (1010, 1530)	1290 (778, 1880)	$p < 0.0001$, AB, AC, AD, BC, BD
Adiol	0.32 (0.22, 0.58)	0.62 (0.44, 0.83)	0.34 (0.29, 0.6)	0.64 (0.41, 0.86)	$p = 0.0195$, AB, AD
AdiolC	58 (37, 91)	171 (124, 282)	328 (318, 393)	387 (308, 597)	$p < 0.0001$, AB, AC, AD
Prog17	17 (8.8, 23)	23 (13, 29)	19 (19, 66)	27 (24, 28)	$p = 0.0643$, AC, AC, AD
E1	15 (5.1, 28)	25 (18, 32)	36 (28, 55)	50 (35, 65)	$p = 0.0036$, AC, AC, AD
E1C	44 (29, 98)	159 (111, 191)	472 (282, 731)	623 (390, 728)	$p < 0.0001$, AB, AC, AD, BD
E2	12 (6.2, 20)	36 (25, 59)	23 (20, 49)	36 (28, 46)	$p < 0.0001$, AB, AC, AD
E2C	11 (8.6, 14)	50 (34, 62)	31 (27, 42)	33 (22, 38)	$p < 0.0001$, AB, AC, AD
E3	9.8 (5.9, 14)	24 (22, 28)	20 (16, 48)	31 (23, 37)	$p = 0.0053$, AB, AC, AD
E3C	50 (43, 74)	476 (300, 625)	524 (331, 1430)	348 (261, 499)	$p < 0.0001$, AB, AC, AD
E116α	0.32 (0.18, 0.48)	0.66 (0.39, 0.74)	0.58 (0.37, 0.8)	1.4 (1.1, 1.4)	$p < 0.0001$, AB, AD, BD, CD
P3α5αC	451 (299, 617)	1600 (1050, 2110)	2070 (1710, 3040)	1090 (803, 1370)	$p < 0.0001$, AB, AC, AD
P3β5αC	138 (80, 186)	488 (288, 821)	805 (429, 1380)	436 (285, 569)	$p < 0.0001$, AB, AC, AD
P3α5β	8.5 (7.1, 11)	9.6 (6.3, 12)	7.6 (7.2, 9.3)	4.4 (3, 6.2)	$p = 0.0207$, AD, BD
P3α5βC	226 (161, 370)	561 (384, 1030)	794 (591, 1070)	475 (405, 573)	$p < 0.0001$, AB, AC, AD
P3β5β	0.86 (0.58, 1.2)	0.71 (0.64, 0.81)	0.58 (0.52, 0.75)	0.36 (0.19, 0.66)	$p = 0.045$, AD
P3β5βC	21 (17, 30)	56 (47, 62)	68 (60, 104)	43 (33, 52)	$p < 0.0001$, AB, AC, AD, CD
Preg20α	1.1 (0.79, 1.7)	1.6 (1.3, 1.9)	2.4 (1.6, 3.7)	1.8 (1, 2)	$p = 0.0158$, AC, AC
Preg20αC	228 (185, 293)	1030 (855, 1280)	1090 (786, 1500)	849 (656, 1180)	$p < 0.0001$, AB, AC, AD
Prog20α	53 (22, 83)	97 (63, 186)	150 (94, 186)	64 (46, 94)	$p = 0.0063$, AB, AC, CD
Prog20αC	18 (11, 34)	38 (30, 59)	38 (34, 71)	35 (27, 53)	$p = 0.0101$, AB, AC, AD
P5α20αC	25 (19, 43)	98 (69, 127)	82 (46, 100)	52 (35, 69)	$p < 0.0001$, AB, AC, AD
P3β5α20αC	2010 (1130, 2700)	9600 (5440, 12400)	7670 (4570, 10500)	4010 (2500, 5410)	$p < 0.0001$, AB, AC, AD
P3β5β20αC	252 (159, 427)	814 (598, 1040)	946 (594, 1130)	587 (359, 867)	$p < 0.0001$, AB, AC, AD
A3α5αC	51 (30, 165)	133 (74, 167)	173 (137, 400)	384 (201, 428)	$p = 0.0011$, AC, AC, AD, BD
A3β5αC	10 (6.5, 16)	28 (17, 36)	77 (46, 141)	95 (75, 175)	$p < 0.0001$, AB, AC, AD, BC, BD
A3α5β	0.071 (0.048, 0.11)	0.081 (0.054, 0.1)	0.09 (0.07, 0.13)	0.15 (0.11, 0.17)	$p = 0.0489$, AD, BD
A3α5βC	4.5 (2.1, 10)	8.6 (6.8, 11)	17 (15, 27)	31 (27, 46)	$p < 0.0001$, AC, AC, AD, BD
A3α5α17βC	7.3 (4.6, 9.7)	29 (22, 39)	26 (22, 33)	25 (17, 38)	$p < 0.0001$, AB, AC, AD
A3β5α17βC	4.3 (2.8, 7.1)	17 (8.3, 18)	17 (13, 21)	19 (12, 22)	$p < 0.0001$, AB, AC, AD
A3α5β17βC	1.2 (0.92, 2.3)	4.4 (2.4, 12)	4 (3.1, 7.5)	5.6 (3.9, 9.8)	$p < 0.0001$, AB, AC, AD
A3β5β17βC	0.36 (0.2, 0.49)	1.2 (0.66, 1.7)	1.1 (0.86, 1.2)	0.52 (0.23, 1)	$p < 0.0001$, AB, AC, BD, CD
Preg7β	0.04 (0.016, 0.071)	0.088 (0.039, 0.17)	0.031 (0.02, 0.12)	0.3 (0.1, 0.44)	$p < 0.0001$, AD, BD, CD
DHEA7α	0.98 (0.66, 1.3)	1.1 (0.84, 1.5)	0.97 (0.86, 1.2)	1.7 (1.3, 2)	$p = 0.0067$, AD, BD, CD
DHEA7β	0.094 (0.074, 0.32)	0.32 (0.22, 0.36)	0.38 (0.27, 0.53)	0.39 (0.33, 0.49)	$p = 0.0075$, AC, AC, AD
AT7α	0.011 (0.0056, 0.021)	0.06 (0.04, 0.09)	0.041 (0.029, 0.081)	0.068 (0.045, 0.17)	$p < 0.0001$, AB, AC, AD
AT7αC	0.01 (0.004, 0.01)	0.06 (0.031, 0.069)	0.16 (0.06, 0.21)	0.11 (0.08, 0.25)	$p < 0.0001$, AB, AC, AD
AT7β	0.014 (0.0054, 0.035)	0.062 (0.05, 0.089)	0.06 (0.047, 0.093)	0.058 (0.035, 0.073)	$p < 0.0001$, AB, AC, AD
AT7βC	0.004 (0.002, 0.01)	0.03 (0.015, 0.048)	0.088 (0.029, 0.15)	0.12 (0.097, 0.25)	$p < 0.0001$, AB, AC, AD, BD
Preg16α	0.45 (0.3, 0.8)	0.71 (0.57, 0.77)	1.6 (1.1, 1.8)	1.2 (0.81, 1.7)	$p < 0.0001$, AC, AC, AD, BC, BD
Preg16αC	0.3 (0.19, 0.61)	0.8 (0.48, 1.6)	1.2 (0.45, 2.9)	2.1 (1.2, 3.5)	$p < 0.0001$, AB, AC, AD
DHEA16α	0.75 (0.43, 0.92)	1.3 (0.9, 1.5)	2.3 (1.8, 2.9)	6.3 (2.8, 9.1)	$p < 0.0001$, AB, AC, AD, BD
DHEA16αC	44 (19, 67)	46 (32, 61)	118 (50, 565)	218 (119, 425)	$p < 0.0001$, AC, AC, AD, BD

[a] K–W, Kruskal–Wallis.
[b] Statistical significance ($p < 0.05$).

birth were not associated with an imbalance in steroid levels. As shown above, the group of women after the 38th week of gestation did not suffer from any serious complications. Therefore, we can assume that the steroid levels in all subjects reflected to a great extent the GA regardless of the onset of labor. We realize that the prediction model completed from our data is inappropriate for prediction of the term in the case of unexpected complications inducing preterm labor. On the other hand, this model could be helpful in the assessment of the term based on regular hormonal changes. Finally, we can estimate which steroids are most closely associated with GA and may serve as the best predictors for the term.

It is clear that the availability of biological fluids in pregnancy is different. Maternal plasma is easily available and sample collecting does not bring serious complication for the mother or fetus, however collecting samples of the amniocentesis, and even more so the cordocentesis, is much more invasive and therefore more hazardous. Therefore, it may be useful to know whether the maternal plasma possesses an informative value comparable to the fetal plasma or amniotic fluid. Hence, we evaluated the associations between GA at labor and the levels of steroids separately for each body fluid and we compared the predictivity of steroids in four types of biological materials. For this purpose, multivariate regression was applied (for details see Section 2.9). Initially, we have included all steroids measured. In the second step those, which did not significantly correlate with the common predictive component for GA (dependent variable) and steroid levels (independent variable), were excluded. The steroids exhibiting values close to −1 or 1 are strongly negatively or strongly positively correlated with GA and therefore will be good predictors for the term of labor. Alternatively, the steroids without the significant predictive value (with insignificant component loadings) should be excluded from

the model because they increase the proportion of unexplained variability and may deteriorate the quality of the regression model.

3.3. Predictive value for estimation of term

Concerning the comparison of the predictivity of different biological fluids, it is best reflected by component loadings of GA showing that the predictivity decreases in the sequence UV ($R = 0.950$), UA ($R = 0.945$), MV ($R = 0.895$), and AF ($R = 0.891$). The squares of the component loadings express the proportion of variability in GA, which is shared with the common predictive component. The correlations between the predicted and the actual GA at labor are illustrated in Fig. 6. The correlations of the individual steroids and GA with the common predictive component are shown in Fig. 7. Here the final model is illustrated as a bar graph displaying significant component loadings in the form of correlation coefficients with a common predictive component for GA and steroid levels for the individual body fluids. The missing bars in Fig. 7 symbolize insignificance of the corresponding steroids in the initial model and the absence of them in the final model. The loadings are computed to reach maximum correlations between GA and steroid levels.

The data in Fig. 7 and Table 4 show that the conjugated 3βOH5S, the key products of the fetal zone of the fetal adrenal being in all probability under the control of placental CRH [65], were strong predictors of the GA in all body fluids. From estrogens, E1 showed a comparable predictivity value for UA and UV and less for MV and AF. E1C and E116α were the best predictors in MV. E2 had high predictivity in UA, UV, and MV but not in AF. E3 was most efficient in UV, while E3C was the best predictor in AF. Unconjugated progesterone reduced metabolites including pregnanolone isomers had low predictive value. On the other hand the conjugated pregnanolone isomers positively correlated with the GA at labor, although less pronouncedly than the conjugated 3βOH5S or estrogens. Again, better predictive values for the latter substances were in UA and UV with the exception of P3α5αC which showed the best correlation in MV. Rather poor dependence of free pregnanolone isomers on GA but good predictive values of their conjugates indicate escalating sulfation of the free pregnanolone isomers with increasing GA, which we proved in a previous study [43]. As discussed in the study, this finding may have consequences in the regulation of neurosteroid levels near term. The free 20α-hydroxy-steroids also had poor predictive values with the exception of Preg20α, being a predictor in UV, and P3α5β20α negatively correlating with GA in UA and UV. Conjugated 20α-hydroxy-steroids proved to have better predictive value. Preg20αC positively correlated with GA in all body fluids under investigation. Of the remaining steroids, A3β5αC was excellent predictor in MV. High predictive value was also found in most 7α/β- and 16α-hydroxy-metabolites of 3βOH5S, probably due to their stability in body fluids.

In conclusion, a novel method for multi-component analysis of the steroid metabolome in body fluids in late pregnancy was accomplished and validated. Additionally, we revealed optimum combinations of steroids for the construction of highly efficient theoretic models for prediction of the onset of labor. The results obtained from fetal blood were found to be better for the prediction of the term. However, maternal blood samples were also found suitable for determination and are therefore the best solution for further practice.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2009.10.012.

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