Measurement of amniotic fluid steroids of midgestation via LC–MS/MS

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1. Introduction

Due to their accessibility and ease of use, quantitative immunoassays played a major role in endocrine research in the last decades [1], helping to further uncover the complex relations in the field of steroid research. However, only single hormones could be measured from a sample at a time. Hence samples had to be split or chromatographically separated and various kits had to be used for the quantification of multiple hormones.

Moreover, it became evident that immunoassays are sometimes flawed by matrix effects and cross-reactivity [2–6], limiting both their sensitivity and specificity, respectively. These effects became strongly apparent when using ELISAs and RIAs for measuring steroids, which share common structural properties (e.g., glucocorticoids and estrogens) [4].

More recently, tandem mass spectrometry was recognized as a highly reliable and sensitive method for profiling of steroids. This technique avoids the issue of antibody cross-reactivity [7–9]. As an advantage over classic immunoassays LC–MS/MS allows for the analysis of multiple hormones and their derivatives in a single

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probe [10,11]. Additionally, LC–MS/MS provides high throughput analysis via process automation. Today, improved robustness and sensitivity of mass spectrometry techniques have led to the development of reliable methods even for peptide quantification, with new applications for hormone detection in tissue [12–16].

Wudy et al. [17] were the first to publish gas-chromatographic mass spectrometric (ID/GC–MS) reference data on the concentrations of steroids in amniotic fluid (AF) of midgestation. The composition of AF changes with gestational age due to the progress of fetal development [18]: during embryogenesis, AF composition closely resembles fetal plasma. With further development of fetal membranes and the placental barrier, free diffusion of AF occurs between the fetus and the AF across the skin from 10–20 weeks of gestation before the onset of skin keratinization thereafter. Hence AF analysis during this time might be of value for the assessment of fetal (patho-) physiology [18]. In this respect, ID/GC–MS via amniocentesis has been introduced as a reliable tool in the prenatal diagnosis of congenital adrenal hyperplasia (CAH) [19,20]. Besides prenatal identification and fetal treatment monitoring of CAH, ID/GC–MS data of Wudy et al. [17] revealed gender specific differences in the AF steroid profile, with higher levels of testosterone and androstenedione in the AF fluid of male fetuses.

Hill et al. [11] recently introduced GC–MS as novel method for multi-component analysis of the steroid metabolome in amniotic fluid (AF), umbilical vein blood and maternal blood. Taking advantage of the high grade of separation of the MS method, they were able to sufficiently establish models for the prediction of the onset of labor by analyzing the levels of 40 unconjugated steroids and 29 steroid polar conjugates in AF at the second stage of labor at term.

While sufficient data exist for GC–MS measurement of steroids in AF at various stages of gestation, no reference values for steroid concentrations in AF exist for LC–MS/MS in healthy subjects of midgestation. Furthermore, while LC–MS/MS analysis allows for steroid profiling of AF via detection of multiple steroids, it remains unknown, whether this approach yields higher accuracy and sensitivity in the prenatal determination of gender compared to the determination of testosterone in AF alone. Hence, we set out to characterize the LC–MS/MS technique in a healthy cohort providing reference data, including cross-validated logistic regression modelling to identify the reliability of prenatal gender verification via LC–MS/MS.

2. Materials and methods

2.1. Study design

In this study, a total of 172 pregnant women with a singleton pregnancy were enrolled, giving birth to 78 male and 94 female fetuses. Patient inclusion was from 09/14/04 until 01/23/08. For patient recruitment, the Department of Obstetrics at the University of Erlangen closely cooperated with an associated prenatal outpatient clinic in Nürnberg. AF measurements and statistical analysis were carried out at the Department of Pediatrics and the Department of Medical Informatics at the University of Erlangen. Amniotic fluid was gathered during genetic amniocentesis at 16 weeks of gestation (range: 14 + 3 − 18 + 2 weeks). Exclusion criteria were syndromal disorders, gestational diabetes, maternal smoking, spontaneous abortion/fetal demise, or a postnatal diagnosis of renal disease. Biometry was conducted prior to the procedure. Amniotic fluid volume and fetal anatomy were normal in all patients. Gestational age was determined by ultrasound measurements before the 14th week of gestation [21]. Estimation of fetal weight (EFW) was calculated as described by Hadlock et al. [22] using the equation: $EFW = 10\^(1.3596 - 0.0000386 \times A \times F + 0.00064 \times H + 0.0000061 \times B \times A + 0.00424 \times A + 0.0174 \times F)$, where A = AC = abdominal circumference (mm), B = BPD = biparietal diameter (mm), F = FL = femur length (mm) and H = HC = head circumference (mm). Auxologic data and clinical characteristics are shown in Table 1. The study was approved by the ethics committee of the University of Erlangen and conducted in accordance with the Declaration of Helsinki and principles of Good Clinical Practice. Written informed consent was obtained from each patient.

2.2. Analysis of amniotic fluid

One additional milliliter of amniotic fluid was drawn during amniocentesis. The AF steroid concentration of each sample was determined by a validated LC–MS/MS method. The summary of the analytical performance is shown in Supplementary Table 1. LC–MS/MS was performed using a modified online SPE-HPLC-MS/MS assay developed by Rauh et al. [23,24] allowing quantitative analysis of steroid hormones in 100 μL amniotic fluid with atmospheric pressure chemical ionization in the positive ion mode. Sample preparation was equivalent to serum samples. Briefly, internal standard solution (100 μL methanol/ammonium acetate solution 1:1, corticosterone-d8: 182 μg/L, cortisol-d4: 50 μg/L, DHEA-S-d5: 2 mg/L, 17-OH-progesterone-d8: 30 μg/L, progesterone-d9: 5 μg/L) was added to 100 μL AF samples, respectively, and was allowed to equilibrate for 15 min. The samples were then precipitated with 200 μL methanol/zinc sulfate (25 g/L, 1:1, v/v) solution. After vigorous vortex mixing, the samples were centrifuged at 18,000 × *g* for 10 min; 250 μL of the clear supernatant were transferred into microtiter plates (polypropylene, Greiner BIO-One, Frickenhausen, Germany) and placed in the autosampler equipped with a 96-well plate cooling stack set to 15 °C. The online SPE was performed by a Chromolith extraction column (RP-18e, 50 × 4.6 mm, Merck), which was coupled to a Chromolith HPLC column (RP-18e, 100 × 2.1 mm, Merck). The autosampler was a HTC PAL (CTC Analytics) fitted with a 250 μL sample loop. The HPLC-system consisted of a Shimadzu LC-20AD HPLC unit, a quaternary pump (HPLC1200 series, Agilent Technologies, Waldbronn, Germany) and a twelve port switching valve (VICI, Valco Instruments, Houston, USA). The injection volume was 200 μL. After 1 min the bound material was eluted from the extraction cartridge in backflush mode onto the analytical column at a flow rate of 1 mL/min. One minute later, the Valco valve position was switched again to allow the extraction column to be re-equilibrated. The total run time was 9 min.

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For scheduled MRM based mass spectrometric detection an API 4000 QTRAP® mass spectrometer (AB Sciex, Toronto, Canada) was

Table 1
Clinical characteristics of the analyzed cohort. AF = amniotic fluid.

	Male	Female	*p*-value
Gestational age at AF analysis in days			
mean ± SD	112.32 ± 5.87	111.59 ± 4.98	0.655
Median (min-max)	111.5 (101-128)	111 (101-123)	
Estimated fetal weight in grams (Hadlock)			
mean ± SD	152.06 ± 31.16	146.30 ± 22.37	0.312
Median (min-max)	145.32 (57.87-230.85)	141.76 (110.72-200.24)	
Birth weight in grams			
mean ± SD	3449.10 ± 537.90	3313.94 ± 469.90	0.046
Median (min-max)	3455 (2270-4800)	3280 (2400-5540)	
Gestational age at birth in days			
mean ± SD	276.68 ± 10.15	275.55 ± 10.16	0.573
Median (min-max)	278 (247-297)	278 (247-294)	

used. The following hormones were measured: androstenedione, corticosterone, cortisol, cortisone, deoxycorticosterone, 11-deoxycortisol, dehydroepiandrosterone (DHEA), dehydroepiandrosterone-sulfate (DHEA-S), 17-hydroxyprogesterone (17-OHP), progesterone and testosterone. Deuterated steroids were used as internal standards and added to each sample preparation mixture. Detailed analytical settings are displayed in Supplementary Table 1. Representative HPLC–MS/MS chromatograms of a calibrator and of a female and male sample are given in Supplementary Figs. 1 and 2, respectively. A standard curve of seven calibration standards was constructed on each day of analysis. The calibrators were prepared in methanol: Dulbecco's phosphate buffered saline (Gibco, Life Technologies, Darmstadt, Germany) (1:1). Calibration curves were calculated using linear least squares regression according to the equation $y = a + bx$, where y is the peak–area ratio of substance to internal standard and x the analyte concentration of the calibrator sample. $1/x$ weighting was used to ensure maximum accuracy at the lower concentrations.

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2.3. Validation of the LC–MS/MS method

The linearity of the method was evaluated across the calibration range (see Supplementary Table 2). The assay acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except at the lower limit of quantification (LLOQ) which was set at 20%. The calibration curve was required to demonstrate a correlation coefficient of 0.990 or higher. The lower limit of quantification was defined as the lowest concentration with a coefficient of variation of $\leq$20%. Three levels (with exception of deoxycorticosterone) of commercial quality control (QC) samples (Chromsystems, Gräfelfing/Munich, Germany) were measured to evaluate within-run and between-run accuracy and precision. QC samples of each level were measured six times on three different days. Relative extraction recovery was evaluated at five concentrations over the whole concentration range in five different AF specimens. The relative recoveries were calculated by linear regression. The set-up for this validation experiment was spiking of the target analytes at five different concentrations and internal standards into AF samples and phosphate buffered saline/MeOH, as reference [25,26].

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To evaluate the effect of the sample matrix on MS response we compared the instrument response for isotope labeled steroids in aqueous calibrator solutions and five different specimens which had been spiked to give a nominal concentration of 100%, 33% and 10% of the below mentioned concentration: androstenedione C13-3 (12.5 μg/L), cortisol-d4 (62.5 μg/L), cortison-d7 (50.0 μg/L), corticosterone-d8 (125 μg/L), DHEA-d5 (125 μg/L), DHEAS-d5 (750 μg/L), 11-deoxycortisol-d5 (12.5 μg/L), 17-OH-progesterone-d8 (37.5 μg/L), progesterone-d9 (18.8 μg/L) and testosterone C13-3 (8.75 μg/L). The effect of the matrix was assessed by calculating the process efficiency (PE), see Supplementary Table 2. The process efficiency is a quantitative assessment of ion suppression or enhancement and the recovery of the extraction process and was calculated as a ratio of the peak area of spiked AF to the peak area of an aqueous calibrator solution of the equivalent concentration in 50% methanol, conducted seven fold. The peak area ratio in the presence and absence of matrix ions may indicate ion suppression or ion enhancement (PE (%) = matrix effect * recovery of the extraction process /100) [25,26]. Blank samples were analyzed before and after the standard curve to verify and ensure sufficient cleaning between the injections. Furthermore, storage stability of AF samples ($n = 5$) in the autosampler at 14 °C was tested.

2.4. Statistical analysis

Results were expressed as mean ± standard deviation (SD), median, minimum and maximum. The level of significance for statistical hypothesis tests was set to alpha = 0.05. Hormone levels in subgroups (e.g., male and female fetuses) were compared using Mann Whitney *U* tests. To evaluate the accuracy of hormone levels in predicting gender, logistic regression modelling was used. Specifically, we considered two logistic regression models: The first model (termed "testosterone model" in the following) used gender (male/female) as outcome variable and testosterone as the only predictor. The second model (termed "combined model") used gender as outcome variable and several hormones (androstenedione, cortisol, corticosterone, cortisone, deoxycorticosterone, 11-deoxycortisol, DHEA, DHEA-S, 17-OHP, progesterone and testosterone) as predictor variables. To obtain valid estimates of prediction accuracy, cross-validation was applied. For cross-validation, 100 samples of size $n = 113$ (thus containing approximately two thirds of the observations) were drawn randomly without replacement from the data. These samples were used as learning samples to fit the logistic regression models described above. Prediction accuracy of the models was evaluated on the respective 100 sets of observations that were not included in the learning samples. For each logistic regression model (testosterone model, combined model), this procedure yielded 100 estimates of classification accuracy (computed as the percentage of newborns with correctly predicted gender), sensitivity (computed as the percentage of male newborns that were correctly predicted as males) and specificity (computed as the percentage of female newborns that were correctly predicted as females). Generally, a child was classified as "male" if its predicted probability of being male (obtained from logistic regression) was larger than 0.5. Statistical analyses were performed using the R Software for Statistical Computing (version 3.1.0) [27].

3. Results

3.1. Patients

The characteristics of the study population are displayed in Table 1. There was no difference in estimated fetal weight obtained via the Hadlock formula between genders. While no significant difference in the duration of pregnancy was found, female newborns were of significantly lighter body weight at birth. However, when referring to the corresponding birth weight percentiles [28], there were no differences between male and female neonates (data not shown). Gestational age at sampling did not significantly differ between groups. We did not find significant correlations of auxologic data with steroid levels in amniotic fluid (data not shown).

3.2. Steroids in amniotic fluid of midgestation

The summary of the analytical performance is shown in Supplementary Table 2. Acceptable accuracy and precision results were achieved for all analytes except for deoxycorticosterone with slightly higher coefficients for the QC samples. There was no evidence of ion suppression and matrix interference in AF. Steroids were stable for at least 40 h inside the autosampler at 14 °C (data not shown). Levels of steroids in AF are displayed in Table 2. The levels of androstenedione ($p < 0.001$) and testosterone ($p < 0.001$) were significantly higher in the AF of male fetuses. The AF levels of cortisol ($p = 0.057$), cortisone, corticosterone ($p = 0.055$), deoxycorticosterone, 11-deoxycortisol, 17-hydroxyprogesterone, DHEA and DHEA-S did not reveal significant sex specific differences. Progesterone AF levels showed a high overlap in their range in both

Table 2
Steroids in amniotic fluid of midgestation.

	Male (μg/L)	Female (μg/L)	*p*-value
Androstenedione			
mean ± SD	0.89 ± 0.49	0.39 ± 0.26	<0.001
Median (min-max)	0.78 (0.09-3.11)	0.33 (0.09-1.65)	
Corticosterone			
mean ± SD	0.62 ± 0.4	0.53 ± 0.42	=0.055
Median (min-max)	0.56 (0.05-1.72)	0.38 (0.01-2.37)	
Cortisol			
mean ± SD	7.34 ± 3.01	6.38 ± 2.73	=0.057
Median (min-max)	6.75 (0.50-16.95)	6.32 (0.67-13.60)	
Cortisone			
mean ± SD	18.43 ± 6.35	17.14 ± 6.52	=0.206
Median (min-max)	18.3 (1.7-45.4)	16.6 (3.3-34.6)	
Deoxycorticosterone			
mean ± SD	0.16 ± 0.07	0.14 ± 0.06	=0.112
Median (min-max)	0.15 (0.03-0.34)	0.14 (0.01-0.3)	
11-Deoxycortisol			
mean ± SD	0.52 ± 0.38	0.61 ± 0.46	=0.354
Median (min-max)	0.42 (0.04-1.71)	0.53 (0.03-2.34)	
DHEA			
mean ± SD	0.64 ± 0.48	0.56 ± 0.36	=0.47
Median (min-max)	0.54 (0.01-2.39)	0.53 (0.04-2.11)	
DHEA-S			
mean ± SD	9.73 ± 8.61	8.67 ± 6.45	=0.344
Median (min-max)	7.68 (0.38-65.1)	7.17 (0.38-43.3)	
17-OHP			
mean ± SD	1.81 ± 0.73	1.73 ± 0.87	=0.248
Median (min-max)	1.73 (0.67-3.64)	1.59 (0.06-4.32)	
Progesterone			
mean ± SD	98.24 ± 63.76	81.19 ± 52.86	=0.061
Median (min-max)	85.75 (4.27-376.0)	66.85 (8.85-341.0)	
Testosterone			
mean ± SD	0.30 ± 0.15	0.02 ± 0.02	<0.001
Median (min-max)	0.28 (0.01-0.77)	0.01 (0-0.07)	

groups, with no significant gender difference (range males 4.27–376.0 μg/L; range females 8.85–341.0 μg/L; $p = 0.061$).

3.3. Cross-validated logistic regression modelling

The results of the statistical analysis are displayed in Table 3. In case of the testosterone model, all performance measures were close to 1 and thus very high (mean cross-validated classification accuracy = 0.9805, mean cross-validated sensitivity = 0.9637, mean cross-validated specificity = 0.9950). This result shows that testosterone can be used to obtain highly accurate predictions of gender both in the female and in the male groups of fetuses. In case of the combined model (where other hormones were added to the set of predictors, in addition to testosterone), performance measures were still high but decreased slightly when compared to the testosterone model (mean cross-validated classification accuracy = 0.9488, mean cross-validated sensitivity = 0.9555, mean cross-validated specificity = 0.9761). Hence, including additional hormones in the model did not prove beneficial with regard to the prediction of gender. The high predictive power of testosterone for the prediction of gender is illustrated in Fig. 1, where testosterone levels of the complete data set are plotted against gestational age. The “optimal” cut-off value for testosterone (defined as the testosterone level that resulted in a predicted probability of 0.5 when logistic regression was applied to the complete data) was 0.074 μg/L (Fig. 1).

4. Discussion

In our study we characterized LC–MS/MS as a valuable tool for AF steroid analysis at midgestation. The analysis of the steroid profile in AF of healthy fetuses provided LC–MS/MS reference data. Moreover, cross-validated logistic regression modeling allowed us to analyze the reliability of LC–MS/MS steroid profiling of AF for

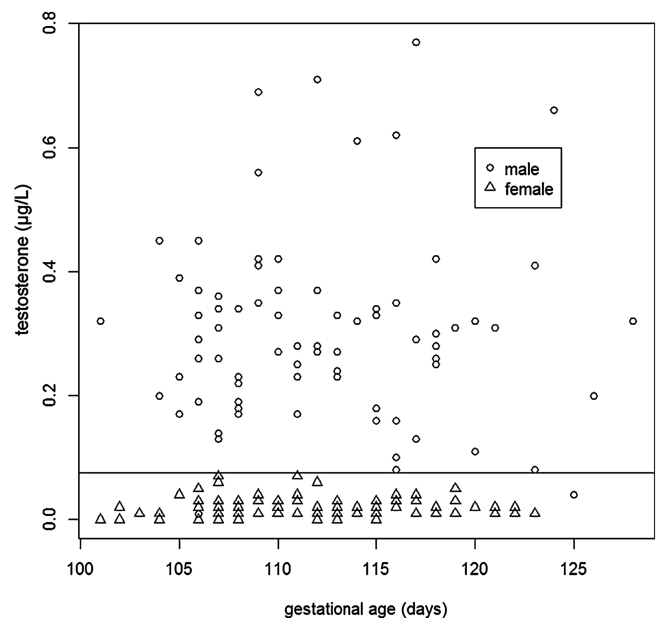

Fig. 1. Testosterone levels of the complete data set plotted against gestational age. Horizontal line represents the testosterone cut-off level that resulted in a predicted probability of 0.5 when logistic regression was applied: 0.074 μg/L. Circles: males, triangles: females.

Table 3
Cross-validated logistic regression modeling.

		Minimum	1st Quartile	Median	Mean	3rd Quartile	Maximum	*p*-value
Accuracy of prediction								
	Combined model	0.8644	0.9322	0.9492	0.9488	0.9661	1.000	
	Testosterone model	0.9322	0.9661	0.9831	0.9805	1.000	1.000	<0.001
Sensitivity								
Male	Combined model	0.8571	0.9310	0.9600	0.9555	0.9658	1.000	
	Testosterone model	0.8261	0.9370	0.9643	0.9637	1.000	1.000	0.009
Specificity								
Female	Combined model	0.8276	0.9580	0.9718	0.9761	1.000	1.000	
	Testosterone model	0.9375	1.000	1.000	0.9950	1.000	1.000	<0.001

gender verification, identifying sole testosterone measurement as the most specific predictor of male gender at birth in healthy patients.

4.1. Analysis of steroids in amniotic fluid of midgestation

As detailed national GC–MS analysis data of midgestation AF pregnancies exist by Wudy et al. [17], we refer to these findings when discussing our LC–MS/MS data:

Alike the GC–MS analysis of AF at midgestation by Wudy et al. [17], we did not observe significant gender specific differences in the range of AF levels of 17-OHP (males, 0.67–3.64 μg/L; females, 0.06–4.32 μg/L; $p < 0.248$) and DHEA (males, 0.01–2.39 μg/L; females, 0.04–2.11 μg/L; $p < 0.47$) AF levels. The levels of these hormones were comparable of values gained via GC–MS [17] for both genders; however, GC–MS showed a wider range for 17-OHP (range 0.21–4.96 μg/L) [17,19,29–32] and a narrower range for DHEA (range 0.19–1.77 μg/L) in healthy fetuses. This finding is of clinical interest, as for the prenatal identification of congenital adrenal hyperplasia (CAH) via GC–MS by 17-OHP measurement in AF a cut-off value of 4.96 μg/L was established [17]. However, our maximum 17-OHP AF level was lower (4.32 μg/L) in our healthy subjects.

In contrast to our 17-OHP findings, studies by Warne et al. [33] and Forest et al. [34], who investigated AF between 9 and 19 weeks gestation by radioimmunoassay (RIA), showed fetal gender to significantly influence AF 17-OHP and DHEA levels: by RIA [34] female 17-OHP AF levels (mean 1.21 μg/L) significantly ($p < 0.05$) exceeded male levels (mean 0.99 μg/L), while by our LC–MS/MS measurements 17-OHP was detected at a higher level (males, mean 1.81 ± 0.73 μg/L; females, mean 1.73 ± 0.87 μg/L). Similarly, DHEA AF concentrations (males, mean 0.21 μg/L; females, mean 0.27 μg/L) determined by RIA [34] showed a significant gender difference ($p < 0.05$) and were also lower than the respective levels measured by LC–MS/MS (males, 0.64 ± 0.48 μg/L; females, 0.56 ± 0.36 μg/L; $p < 0.47$).

As previously described for GC–MS [17], the levels of androstenedione ($p < 0.001$) and testosterone ($p < 0.001$) in our study were significantly higher in the AF of male fetuses when measured by LC–MS/MS: For androstenedione medians and ranges were similar for both GC–MS (males 0.93 μg/L; females, 0.53 μg/L) [17] and LC–MS/MS (males, 0.78 μg/L; females, 0.33 μg/L) method. The androstenedione range showed a considerable overlap for GC–MS (males, 0.29–1.98 μg/L; females, 0.00–2.71 μg/L) [17] and LC–MS/MS (males, 0.09–3.11 μg/L; females, 0.09–1.65 μg/L). For testosterone our LC–MS/MS method showed a clear reduction of overlap in ranges (males, 0.01–0.77 μg/L; females 0–0.07 μg/L), when compared to GC–MS (males, 0.00–0.50 μg/L; females: 0.00–0.27 μg/L) [17]. In concordance with these findings, Forest et al. [34] found significantly increased androstenedione and testosterone concentrations via RIA analysis in AF of male (mean 0.66 μg/L and 0.23 μg/L, respectively) as compared to AF of female fetuses (mean 0.39 μg/L and 0.049 μg/L, respectively ($p < 0.001$ for both).

Similar to RIA analysis [34], the levels of the AF glucocorticoids cortisol (males, 7.34 ± 3.01 μg/L; females, 6.38 ± 2.73 μg/L) and cortisone (males, 18.43 ± 6.35 μg/L; females, 17.14 ± 6.52 μg/L) LC–MS/MS concentrations did not show a significant gender difference at midgestation. RIA AF concentrations of cortisol (males, mean 4.66 μg/L; females, mean 4.97 μg/L) and cortisone (males, mean 15.00 μg/L; females, mean 17.37 μg/L) were comparable to our measured concentrations.

In addition to Wudy et al. [17], we supply LC–MS/MS AF reference levels of the steroids DHEA-S, 11-deoxycortisol, corticosterone and deoxycorticosterone. AF levels of 11-deoxycortisol, corticosterone and deoxycorticosterone have been determined via RIA in 1993 by Dörr and Sippel [20] with similar results.

4.2. Analysis of prenatal gender prediction using cross-validation statistics

The improved predictive performance of the testosterone model compared to the combined model can be explained by the additional variance that is introduced by addition of the other hormones to the logistic regression model. As seen from Fig. 1, testosterone alone was sufficient to yield an almost perfect separation of female and male children in our data (horizontal line at testosterone level 0.074 μg/L in Fig. 1). Consequently, adding the other hormones to the logistic regression model could not further improve this very high prediction accuracy of testosterone but only introduced additional variance in the parameter estimates of the combined logistic regression model. This additional variance in turn resulted in a decrease in prediction accuracy and could presumably be reduced by a larger sample size.

4.3. Limitations

Our study only analyzed the AF steroid levels in healthy subjects. In fetuses with CAH caused by 21-hydroxylase deficiency the steroid profile is significantly altered:

17-OHP levels usually exceed the threshold of 4.96 μg/L and androstenedione levels peak to 2 μg/L and 2.5 μg/L in males and females, respectively, as measured by GC–MS [17]. Moreover, dexamethasone treatment, as used in prenatal CAH treatment [20], further strongly alters AF steroid levels. Therefore, the use of our LC–MS/MS AF steroid reference values remains restricted to healthy fetuses at early gestation only. Interestingly however, Wudy et al. [17] found no differences in steroid concentrations between the first and second halves of the midgestational period via GC–MS. We further emphasize that the aim of our study was to characterize LC–MS/MS as a method for the determination of steroids in AF and to analyze its predictive value for the determination of gender. Our study was not intended to establish LC–MS/MS as a clinical tool for routine gender determination. Prenatal ultrasound and DNA analysis of fetal cells in maternal blood clearly remain the methods of choice due to their low risk for the fetus.

4.4. Future aspects

While the focus of our study was the analysis of AF steroids, recent studies sufficiently used mass-spectrometric analysis to uncover the AF proteom [35] and to determine AF metabolomics [36]. With applications of the MS method continuously expanding, future fields of AF research are evolving.

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