

## Sex Differences in Reproductive Hormones During Mini-Puberty in Infants With Normal and Disordered Sex Development

Trine Holm Johannsen,<sup>1,2</sup> Katharina Maria Main,<sup>1,2</sup> Marie Lindhardt Ljubicic,<sup>1,2</sup> Tina Kold Jensen,<sup>1,2,3,4</sup> Helle Raun Andersen,<sup>3</sup> Marianne Skovsager Andersen,<sup>5</sup> Jørgen Holm Petersen,<sup>1,2,6</sup> Anna-Maria Andersson,<sup>1,2</sup> and Anders Juul<sup>1,2</sup>

<sup>1</sup>Department of Growth and Reproduction, Rigshospitalet, University Hospital of Copenhagen, DK-2100 Copenhagen, Denmark; <sup>2</sup>International Center for Research and Research Training in Endocrine Disruption of Male Reproduction and Child Health, Rigshospitalet, University of Copenhagen, DK-2100 Copenhagen, Denmark; <sup>3</sup>Department of Environmental Medicine, Institute of Public Health, University of Southern Denmark, DK-5000 Odense, Denmark; <sup>4</sup>Hans Christian Andersen Children's Hospital, Odense University Hospital, DK-5000 Odense, Denmark; <sup>5</sup>Department of Endocrinology, Odense University Hospital, DK-5000 Odense, Denmark; and <sup>6</sup>Department of Biostatistics, University of Copenhagen, DK-1353 Copenhagen, Denmark

**Context:** The early activation of the hypothalamic-pituitary-gonadal axis during infancy can be used in the evaluation of infants suspected of disorders of sex development (DSD). However, few data exist on sex-specific reference ranges for these hormones during early life.

**Objective:** To evaluate sex differences in reproductive hormone concentrations in serum from healthy infants to define sex-specific cutoff values and to apply these in infants with DSD.

**Design:** A cross-sectional study.

**Setting:** A tertiary center for pediatric endocrinology at the University Hospital of Copenhagen.

**Patients or Other Participants:** Healthy infants (1840) and patients with DSD (27), aged 2 to 5 months.

**Main Outcome Measures:** Serum concentrations of LH, FSH, testosterone (T), estradiol, sex hormone-binding globulin (SHBG), inhibin B, anti-Müllerian hormone (AMH), dehydroepiandrosterone (DHEA), DHEA sulfate (DHEAS), 17-hydroxyprogesterone (17-OHP), androstenedione, and LH/FSH ratio.

**Results:** LH and FSH concentrations showed overlap between sexes, with LH being highest in boys and FSH being highest in girls. The LH/FSH ratio separated infant boys from girls with minimal overlap at a cutoff value of 0.32. Inhibin B and AMH concentrations were markedly higher in boys compared with girls, with minimal or no overlap. In infants with Klinefelter syndrome, 45,X/46,XY mosaicism and male phenotype, and Turner syndrome, the LH/FSH ratio matched the gender of rearing. However, infants with complete androgen insensitivity syndrome had LH/FSH ratios within the male range.

**Conclusions:** Reference ranges for reproductive hormones and LH/FSH ratio during mini-puberty were established in this study. The classifiers that best separated sex in mini-puberty were AMH, LH/FSH ratio, and T. Use of the LH/FSH ratio may add valuable information in the workup of infants suspected of DSD. (*J Clin Endocrinol Metab* 103: 3028–3037, 2018)

A transient postnatal activation of the hypothalamic-pituitary-gonadal axis, also termed mini-puberty, occurs in healthy infants (1). This rise in gonadotropins, which peaks when the infant is between 1 week and 3 months of age (2), appears to show a marked sexual dimorphism with preponderance of LH in boys and of FSH in girls (2, 3). A subsequent rise in serum concentrations of the gonadal hormones is also seen in both sexes, with preponderance of testosterone (T), inhibin B, and anti-Müllerian hormone (AMH) in boys and estradiol in girls (3–9). Concentrations of LH, FSH, and T in boys and of LH and T in girls decrease to prepubertal levels in the following months (2, 3), whereas the elevation of FSH and estradiol is more prolonged in girls (2, 10).

As concentrations of gonadotropins and gonadal hormones are measurable during mini-puberty, this period represents an important diagnostic window in clinical workup of infants suspected of disorders of sex development (DSD). Newborns with DSD comprise different congenital conditions in which development of chromosomal, gonadal, or anatomical sex is atypical (11). Ambiguity of genital appearance is seen in the most severe cases and may be found in virilized 46,XX females or undervirilized 46,XY males.

The apparently sexual dimorphic pattern of gonadotropins and reproductive hormone levels during mini-puberty can help in the evaluation of a child with DSD. However, little evidence exists on the sex-specific reference ranges for these hormones during the first months of life. Thus, based on a large group of healthy infants, the aims of this study were to establish reference ranges for circulating concentrations of gonadotropins, gonadal, and adrenal hormones in mini-puberty and to establish sex-specific cutoff values to separate males from females.

## Materials and Methods

### Healthy infants

In total, 1840 children (1041 boys, 799 girls) were recruited from three different cohorts: cohort 1 (6, 12) is a prospective mother-child cohort of healthy women of Danish origin recruited in the first trimester of pregnancy. Women were recruited from three university hospitals in Copenhagen from 1997 to 2002. From this study, 1278 children (733 boys, 545 girls) were included. Cohort 2 (13) is a prospective cohort of children of pregnant women used in greenhouses in Funen, Denmark. Women were recruited at the Department of Occupational and Environmental Medicine at Odense University Hospital from 1996 to 2001. From this study, 117 children (64 boys, 53 girls) were included. Cohort 3 (14) is a prospective mother-child cohort. Women were recruited from the Municipality of Odense, Denmark, from 2010 to 2012. From this study, 445 children (244 boys, 201 girls) were included. Data from the three cohorts were pooled.

Only children born at term were included, *i.e.*, born between 37 weeks + 0 days (259 days) and 41 weeks + 6 days (293 days). Each infant contributed with one blood sample. The mean age at blood sampling was 3.3 months [2.5 to 97.5 percentiles (p2.5 to 97.5): 2.5 to 4.7 months] in boys, ranging from 2.1 to 5.0 months, and 3.3 months (p2.5 to 97.5: 2.5 to 4.6) in girls, ranging from 2.3 to 5.0 months. No statistically significant difference in age at blood sampling was observed between boys and girls ( $P = 0.64$ ). The hormone data, except the LH/FSH ratio, on healthy infants have been presented separately in previous studies (6–8, 12, 15), but the data have not been presented in a combined form previously. Not all analytes were measured in all healthy infants.

### Infants with disorders of sex development

Twenty-seven patients with DSD at the age of two to five months were included. Data from 13 infant boys with nonmosaic Klinefelter syndrome (47,XXY karyotype) were included. Some of these data [LH, FSH, T (RIA), sex hormone-binding globulin (SHBG), inhibin B] but not all (LH/FSH ratio, estradiol, AMH) have previously been reported (16). Data from eight patients with 45,X/46,XY mosaicism and male phenotype were included. Some of these data (LH, FSH, T, estradiol, inhibin B) but not all (LH/FSH ratio, SHBG, AMH) have been presented before (17). Data from four infant girls with Turner syndrome (miscellaneous karyotypes) were included. Some of these hormone data (LH, FSH, estradiol, AMH, inhibin B) but not all (LH/FSH ratio, T, SHBG) have previously been reported (8, 18). Data from two patients with complete androgen insensitivity syndrome (CAIS) have not been presented before. Not all analytes were measured in all patients.

### Hormone analyses

For the healthy infants, nonfasting blood samples were drawn from an antecubital vein from 8:00 AM to 6:00 PM, clotted, and centrifuged, and serum was stored at  $-20^{\circ}\text{C}$  for a maximum of 5 years before analysis. All hormone analyses were performed at the Department of Growth and Reproduction, Rigshospitalet, Copenhagen, Denmark.

Concentrations of LH ( $n = 1289$ ), FSH ( $n = 1289$ ), and SHBG ( $n = 1278$ ) were analyzed by time-resolved fluoroimmunoassays (AutoDELFIA; PerkinElmer, Turku, Finland). RIAs were used to determine concentrations of T (Coat-A-Count; Diagnostic Products, Los Angeles, CA;  $n = 848$ ) and estradiol (Direct  $^{125}\text{I}$  Estradiol; Pantex, Santa Monica, CA; previously named Immunodiagnostic Systems, Bolton, UK) ( $n = 1,299$ ). Double antibody enzyme-immunometric assays were used to determine Inhibin B (Oxford Bio-Innovation, Oxfordshire, UK; later named Serotec, Oxford, UK) (19) ( $n = 1,258$ ), and AMH (Immunotech, Beckman Coulter, Marseilles, France) ( $n = 466$ ). Together with dehydroepiandrosterone (DHEA;  $n = 715$ ), DHEA sulfate (DHEAS;  $n = 716$ ), androstenedione ( $n = 716$ ), and 17-hydroxyprogesterone (17-OHP;  $n = 716$ ), serum concentrations of T ( $n = 716$ ) additionally were measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS), as previously described (20).

The limits of detection (LODs) were the following: LH = 0.05 IU/L, FSH = 0.05 IU/L, T (RIA) = 0.23 nM, estradiol = 18.1 pM, SHBG = 0.23 nM, inhibin B = 20 pg/mL, AMH = 2 pM, T (LC-MS/MS) = 0.10 nM, DHEA = 0.88 nM, DHEAS = 48 nM, androstenedione = 0.18 nM, and 17-OHP = 0.19 nM.

Values below the LOD were replaced with the hormone-specific LOD/2. The percentages of samples with concentrations below LOD are shown in Figs. 1–3.

The interassay coefficients of variations were <6% for LH, FSH, and SHBG, <10% for T (RIA), <13% for estradiol, <18% for inhibin B, <12% for AMH, 9% for T (LC-MS/MS), 11% for DHEA, 5% for DHEAS, 8% for androstenedione, and 9% for 17-OHP.

## Statistical analyses

Concentrations of all hormones were plotted as functions of age in months. Individual LH/FSH ratios ( $n = 1284$ ) were calculated as the serum concentrations of LH divided by the serum concentrations of FSH. Free T (FT; LC-MS/MS) was calculated according to Vermeulen *et al.* (21) ( $n = 267$ ).

Based on visual inspection, some of the hormone concentrations seemed to change within the age range. Thus, age was subdivided into two groups: aged 2.0 to <3.5 months and 3.5 to 5.0 months. Age, hormone concentrations, and ratios were logarithm (log) transformed and reported as back-transformed geometric means and p2.5 to 97.5. Statistical differences in age and hormone concentrations within and between sexes were analyzed using independent sample *t* test.

The decrease in serum concentration of reproductive hormones and the LH/FSH ratio as a function of age were calculated using a general linear model, with reproductive hormones and the LH/FSH ratio as dependent variables (log transformed) and age as an independent variable (not transformed).

The correlation of serum-T measurements by RIA and LC-MS/MS was calculated using bivariate Spearman correlation on untransformed data.

Based on receiver operating characteristic (ROC) curves, the appropriateness of hormone concentrations and ratios as classifiers for separation of sexes during mini-puberty were evaluated. The ROCs were used to determine the areas under the curves and their corresponding 95% confidence intervals and to establish the cutoff values that resulted in the highest sensitivity (true positive rate) and specificity (true negative rate). The cutoff values were assessed with equal weightings of sensitivity and specificity. Accuracy of the tests' performance was defined as the

number of correctly classified children, according to cutoff values relative to the total number of children.

$P < 0.05$  was considered statistically significant. The statistical analyses were performed using IBM Statistics SPSS, version 22 (IBM, Armonk, NY).

## Ethical considerations

For all healthy participants, a written, informed consent form was signed by the parents before study participation. All cohorts were approved by the regional Ethics Committee (cohort 1: no. [KF] 01-030/97; cohort 2: no. S-20070068; and cohort 3: no. S-20090130) and the Danish Data Protection Agency (cohort 1: no. 2003-41-2996; cohort 2: no. 2014-41-2748; and cohort 3: nos. 13/14088 and 15/15326). Access to patient data was approved by the Danish Patient Safety Authority (no. 3-3013-1376/1) and the Danish Data Protection Agency (no. 2012-58-0004, I-Suite no. 04204).

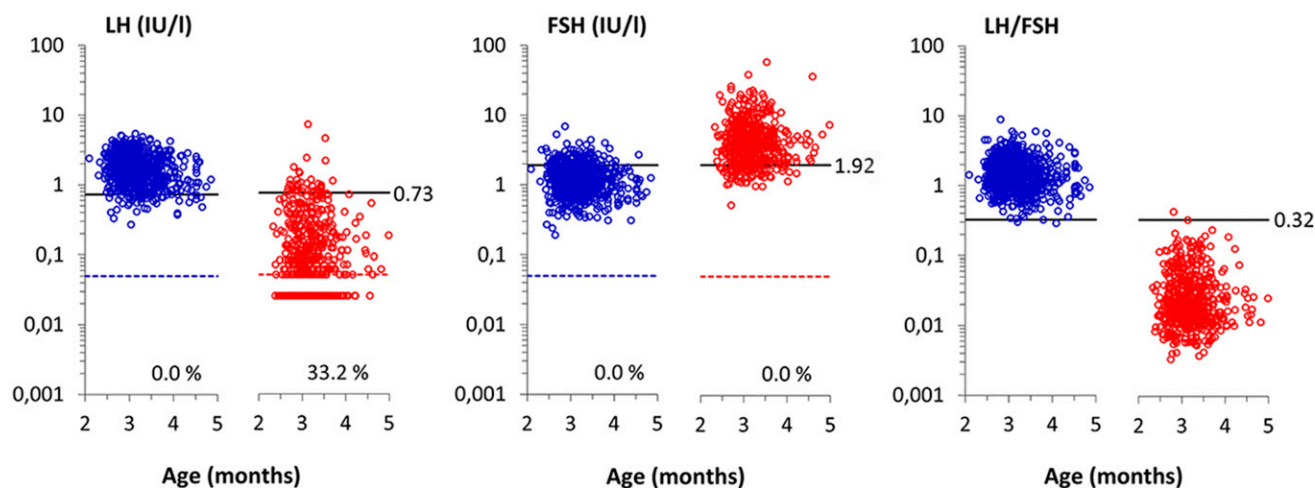
## Results

### Sex differences in hormone concentrations and LH/FSH ratios

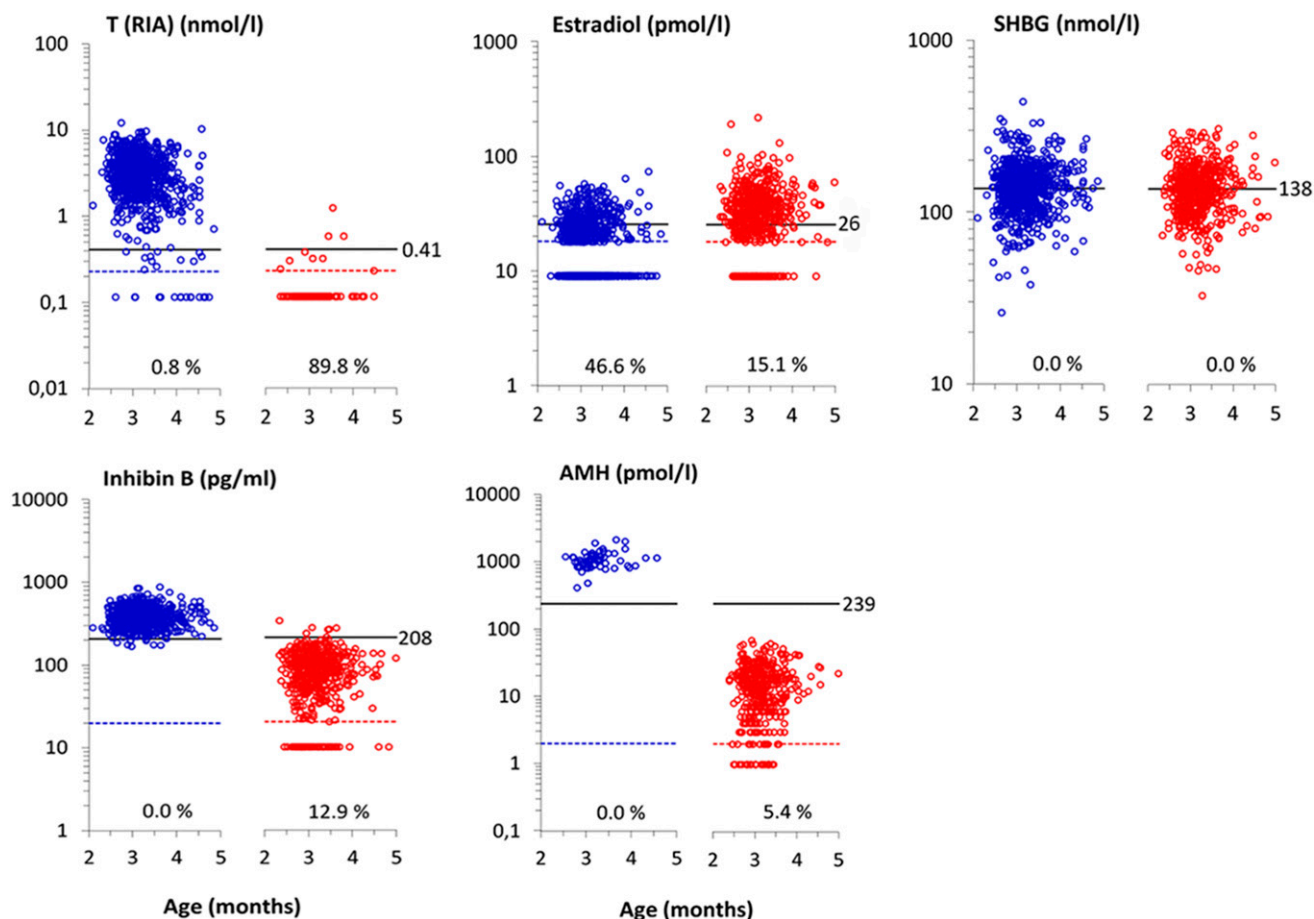
Serum concentrations of hormones and the LH/FSH ratio as functions of age are shown graphically in Figs. 1–3. Boys had significantly higher serum concentrations of LH, total T (RIA and LC/MS-MS), inhibin B, AMH, 17-OHP, and calculated FT than girls (all  $P < 0.001$ ). Girls had significantly higher serum concentrations of FSH, estradiol, and DHEA than boys (all  $P < 0.001$ ). The LH/FSH ratio was significantly higher in boys [1.40 (p2.5 to 97.5: 0.50 to 3.87)] than in girls [0.02 (0.01 to 0.14);  $P < 0.001$ ].

### Age-group differences in hormone concentrations and LH/FSH ratios

Data on concentrations of the hormones and LH/FSH ratio by age groups are shown in Table 1. The boys



**Figure 1.** Serum concentrations of LH, FSH, and the LH/FSH ratio in boys (blue) and girls (red) during mini-puberty. The concentrations are shown on a log with base 10 (log<sub>10</sub>)-transformed y-axis (dotted lines, LOD; solid lines, cutoff value for separating boys from girls; %, percentage of measurements below LOD).



**Figure 2.** Serum concentrations of T (RIA), estradiol, SHBG, inhibin B, and AMH in boys (blue) and girls (red) during mini-puberty. The concentrations are shown on a log10-transformed y-axis [dotted lines, LOD (SHBG, not shown); solid lines, cutoff value for separating boys from girls; %, percentage of measurements below LOD].

with an age below 3.5 months had significantly higher concentrations of LH, total T (RIA and LC-MS/MS), DHEA, DHEAS, androstenedione, and 17-OHP than the boys aged 3.5 months or above. These hormone concentrations decreased by 17% to 49%, highest for total T (LC-MS/MS) per month, from 2 to 5 months of age. The LH/FSH ratio was also significantly higher in boys below 3.5 months of age compared with the older boys, with a monthly decrease of 11%. Conversely, significantly higher concentrations of SHBG were observed in the older boys as compared with the boys below 3.5 months of age, with a monthly increase of 5%.

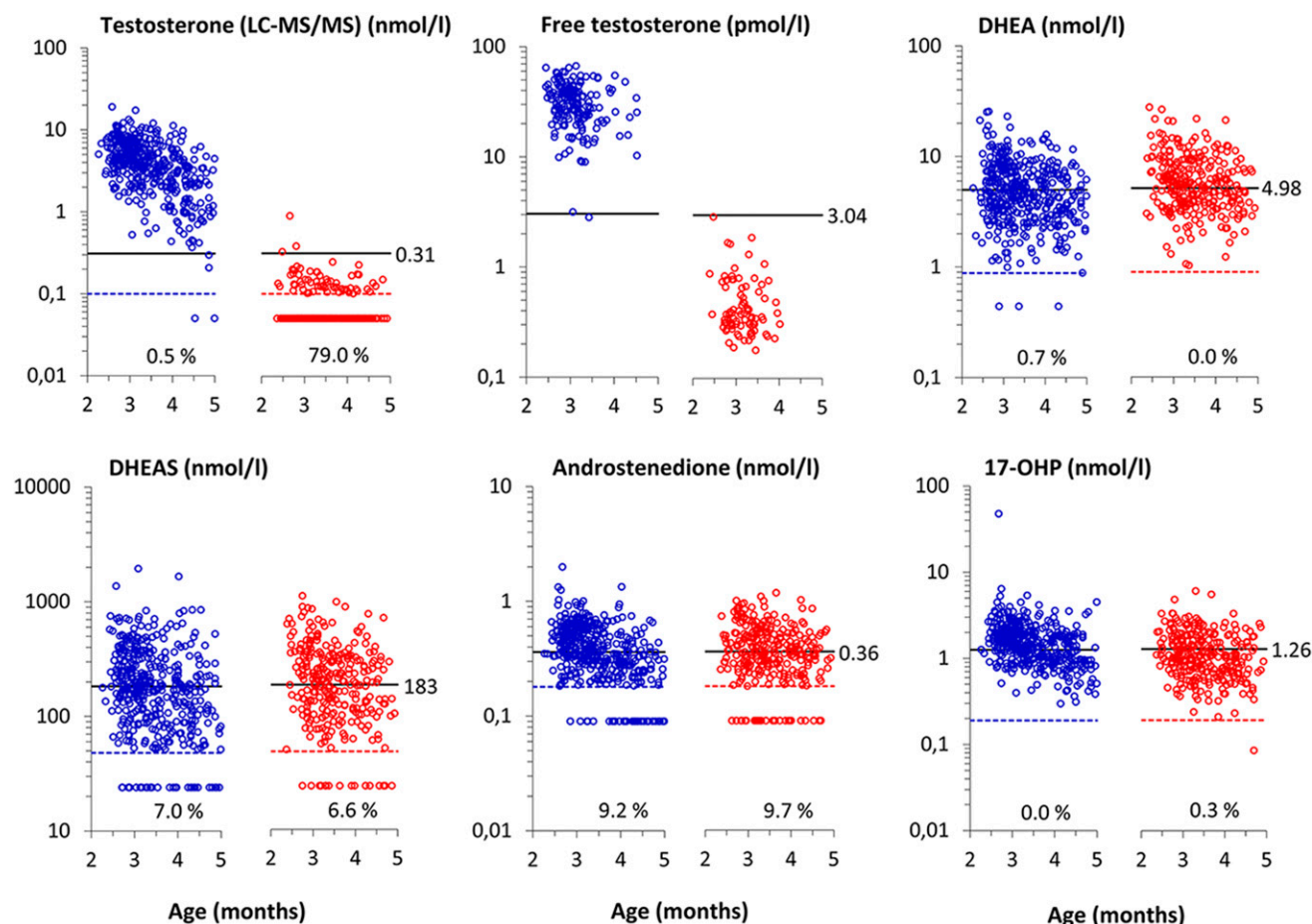
The girls with an age below 3.5 months had significantly higher concentrations of DHEAS, androstenedione, and 17-OHP than the girls aged 3.5 months or above, corresponding to decreases of 12% to 26% per month, highest for DHEAS. Conversely, significantly higher concentrations of AMH were observed in the older girls compared with the girls below 3.5 months of age, with a monthly increase of 31%. The LH/FSH ratio did not differ between age groups in girls.

### Discrimination between sexes

Results obtained from the ROC analyses are shown in Table 2. The hormones with the highest performance in discriminating between sexes were AMH (accuracy = 100%), the LH/FSH ratio (accuracy = 99.8%), and T measured by LC-MS/MS technique (calculated FT: accuracy = 99.6% and total T: accuracy = 99.0%).

### Hormone levels in infants with DSD

All LH, FSH, T, and AMH values from the infants with Klinefelter syndrome were within male reference ranges, but one had an LH/FSH ratio and an inhibin B concentration below the sex-specific cutoff values (Fig. 4). All hormone concentrations and the LH/FSH ratio were within male reference range in the male patients with 45, X/46,XY mosaicism, but one had a T (RIA) concentration below the sex-specific cutoff value. The infant girls with Turner syndrome had undetectable levels of T and undetectable to low concentrations of estradiol, inhibin B, and AMH. Their LH and FSH concentrations were above the female reference range, but their LH/FSH ratios were within the female reference range. The LH/FSH ratio and



**Figure 3.** Serum concentrations of T (LC-MS/MS), calculated FT, DHEA, DHEAS, androstenedione, and 17-OHP in boys (blue) and girls (red) during mini-puberty. The concentrations are shown on a log10-transformed y-axis (dotted lines, LOD; solid lines, cutoff value for separating boys from girls; %, percentage of measurements below LOD).

inhibin concentrations were within the male reference range in the patients with CAIS.

### Correlation of T measurements in infant boys by RIA and LC-MS/MS methods

In total, 182 boys had T concentrations measured by both RIA and LC-MS/MS (Fig. 5). The two methods correlated significantly ( $r_s = 0.92$ ,  $P < 0.001$ ) with a median ratio of  $T(\text{RIA})/T(\text{LC-MS/MS}) = 0.67$  (interquartile range: 0.58 to 0.75).

## Discussion

This is a large study reporting reference values of pituitary and gonadal reproductive hormones in a cohort of healthy infants examined during mini-puberty by highly sensitive analytical methods. Furthermore, this study shows the accuracy and thus, validity of the separation of boys and girls, based on these measurements in a large group of healthy infants.

Boys had significantly higher concentrations of LH and lower concentrations of FSH than girls, which is in line

with previous studies (2, 3). A cutoff value for the LH/FSH ratio of 0.32 separated boys from girls with an accuracy of 99.8%, suggesting that the LH/FSH ratio is an excellent predictor for sex during mini-puberty. This supports a previous study of healthy, newborn infants (4).

In the current study, the appropriateness of gonadotropins and the LH/FSH ratio in the classification of patients with DSD was examined. Although the number of included patients in the current study was sparse, both gonadotropins (100% of patients correctly classified) and the LH/FSH ratio (24 of 25 patients; *i.e.*, 96% of patients correctly classified) seemed to be excellent predictors of sex in patients with sex chromosome DSD. The appropriateness of the ratio was additionally demonstrated in CAIS patients, as their LH/FSH ratio was within the male range. The applicability of a gonadotropin ratio of 0.32 in examining the presence of testicular tissue was investigated by re-evaluating previously published data by Bouvattier *et al.* (1), as they used similar LH and FSH assays. In their group of patients with androgen insensitivity syndrome, followed during the first 90 days of life, this cutoff value for the LH/FSH

**Table 1. Group Differences in Hormone Concentrations and LH/FSH Ratios in Healthy Infants Aged 2.0 to <3.5 Months and 3.5 to 5.0 Months**

	LOD	Age, Mo	Males, n	Females, n	Cutoff	Males, Gmean (p2.5-97.5)	Females, Gmean (p2.5-97.5)	P Value, Male vs Female	P Value, <3.5 vs ≥3.5 Mo	
									Male	Female
LH, IU/L	0.05	2.0–3.5	581	432	0.73	1.71 (0.62–4.08)	<LOD (<LOD–0.98)	<0.001	<0.001	0.46
		3.5–5.0	166	110		1.40 (0.54–3.32)	<LOD (<LOD–1.25)	<0.001		
FSH, IU/L	0.05	2.0–3.5	578	435	1.92	1.19 (0.41–3.02)	3.98 (1.23–17.4)	<0.001	0.13	0.87
		3.5–5.0	165	111		1.11 (0.42–2.68)	3.93 (1.30–17.7)	<0.001		
LH/FSH ratio	—	2.0–3.5	578	431	0.32	1.44 (0.54–3.88)	0.02 (0.01–0.14)	<0.001	0.005	0.41
		3.5–5.0	165	110		1.26 (0.44–3.91)	0.02 (0.01–0.19)	<0.001		
T (RIA), nM	0.23	2.0–3.5	592	74	0.41	3.04 (0.69–7.60)	<LOD (<LOD–0.40)	<0.001	<0.001	0.24
		3.5–5.0	168	14		1.97 (<LOD–7.01)	<LOD (<LOD–NA)	<0.001		
Estradiol, pM	18.1	2.0–3.5	571	455	26	<LOD (<LOD–47)	29 (<LOD–79)	<0.001	0.45	0.31
		3.5–5.0	160	113		<LOD (<LOD–49)	31 (<LOD–98)	<0.001		
SHBG, nM	0.23	2.0–3.5	579	427	138	135 (66–268)	133 (67–264)	0.51	0.04	0.09
		3.5–5.0	162	110		143 (71–262)	141 (72–282)	0.76		
Inhibin B, pg/mL	20	2.0–3.5	571	423	208	379 (229–631)	62 (<LOD–184)	<0.001	0.98	0.47
		3.5–5.0	158	106		379 (222–662)	67 (<LOD–174)	<0.001		
AMH, pM	2	2.0–3.5	48	339	239	1013 (425–1810)	11 (<LOD–49)	<0.001	0.10	0.007
		3.5–5.0	12	67		1183 (797–NA)	15 (2–46)	<0.001		
T (LC-MS/MS), nM	0.10	2.0–3.5	251	165	0.31	4.75 (1.35–11.3)	<LOD (<LOD–0.21)	<0.001	<0.001	0.57
		3.5–5.0	175	125		2.25 (0.32–9.65)	<LOD (<LOD–0.17)	<0.001		
FT (LC-MS/MS), pM	—	2.0–3.5	157	72	3.04	29.8 (8.96–64.3)	0.43 (0.19–2.04)	<0.001	0.38	0.96
		3.5–5.0	25	13		26.9 (10.2–NA)	0.43 (0.23–NA)	<0.001		
DHEA, nM	0.88	2.0–3.5	250	165	4.98	4.99 (1.17–18.0)	5.71 (1.51–20.2)	0.03	0.002	0.08
		3.5–5.0	175	125		4.12 (1.28–13.6)	5.07 (1.90–15.4)	0.002		
DHEAS, nM	48	2.0–3.5	251	165	183	191 (<LOD–723)	192 (<LOD–830)	0.99	<0.001	0.04
		3.5–5.0	175	125		134 (<LOD–817)	157 (<LOD–749)	0.12		
Androstenedione, nM	0.18	2.0–3.5	251	165	0.36	0.43 (0.18–0.98)	0.36 (<LOD–0.95)	0.003	<0.001	0.04
		3.5–5.0	175	125		0.26 (<LOD–0.75)	0.32 (<LOD–0.86)	0.005		
17-OHP, nM	0.19	2.0–3.5	251	165	1.26	1.70 (0.75–4.32)	1.16 (0.36–3.23)	<0.001	<0.001	0.001
		3.5–5.0	175	125		1.14 (0.40–3.51)	0.90 (0.24–2.52)	<0.001		

Abbreviations: Cutoff, the hormone concentration in serum or hormone ratio that best separates sexes; Gmean, geometric mean; NA, not available.

ratio correctly predicted the presence of testicular tissue in all 14 patients with measurable LH and FSH concentrations at day 90.

AMH, inhibin B, and T were found in significantly higher concentrations in boys than in girls, whereas concentrations of estradiol were highest in girls. However, a significant overlap between sexes was seen, which may be explained by the large coefficient of

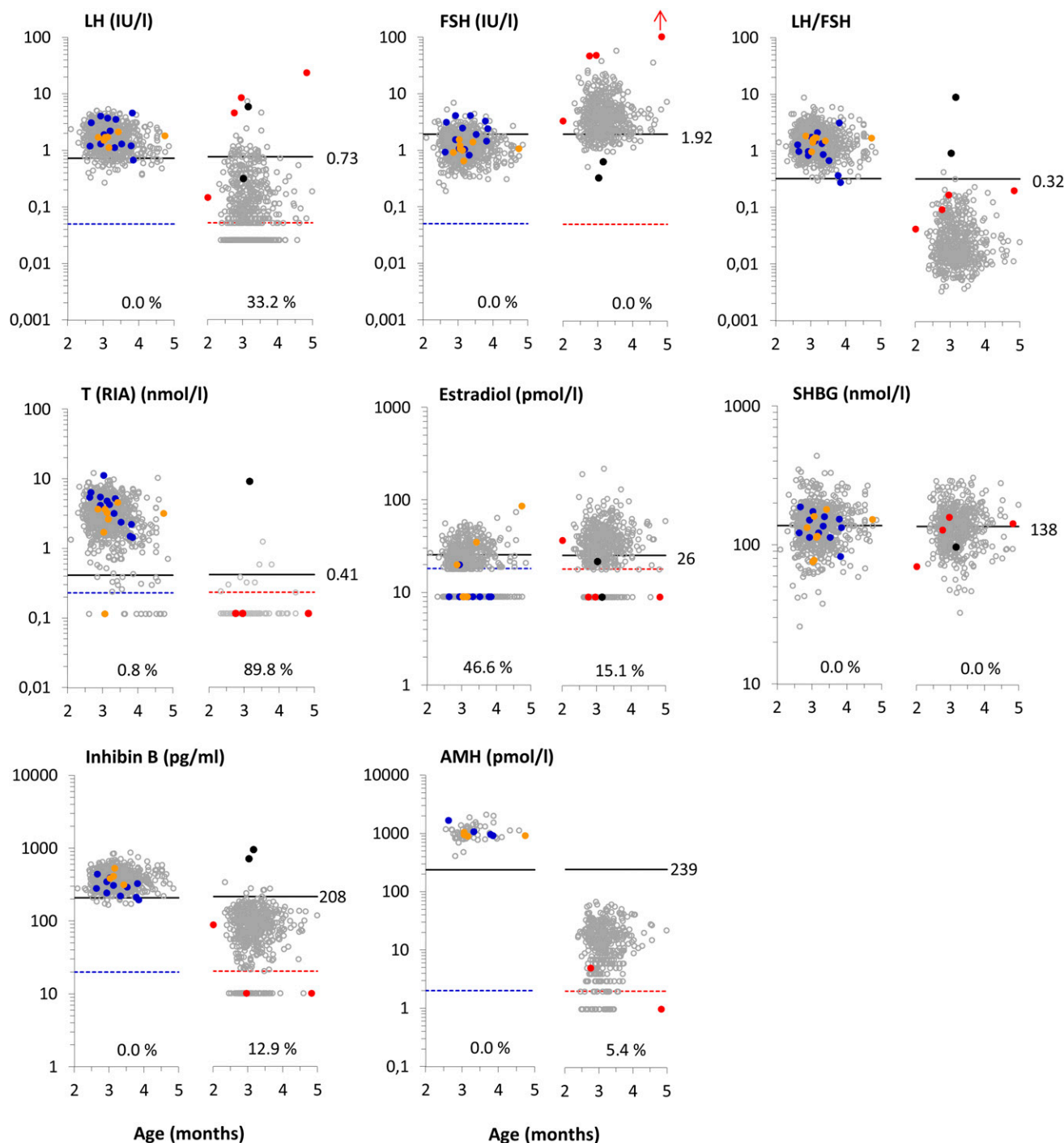
variation for the estradiol assay. The AMH and inhibin B concentrations are 100 times (7, 8) and 10 times, respectively, higher (3) in boys than in girls, and inhibin B concentrations during mini-puberty reach higher concentrations than those observed in adult men (3). AMH determination had the highest classifier performance in discriminating boys from girls, but also, inhibin B, T (both LC-MS/MS and RIA), and calculated FT performed well

**Table 2. Serum Hormone Concentrations and LH/FSH Ratios as Classifiers to Separate Sexes in Mini-Puberty**

	Cutoff Value	AUC (95% CI)	TPR, %	TNR, %	PPV, %	NPV, %	ACC, %
LH, IU/L	>0.73	0.98 (0.98–0.99)	93.7	93.9	95.5	91.5	93.8
FSH, IU/L	≤1.92	0.94 (0.92–0.95)	86.3	86.1	82.1	89.5	86.2
LH/FSH ratio	>0.32	1.00 (1.00–1.00)	99.7	99.8	99.9	99.6	99.8
T (RIA), nM	>0.41	0.99 (0.98–0.99)	96.6	96.6	99.6	76.6	96.6
Estradiol, pM	≤26	0.76 (0.73–0.79)	67.6	70.3	63.9	73.6	69.1
SHBG, nM	>138	0.51 (0.48–0.55)	52.1	50.8	59.4	43.5	51.6
Inhibin B, pg/mL	>208	1.00 (1.00–1.00)	98.6	98.7	99.0	98.1	98.6
AMH, pM	>239	1.00 (1.00–1.00)	100.0	100.0	100.0	100.0	100.0
T (LC-MS/MS), nM	>0.31	1.00 (0.99–1.00)	99.1	99.0	99.3	98.6	99.0
FT (LC-MS/MS), pM	>3.04	1.00 (1.00–1.00)	99.5	100.0	100.0	98.8	99.6
DHEA, nM	≤4.98	0.57 (0.53–0.61)	54.1	54.1	44.6	63.4	54.1
DHEAS, nM	≤183	0.52 (0.48–0.57)	52.1	52.1	42.5	61.5	52.1
Androstenedione, nM	>0.36	0.51 (0.47–0.56)	50.9	51.0	60.4	41.5	51.0
17-OHP, nM	>1.26	0.66 (0.62–0.70)	61.7	61.7	70.3	52.3	61.7

Abbreviations: “>” and “≤,” direction of male values; ACC, accuracy = (true positive + true negative)/total population; AUC, area under the curve; CI, confidence interval; Cutoff Value, the hormone concentration or hormone ratio that best separates sexes; NPV, negative predictive value = true negative/(true negative + false negative); PPV, positive predictive value = true positive/(true positive + false positive); TNR, true negative rate (specificity) = true negative/(true negative + false positive); TPR, true positive rate (sensitivity) = true positive/(true positive + false negative).



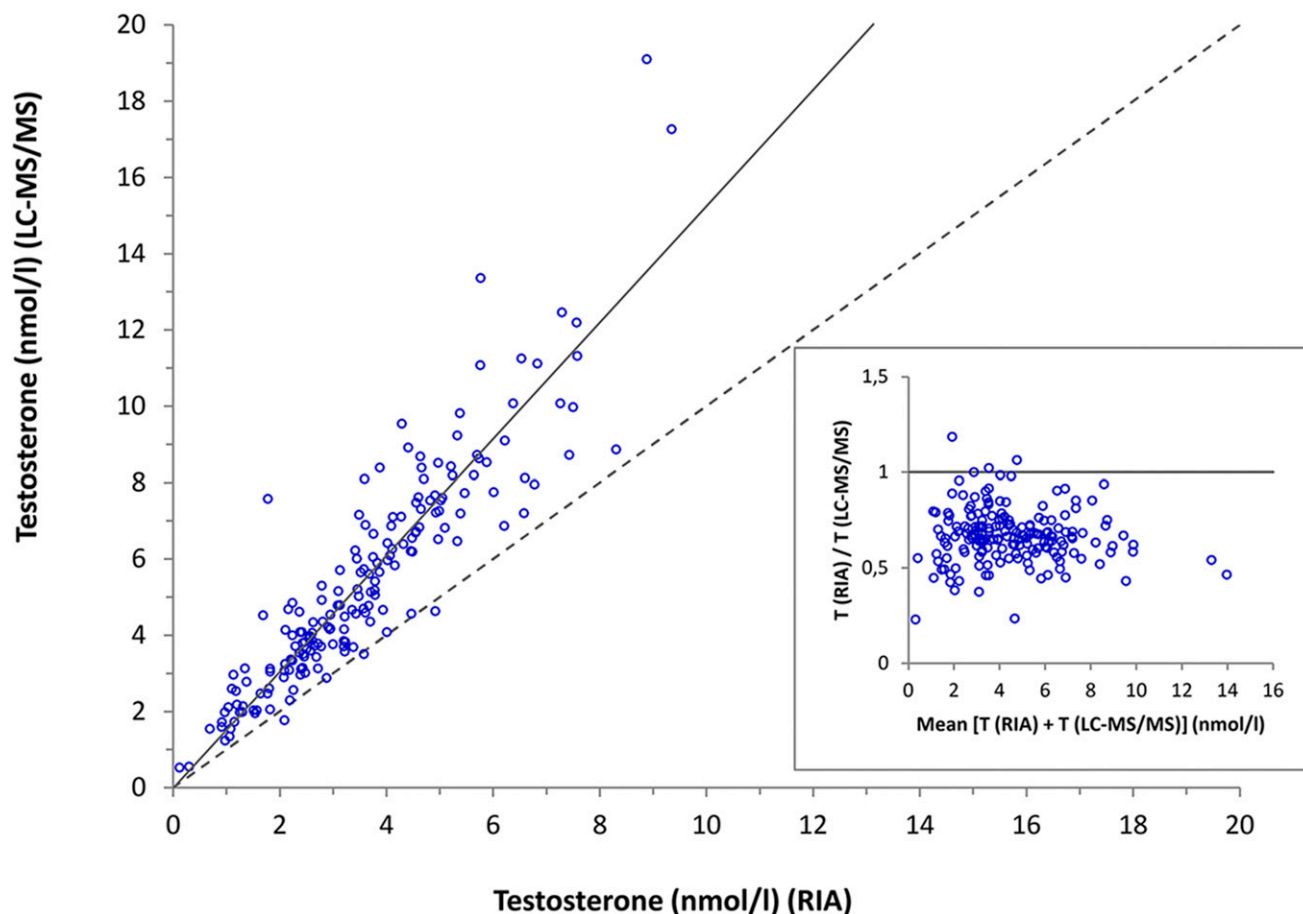


**Figure 4.** Serum concentrations of LH, FSH, the LH/FSH ratio, T (RIA), estradiol, SHBG, inhibin B, and AMH in 13 patients with Klinefelter syndrome (blue), 8 patients with 45,X/46,XY mosaicism and male phenotype (orange), 4 patients with Turner syndrome (red), and 2 patients with CAIS (black) during mini-puberty compared with sex-specific reference ranges. Not all analytes were measured in all patients. The concentrations are shown on a log10-transformed y-axis [dotted lines, LOD (SHBG not shown); solid lines, cutoff values; %, percentage of measurements below LOD].

as classifiers. The patients with sex chromosome disorders were all correctly classified by AMH, inhibin B, and T cutoff values bar two: one male infant with Klinefelter syndrome and an inhibin B concentration below the sex-specific cutoff and one male infant with 45,X/46,XY mosaicism and a T concentration below the sex-specific cutoff. Furthermore, the female patients with CAIS had

inhibin B concentrations within the male range. In patients with nonpalpable gonads or ambiguous genitalia, the use of these classifiers in mini-puberty thus represents important tools in the evaluation of gonadal function.

The clinical use of AMH measurements in patients with DSD has previously been reported. Thus, Lee *et al.* (22) reported a sensitivity of 92% and a specificity of



**Figure 5.** Correlation of T measurements by RIA and LC-MS/MS in 182 boys during mini-puberty (dotted line, identity line; solid line, tendency line). Inset: Bland-Altman plot of the ratio of concentrations of T measured by RIA and LC-MS/MS as a function of the mean T concentrations (nM). Solid line,  $T(\text{RIA})/T(\text{LC-MS/MS})$  ratio = 1.

98% for AMH in detecting the absence of testicular tissue in children with virilization at birth and nonpalpable gonads. Furthermore, when evaluating AMH as a marker for the presence of testicular tissue in phenotypic female patients, Misra *et al.* (23) reported a sensitivity of 92.9% and a specificity of 100%. Additionally, Hafez *et al.* (24) reported AMH and inhibin B measurements as predictors for the presence of functioning testicular tissue with an accuracy of 85% and 87%. Compared with these previous reports, higher sensitivity and specificity were observed in our large group of healthy infants. This may, at least in part, be explained by a more age-homogenous group in our study.

Sexual dimorphism of T concentrations in mini-puberty is previously reported (3, 5). During the first months of life, androgen production both has a gonadal and an adrenal origin (5, 25). In male infants, T production primarily takes place in the testes (25), whereas the weaker androgens have their origin in the fetal adrenal zone, which regresses within the first 3 months of life, thus decreasing the production of adrenal androgens in both sexes (25, 26). In line with this, a significant decrease in T was

observed for both males and females. The reported cutoff value of T was calculated for the full age range with the knowledge that in infants below 3.5 months, it would have been higher and in infants aged 3.5 months or above, it would have been lower. This has to be taken into account in the evaluation of a child with a suspected endocrine disorder. Furthermore, despite a close correlation between the T measurements when measured by RIA and LC-MS/MS, the absolute difference in T concentrations between these two methods was high, emphasizing the importance of taking the assay specifications into account for clinical use.

The strengths of this study include the following: the (1) large number of healthy infant boys and girls, (2) demonstration of the appropriateness of using the LH/FSH ratio in the diagnosis of endocrine disorders in infancy, and (3) use of highly sensitive analytic methods. The limitations of the study include the following: that (1) the study populations consisted of primarily Danish infants, so caution needs to be applied in other ethnicities, (2) the study population consisted of infants aged 2 to 5 months; thus, we do not know whether the observed



sex differences are similar right after birth when the clinical workup of patients with DSD may take place, (3) the tests' performances were restricted to the specific analytical methods used in this study, so cutoff values may need to be established for other assays used in individual laboratories, (4) the samples were stored at  $-20^{\circ}\text{C}$ , which may have caused issues with some of the hormones, and (5) the interassay coefficients of variations were high for estradiol, inhibin B, and AMH, which may reduce comparability across centers.

## Conclusion

In summary, based on highly sensitive methods, reference ranges of gonadotropins and reproductive hormones during mini-puberty were established. The classifiers that best separated sex in mini-puberty were AMH, the LH/FSH ratio, and T. Thus, the implementation of the LH/FSH ratio in the clinical evaluation may add new information in the first-line assessment of an infant suspected of DSD.

## Acknowledgments

We thank the families for participating and the technicians for their skilled work in analyzing the hormones.

**Financial Support:** Cohort 1 was funded by the European Commission through the Biomed 2 Program (BMH4-CT96-0314), Environmental Reproductive Health (QLK4-CT1999-01422), and EXPORED (QLK4-2001-00269); by the Danish Council for Independent Research (Grants 9700833 and 9700909); and by the Svend Andersens Foundation. Cohort 2 was funded by the Danish Environmental Research Program (96.01.015.16.05). Cohort 3 was funded by ReproUnion; EDMaRC; the Novo Nordisk Foundation (NNF15OC0017734 and NNF17OC0029404); the Danish Environmental Protection Agency (the Center on Endocrine Disrupting Chemicals); the Danish Foundation for Scientific Innovation and Technology (09-067180); the Danish Research Council (4004-00352B\_FSS); Ronald McDonald Children Foundation; K. A. Rohde and Wife's Foundation; Odense University Hospital and Region of Southern Denmark; Municipality of Odense; the Danish Council for Strategic Research through Program Commission on Health, Food and Welfare (2101-08-0058), Odense University Hospital Research Foundation, and Odense Patient data Exploratory Network (OPEN); and the Foundation for Research Collaboration between Rigshospitalet and Odense University Hospital.

**Correspondence and Reprint Requests:** Trine Holm Johannsen, MD, Department of Growth and Reproduction, GR5064 and EDMaRC, University Hospital of Copenhagen, Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen, Denmark. E-mail: [trine.holm.johannsen@regionh.dk](mailto:trine.holm.johannsen@regionh.dk).

**Disclosure Summary:** The authors have nothing to disclose.

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