Hormone references for ultrasound breast staging and endocrine profiling to detect female onset of puberty

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**Abbreviations:** CV, coefficient of variation; E₁, estrone; E₂, estradiol; FSH, follicle-stimulating hormone; LC-MS/MS, liquid chromatography tandem-mass spectrometry; LH, luteinizing hormone; PCA, principle component analysis; ROC, receiver operating characteristics curve; SHBG, sex hormone-binding globulin; US, ultrasound.
ABSTRACT

Context: Application of ultrasound (US) to evaluate attainment and morphology of glandular tissue provides a new rationale for evaluating onset and progression of female puberty, but currently no hormone references complement this method. Further, previous studies have not explored the predictive value of endocrine profiling to determine female puberty onset.

Objective: To integrate US breast staging with hypothalamic-pituitary-gonadal hormone references and test the predictive value of an endocrine profile to determine thelarche.

Design Setting and Participants: Cross-sectional sample of 601 healthy Norwegian girls, age 6 to 16 years.

Main outcome measures: Clinical and ultrasound breast evaluations were performed for all included girls. Blood samples were analyzed by immunoassay and ultrasensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) to quantify estradiol (E₂) and estrone (E₁) from the sub-picomolar range.

Results: References for E₂, E₁, LH, FSH and SHBG were constructed in relation to chronological age, Tanner and US breast stages. An endocrine profile index score derived from principle component analysis of these analytes was a better marker of puberty onset than age or any individual hormone, with ROC AUC 0.91 (P<0.001). Ultrasound detection of non-palpable glandular tissue in 14 out of 264 (5.3%) girls with clinically prepubertal presentation was associated with significantly higher median serum levels of E₂ (12.5 vs 4.9 pmol/L; P<0.05) and a distinct endocrine profile (arbitrary units; P<0.001).

Conclusions: We provide the first hormone references for use with US breast staging and demonstrate the application of endocrine profiling to improve detection of female puberty onset.

Keywords: female puberty, estrogen, pediatric endocrinology, references; hormones; principal component analysis
INTRODUCTION

A comprehensive metastudy recently established a statistically significant and still ongoing trend of earlier breast development (thelarche, i.e. puberty onset) in girls, corresponding to almost three months per decade since 1977 (1). This finding underlines the need to compile updated references for puberty development milestones and pertinent hormones used in the diagnosis and management of altered puberty onset in the current pediatric population.

Hypothalamic activation initiates female puberty by enhanced release of pituitary gonadotropins, mainly luteinizing hormone (LH) and follicle-stimulating hormone (FSH) to stimulate gonadal maturation (2). Abnormal circulating levels of FSH and LH in conjunction with gonadal hormones can support the diagnosis of pituitary malfunction, hypogonadism and various disorders of sexual development (DSDs) associated with altered puberty onset (3-5). The main female sex steroids estrone (E1) and estradiol (E2) are involved in development and maintenance of the female phenotype and gonadal function. In particular, E2 is an integral biomarker for assessing female pubertal timing, menstrual status and fertility.

Female puberty onset is traditionally defined by breast formation, i.e. attainment of palpable glandular breast tissue and areolar enlargement, corresponding to Tanner stage B2 (6). In contrast, ultrasound leverages short-wavelength echogenicity of different tissues to render visual representations of internal anatomic structures and morphology. Evaluation of breast glandular tissue by ultrasound is a new approach for assessing female puberty but its clinical utility remains unexplored and compatible hormone references have been lacking. Notably, formation of glandular tissue in early thelarche may be detectable by ultrasound but not palpable by hand (7). In the pediatric subspecialist setting, US breast staging may improve clinical investigations of altered puberty timing since this method allows for storage and objective retrospective assessments of digital images during longitudinal patient follow-
up. Moreover, the procedure is harmless (8,9); perceived by the patient as less invasive than palpation (our unpublished observations), provides better definition of breast maturation than Tanner stages (10) while being able to differentiate between glandular and adipose tissue in overweight girls where excess fat accretion may confound traditional breast staging by palpation and visual inspection (11,12). Age references for both breast staging by clinical Tanner and ultrasound (US) breast examinations are provided (Fig. 1) in line with our previously published population study (7).

In medicine and biology, principle component analysis (PCA) is a statistical approach to stratify patients or identify phenotype clusters by capturing the variance from several variables, or dimensions (13). Previous application of PCA in pediatric research include composite risk index scoring for metabolic syndrome (14) and identification of allergy phenotypes (15). Endocrine profiling by PCA was previously demonstrated to identify distinct subtypes of Cushing syndrome (16), improve the predictive value of newborn screening for congenital adrenal hyperplasia (17) and extract endocrine phenotypes with implications for puberty timing in a longitudinal study of female puberty (18). However, the utility of a reference endocrine profile as diagnostic marker of puberty onset remains unexplored.

From the female pediatric population sample in the cross-sectional Bergen Growth Study 2 (BGS2) in Norway, we aimed to establish hormone references in relation to both traditional Tanner and US breast stages. We leveraged a newly established in-house liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to quantify estrogens in the sub-picomolar range (19) in order to investigate whether ultrasound stratification of clinically prepubertal girls would reveal biochemically distinct phenotypes. Lastly, we explored the predictive value of hormone biomarkers and an endocrine profile to determine female puberty onset.
MATERIALS AND METHODS

Cohort description

Clinical examinations and data collection for the Bergen Growth Study 2 (BGS2) cross-sectional cohort was conducted in 2016. Children in the age interval 6 to 16 years from six schools were voluntarily recruited with parental consent to be examined regarding puberty status. This cohort was described previously (7). Briefly, a total of 703 girls were included in the cohort (participation rate 49.5%), of which 651 donated blood samples. The following participants were excluded: 27 due to chronic disease; 12 due to use of oral contraceptives and 11 due to insufficient blood sample volume.

Clinical and ultrasound evaluation of puberty

Breast maturation was evaluated both clinically (Tanner B) and with ultrasound (US). US images were analyzed after study completion to prevent observer bias. Clinical evaluation was performed in accordance with the schematics proposed by Marshall and Tanner (6) and included breast palpation. An experienced radiologist established the ultrasound staging protocol in line with a previous study (20). Description of the morphological distinctions providing the basis for US breast staging was provided previously (21). A trained nurse performed all clinical and US evaluations, and the most mature breast was examined. Briefly, stage US B0 was characterized as a small hypoechoic area in the retro-areolar area. Unique to stage US B1 was the presence of hyperechoic tissue with a triangular shape. In stage US B2, corresponding to clinical thelarche, the internal breast was characterized by a small hypoechoic centre (linear, round or star-shaped) with surrounding glandular hyperechoic tissue. The presence of a hypoechoic centre was a prerequisite for stages US B3 and US B4, and while the appearance in US B3 was spider-shaped, the centre was defined as increasingly
roundish in US B4. The mature stage US B5 presented as a heterogeneous mass devoid of the hypoechoic centre. As described previously (21), the intra-observer agreement of US B staging was ‘very good’ (Cohen’s kappa 0.84; 95% CI 0.78 – 0.91). Presence of pubic hair was defined as a score of 2 or higher according to the Tanner PH scale (22).

**Blood sample analyses**

Samples were collected from venous blood between (hh:mm) 8:20 and 14:10. Average time of blood draw was 10:57 and cumulative proportion of samples collected according to time of day starting from 08:20 is provided: 09:00 (9%); 10:00 (30%); 11:00 (53%); 12:00 (69%); 13:00 (88%); 14:00 (99%); 14:10 (100%). Serum was stored at -80 °C prior to analysis at the Hormone Laboratory, Department of medical biochemistry and pharmacology, Haukeland University Hospital Hormone laboratory, where personnel were blinded for participant age and pubertal status. The laboratory and its analytical practice are accredited in accordance with NS-EN ISO 15189:2012. The following coefficients of variation (CV) refer to the inter-assay variation. Siemens IMMULITE 2000 XPi was used to analyze basal levels of LH (CV 7% at 10 IU/L), FSH (CV 5% at 17 IU/L) and sex hormone-binding globulin (SHBG; CV 6% at 6.74 µg/mL). E₂ and E₁ were analyzed by an ultrasensitive LC-MS/MS method as recently described (23). Briefly, samples were subjected to liquid-liquid extraction before analysis and quantification by LC-MS/MS. Lower limit of detection (LOD) was 0.28 pmol/L for E₂ and 0.15 pmol/L for E₁. Lower limit of quantification (LOQ) was 0.58 pmol/L for E₂ (CV 9.1% in the range 1.7 - 153.3 pmol/L) and 0.25 pmol/L for E₁ (CV 7.8%, range 1.7 - 143.1 pmol/L). To convert estradiol (E₂) to pg/mL, divide by 3.671. To convert estrone (E₁) to pg/mL, divide by 3.698. To convert SHBG to µg/mL, divide by 8.896.
Hormone reference intervals

Nonparametric reference intervals were established in accordance Clinical & Laboratory Standards Institute (CLSI) EP28-A3c guidelines (24) and the Canadian Laboratory Initiative on Pediatric Reference Intervals (CALIPER) framework (25). Briefly, the central 95% range was defined by the lower 2.5th and upper 97.5th percentile limits. Reference intervals were established from breast stage partitions by resampling to 500 bootstrapped data points using the Analyse-it (Analyse-it, Leeds, UK) extension in Microsoft Excel (Microsoft, Redmond, WA, USA). The Harris-Boyd standard deviate test (26-28) was applied to log-transformed data with approximate Gaussian distributions to determine statistically significant differences between pairwise, successive breast stage partitions. Justified partitioning criteria were met if the numeric Harris-Boyd z score exceeded that of the critical z* sample power test, as mathematically outlined previously (26-29). Continuous hormone reference intervals in relation to chronological age were generated using the CLSI-compliant referenceIntervals package in R (R Development Core Team, Vienna, Austria), based on a moving window of 120 observations as described previously (29).

Endocrine profiling

Data dimensionality reduction by principle component analysis (PCA) was applied to generate a composite endocrine profile score for serum level constellations of hormones E₁, E₂, LH, FSH and SHBG. PCA was applied to hormone data from 403 pre-menarcheal girls (age interval 6.2 - 15.7 years). Participants with missing data for one or more hormones were discarded. Post-menarcheal girls were excluded from PCA in order to omit menstrual cycle hormone fluctuations. The first principal component 1 (PC1) comprised the following loadings: E₁ (0.497), E₂ (0.494), LH (0.475), FSH (0.469) and SHBG (-0.252). This PC1 exhibited an Eigenvalue of 3.6 (i.e. its standard deviation of 1.9 squared) and accounted for 70.0% of the hormone dataset variance. Secondary PCs returned Eigenvalues below 1.0 and...
were accordingly discarded. Participant PC1 scores were thus used to assign individual endocrine profiles in context of the total dataset variance. The PCA was computed in R with code operations provided as supplemental data (30).

**Ethical considerations**

The Bergen Growth Study 2 was approved by the Norwegian Regional Ethics Committee, case references 2015/128/REK and 2015/235/REK. The study design and conduct conformed to good clinical practice and the ethical decrees of the Helsinki Declaration. Children below 16 years of age were only examined with written and informed parental consent and child assent, both of which were instantly revocable. Participants were rewarded with a cinema voucher. Breast palpation or ultrasound performed in the study did not detect any pathology.

**Statistical analyses**

Transition to pubertal breast stage was estimated from age using probit regression for a generalized linear model in R, as described previously (7). Receiver operating characteristics (ROC) curves were generated using R and the optimal ROC cutoff value (Youden index) was computed using with the pROC package, corresponding to the point of the ROC curve where the sum of sensitivity and specificity for distinguishing two groups is highest (31). Statistical significance was defined as: *P<0.05, **P<0.01, ***P<0.001 (two-tailed Mann Whitney U test) and ^z>z^* (Harris-Boyd test).

**RESULTS**

**Hormone references in relation to chronological age**

Serum levels of E$_2$, E$_1$, LH, FSH, SHBG and LH/FSH ratio were plotted against age and reference centiles defining the 95% normal range and median were computed from a moving window of 120 observations (**Fig. 2**). Table format hormone references interpolated
from these models are provided in SI units Supplemental Table 1 (32) and conventional units Supplemental Table 2 (33).

**Hormones reference intervals by puberty stages**

Reference intervals according to breast evaluations by clinical examination (Tanner B stages) and ultrasound (US B stages) were established by bootstrapping breast stage partitions to 500 observations (Table 1). The equivalent table with analytes annotated in conventional units is provided as Supplemental Table 3 (34). Justified partitioning of incremental breast stage reference intervals was verified using the pairwise Harris-Boyd standard deviate test (Z). Where indicated not significant (n.s.), the reference interval overlap of the current and the previous partition was too extensive to warrant partitioning despite the dichotomized segregation by breast stages. By the same logic, standard deviate testing of corresponding Tanner B and US B stage reference intervals (i.e. Tanner B2 vs US B2, with matching increments) showed agreement between the two methods with no statistical evidence to support endocrinological distinctions for any of the five hormones. Median ages (95% CI) for the Tanner B stages were: B1, 8.7 (6.6 - 11.6); B2, 10.6 (8.5 - 13.8); B3, 11.9 (10.2 - 15.1); B4, 14.0 (11.6 - 16.0) and B5, 14.6 (11.7 - 16.0) years. Median ages for the US B stages were: US B0, 8.2 (6.5 - 11.4); US B1, 8.9 (6.8 - 11.9); US B2, 10.6 (8.3 - 13.8); US B3, 12.0 (9.6 - 15.8); US B4, 13.8 (11.2 - 16.0) and US B5, 14.4 (12.1 - 16.0).

**Endocrine profile detects thelarche**

Next, we evaluated the predictive value of age, endocrine profile and individual analytes, respectively, in determining the transition to puberty onset defined by attainment of breast stage Tanner B2+ or US B2+ (Table 2). For these analyses we selected the age interval 8.0 - 12.0 years that defined both the earliest and latest occurrence of Tanner B2 in the dataset. Statistically significant differences were observed for all variables between prepubertal and pubertal groups (P<0.001 for all, Mann-Whitney U). Notably, the endocrine
profile index (i.e. participant PC1 scores) returned the highest area under the ROC curve and best negative predictive value (NPV) to distinguish prepubertal and pubertal girls.

**Ultrasound evaluation allows for refined characterization of early thelarche**

Minor disagreements between Tanner and US breast staging were encountered on the intra-individual level, and we next decided to investigate the possible endocrine implications of these discrepancies. Stratifying each of the established Tanner B stages by the corresponding breast stages obtained by US, we observed no biochemical differences between US B strata in pubertal girls (Tanner B2-5). However, statistically significant endocrine discrepancies were evident upon stratification of clinically prepubertal Tanner B1 girls by US B stages (**Table 3**). Among these 264 prepubertal girls, individually evaluated as Tanner B1, ultrasound breast morphology was radiologically discernable by ultrasound as mainly prepubertal US B0 (139/264 = 52.7%) or US B1 (111/264 = 42.0%), but pubertal attainment of glandular tissue was also evident in a subset of US B2 girls (14/264 = 5.3%). Notably, both age and serum levels of E1 were significantly higher in US B1 relative to US B0 (P<0.05 and P<0.01, respectively, Mann-Whitney U). Comparing the US B1 and US B2 strata by Mann-Whitney U tests, we observed statistically significant differences in serum levels of E2 (P<0.05), FSH (P<0.01) and SHBG (P<0.05), corresponding to a highly distinct endocrine phenotype indicated by the endocrine profile (P<0.001). Importantly, these endocrine differences were observed despite no significant age difference between the US B1 and US B2 subgroups. In this comparison, the endocrine profile emerged as the most statistically significant variable.

**DISCUSSION**

In the current study, 601 healthy Norwegian girls were evaluated by both clinical and US breast evaluations, allowing for exceptional endocrine and methodological comparisons. Overall, the girls in the current Norwegian cohort exhibited median age at occurrence of
Tanner B2 at 10.4 years, US B2 at 10.2 years and menarche at 12.7 years (7), which is comparable to other Western countries (35-38). The cohort was representative of the general Norwegian demography, having a ~90% majority Caucasian population. Agreement between the two breast staging methods was satisfactory as previously described (7,21). Interestingly, our participants perceived ultrasound as a less invasive procedure compared to palpation, and the majority (67.3%) of the girls favored the ultrasound evaluation (our unpublished observations).

Although reference intervals for US and Tanner breast stage were biochemically comparable overall, US enabled detection of glandular tissue in a subset of clinically prepubertal girls with distinct biochemical baseline characteristics. From the stratification of clinically prepubertal (Tanner B1) girls by US B stages in Table 2, we observed significant endocrine profile differences between the sub-strata of prepubertal (US B1) and pubertal (US B2) girls. Although the subgroup of girls with detectable glandular tissue by ultrasound (US B2) was small, this finding implies that endocrine profiling by PCA may provide more sensitivity than any singular hormone in context of detecting thelarche. Further studies are warranted to explore the use-case for tailored endocrine profiles as predictive or diagnostic markers of endocrinopathies including hypogonadism and disorders of sexual development.

The application of endocrine profiling in the current study has limitations and warrants discussion. PCA was applied to capture the total variance of five hormone concentrations from 403 pre-menarcheal girls. The first principal component, PC1, explained 70% of the total dataset variance and thus the PC1 scores associated with each study participant were leveraged as a composite endocrine profile index. The rationale for this endocrine profile was that its constituent hormones are integral components of the pubertal HPG signaling axis and subject to reciprocal regulation. Loading other pertinent hormone dimensions into the endocrine profile would arguably generate additional complexity and
depth. The application of endocrine profiling by PCA cluster analysis was recently applied to extract distinct endocrine phenotype clusters in a longitudinal study of female puberty (18). In contrast, our approach was to generate a reference endocrine profile for normal puberty development. As shown in Table 2, the composite endocrine profile index was an excellent marker of thelarche, but in terms of ROC diagnostic performance it was only marginally better than, or practically equivalent to that of E$_2$ levels alone. However, the occurrence US thelarche morphology in clinically prepubertal girls in Table 3 was marked with a higher degree of statistical confidence by the endocrine profile than by E$_2$ alone. These findings imply that endocrinological investigations of altered puberty timing in some cases may benefit from PCA profiling. Automated computation for endocrine profiling may provide added value to existing laboratory systems or clinical decision trees. In a previous study, Fugl et al. observed that baseline levels of LH was the better analyte variable to determine thelarche (39). Notably, this study included a sample size of only 43 girls, with a late minimum inclusion age of 9.8 years.

A central undertaking in the current work was the construction of reference intervals compatible with previously defined US breast stages (7,20,21). Table 1 was configured to juxtapose reference intervals for clinical Tanner B stages and US B stages. Notably, applying the Harris-Boyd standard deviate test criteria, there were no cases where hormone levels differed significantly between corresponding Tanner B and US B stages throughout puberty or when pooled observations in US B0/1 was compared to Tanner B1. This implies that these two ordinal methods of breast staging are principally compatible in endocrinological terms, with matching breast stages aligning biochemically despite different methodological rationales. However, US enabled stratification of girls nearing thelarche.

Our reference intervals should be interpreted with some precautions. The current references represent average hormone levels throughout the morning and early noon, and do
not account for intra-day variation for gonadotropins and estrogen levels. In this regard, the diurnal rhythm for estrogen, LH and FSH secretion have been extensively studies by others (40-42). Due to logistical constraints, we were unable to obtain fasting morning samples and, in line with similar studies (25,43-46) our references rely on sample power to squelch diurnal secretion patterns and may thus be representative for outpatient clinics. Further, we did not account for latent hormone fluctuations associated with monthly cyclicity in pre-menarcheal girls, and post-menarche data was not stratified by menstruation cycle phase at the time of blood draw.

In conclusion, we have provided the first set of statistically robust hormone references for US breast staging, in comparison to traditional Tanner B stages for female puberty. Our results demonstrate a high degree of agreement between the two methods of puberty staging both in terms of age at stage occurrences and endocrine parameters. However, ultrasound enabled detection of non-palpable glandular tissue in a subset of clinically prepubertal girls, and this phenotype was corroborated by a pubertal endocrine profile. Further, we have demonstrated that index scores from endocrine profiling by PCA represents a useful predictive marker of puberty onset with a possible use-case in detecting pediatric endocrinopathies associated with altered puberty onset.
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Data availability
Restrictions apply to the availability of data generated or analyzed during this study to preserve patient confidentiality or because they were used under license. The corresponding author will on request detail the restrictions and any conditions under which access to some data may be provided.
REFERENCES

1. Eckert-Lind C, Busch AS, Petersen JH, Biro FM, Butler G, Brauner EV, Juul A. Worldwide Secular Trends in Age at Pubertal Onset Assessed by Breast Development Among Girls: A Systematic Review and Meta-analysis. *JAMA Pediatr.* 2020:e195881.

2. Swerdloff RS, Odell WD. Hormonal mechanisms in the onset of puberty. *Postgrad Med J.* 1975;51:200-8.

3. Catli G, Erdem P, Anik A, Abaci A, Bober E. Clinical and laboratory findings in the differential diagnosis of central precocious puberty and premature thelarche. *Turk Pediatri Ars.* 2015;50:20-6.

4. Ahmed SF, Achermann JC, Arlt W, Balen AH, Conway G, Edwards ZL, Elford S, Hughes IA, Izatt L, Krone N, Miles HL, O'Toole S, Perry L, Sanders C, Simmonds M, Wallace AM, Watt A, Willis D. UK guidance on the initial evaluation of an infant or an adolescent with a suspected disorder of sex development. *Clin Endocrinol (Oxf).* 2011;75:12-26.

5. Moshiri M, Chapman T, Fechner PY, Dubinsky TJ, Shnorhavorian M, Osman S, Bhargava P, Katz DS. Evaluation and management of disorders of sex development: multidisciplinary approach to a complex diagnosis. *Radiographics.* 2012;32:1599-618.

6. Marshall WA, Tanner JM. Variations in pattern of pubertal changes in girls. *Arch Dis Child.* 1969;44:291-303.

7. Bruserud IS, Roelants M, Oehme NHB, Madsen A, Eide GE, Bjerknes R, Rosendahl K, Julissson PB. References for Ultrasound Staging of Breast Maturation, Tanner Breast Staging, Pubic Hair, and Menarche in Norwegian Girls. *J Clin Endocrinol Metab.* 2020;105.
8. Ziskin MC. Fundamental physics of ultrasound and its propagation in tissue. *Radiographics*. 1993;13:705-9.

9. Arthurs OJ, Bjorkum AA. Safety in pediatric imaging: an update. *Acta Radiol*. 2013;54:983-90.

10. Calcaterra V, Sampaolo P, Klersy C, Larizza D, Alfei A, Brizzi V, Beneventi F, Cisternino M. Utility of breast ultrasonography in the diagnostic work-up of precocious puberty and proposal of a prognostic index for identifying girls with rapidly progressive central precocious puberty. *Ultrasound Obstet Gynecol*. 2009;33:85-91.

11. Carlson L, Flores Poccia V, Sun BZ, Mosley B, Kirste I, Rice A, Sridhar R, Kangarloo T, Vesper HW, Duke L, Botelho JC, Filie AC, Adams JM, Shaw ND. Early breast development in overweight girls: does estrogen made by adipose tissue play a role? *Int J Obes (Lond)*. 2019;43:1978-87.

12. Yuce O, Sevinc D. Ultrasonographic assessment of pubertal breast development in obese children: compliance with the clinic. *J Pediatr Endocrinol Metab*. 2018;31:137-41.

13. Ringner M. What is principal component analysis? *Nat Biotechnol*. 2008;26:303-4.

14. Eisenmann JC. On the use of a continuous metabolic syndrome score in pediatric research. *Cardiovasc Diabetol*. 2008;7:17.

15. Just J, Gouvis-Echraghi R, Rouve S, Wanin S, Moreau D, Annesi-Maesano I. Two novel, severe asthma phenotypes identified during childhood using a clustering approach. *Eur Respir J*. 2012;40:55-60.

16. Eisenhofer G, Masjkur J, Peitzsch M, Di Dalmazi G, Bidlingmaier M, Gruber M, Fazel J, Osswald A, Beuschlein F, Reincke M. Plasma Steroid Metabolome Profiling
for Diagnosis and Subtyping Patients with Cushing Syndrome. *Clin Chem*. 2018;64:586-96.

17. Lasarev MR, Bialk ER, Allen DB, Held PK. Application of Principal Component Analysis to Newborn Screening for Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab*. 2020;105.

18. Fassler CS, Gutmark-Little I, Xie C, Giannini CM, Chandler DW, Biro FM, Pinney SM. Sex Hormone Phenotypes in Young Girls and the Age at Pubertal Milestones. *J Clin Endocrinol Metab*. 2019;104:6079-89.

19. Bertelsen BE, Kellmann R, Viste K, Bjørnevik AT, Eikesdal HP, Lønning PE, Sagen JV, Almås B. An Ultrasensitive Routine LC-MS/MS Method for Estradiol and Estrone in the Clinically Relevant Sub-Picomolar Range. *J Endocr Soc*. 2020;4: bvaa047.

20. Garcia CJ, Espinoza A, Dinamarca V, Navarro O, Daneman A, Garcia H, Cattani A. Breast US in children and adolescents. *Radiographics*. 2000;20:1605-12.

21. Bruserud IS, Roelants M, Oehme NHB, Eide GE, Bjerknes R, Rosendahl K, Juliasson PB. Ultrasound assessment of pubertal breast development in girls: intra- and interobserver agreement. *Pediatr Radiol*. 2018;48:1576-83.

22. Emmanuel M, Bokor BR. Tanner Stages. *StatPearls*. Treasure Island (FL)2020.

23. Bertelsen B-E, Kellmann R, Viste K, Bjørnevik AT, Eikesdal HP, Lønning PE, Sagen JV, Almås B. An ultrasensitive routine LC-MS/MS method for estradiol and estrone in the clinically relevant sub-picomolar range. *Journal of the Endocrine Society*. 2020.

24. Ep28-a3c - Defining, establishing, and verifying reference intervals in the clinical laboratory. Clinical And Laboratory Standards Institute (CLSI) 2008. Wayne, PA, USA.
25. Adeli K, Higgins V, Trajcevski K, White-Al Habeeb N. The Canadian laboratory initiative on pediatric reference intervals: A CALIPER white paper. *Crit Rev Clin Lab Sci*. 2017;54:358-413.

26. Harris EK, Boyd JC. On dividing reference data into subgroups to produce separate reference ranges. *Clin Chem*. 1990;36:265-70.

27. Fuentes-Arderiu X, Ferre-Masferrer M, Alvarez-Funes V. Harris & Boyd's test for partitioning the reference values. *Eur J Clin Chem Clin Biochem*. 1997;35:733.

28. Horn PS, Pesce AJ, Copeland BE. A robust approach to reference interval estimation and evaluation. *Clin Chem*. 1998;44:622-31.

29. Madsen A, Oehme NB, Roelants M, Bruserud IS, Eide GE, Viste K, Bjerknes R, Almas B, Rosendahl K, Sagen JV, Mellgren G, Juliusson PB. Testicular Ultrasound to Stratify Hormone References in a Cross-Sectional Norwegian Study of Male Puberty. *J Clin Endocrinol Metab*. 2020;105.

30. Figshare Digital Repository 2020. Madsen A, Bruserud IS, Bertelsen BE, et al. Supplemental Data protocol. Deposited July 1, 2020. https://doi.org/10.6084/m9.figshare.12594095.v1

31. Ruopp MD, Perkins NJ, Whitcomb BW, Schisterman EF. Youden Index and optimal cut-point estimated from observations affected by a lower limit of detection. *Biom J*. 2008;50:419-30.

32. Figshare Digital Repository 2020. Madsen A, Bruserud IS, Bertelsen BE, et al. Supplemental Table 1. Deposited July 1, 2020. https://doi.org/10.6084/m9.figshare.12593762.v1

33. Figshare Digital Repository 2020. Madsen A, Bruserud IS, Bertelsen BE, et al. Supplemental Table 2. Deposited July 1, 2020. https://doi.org/10.6084/m9.figshare.12593912.v1
34. Figshare Digital Repository 2020. Madsen A, Bruserud IS, Bertelsen BE, et al. Supplemental Table 3. Deposited July 1, 2020. https://doi.org/10.6084/m9.figshare.12593918.v1

35. Mul D, Fredriks AM, van Buuren S, Oostdijk W, Verloove-Vanhorick SP, Wit JM. Pubertal development in The Netherlands 1965-1997. Pediatr Res. 2001;50:479-86.

36. Biro FM, Greenspan LC, Galvez MP, Pinney SM, Teitelbaum S, Windham GC, Deardorff J, Herrick RL, Succop PA, Hiatt RA, Kushi LH, Wolff MS. Onset of breast development in a longitudinal cohort. Pediatrics. 2013;132:1019-27.

37. Aksglaede L, Sorensen K, Petersen JH, Skakkebaek NE, Juul A. Recent decline in age at breast development: the Copenhagen Puberty Study. Pediatrics. 2009;123:e932-9.

38. Roelants M, Hauspie R, Hoppenbrouwers K. References for growth and pubertal development from birth to 21 years in Flanders, Belgium. Ann Hum Biol. 2009;36:680-94.

39. Fugl L, Hagen CP, Mieritz MG, Tinggaard J, Fallentin E, Main KM, Juul A. Glandular breast tissue volume by magnetic resonance imaging in 100 healthy peripubertal girls: evaluation of clinical Tanner staging. Pediatr Res. 2016;80:526-30.

40. Mitamura R, Yano K, Suzuki N, Ito Y, Makita Y, Okuno A. Diurnal rhythms of luteinizing hormone, follicle-stimulating hormone, testosterone, and estradiol secretion before the onset of female puberty in short children. J Clin Endocrinol Metab. 2000;85:1074-80.

41. Wennink JM, Delemarre-van de Waal HA, Schoemaker R, Schoemaker H, Schoemaker J. Luteinizing hormone and follicle stimulating hormone secretion patterns in girls throughout puberty measured using highly sensitive immunoradiometric assays. Clin Endocrinol (Oxf). 1990;33:333-44.
42. Goji K. Twenty-four-hour concentration profiles of gonadotropin and estradiol (E2) in prepubertal and early pubertal girls: the diurnal rise of E2 is opposite the nocturnal rise of gonadotropin. *J Clin Endocrinol Metab.* 1993;77:1629-35.

43. Jung B, Adeli K. Clinical laboratory reference intervals in pediatrics: the CALIPER initiative. *Clin Biochem.* 2009;42:1589-95.

44. Frederiksen H, Johannsen TH, Andersen SE, Albrethsen J, Landersoe SK, Petersen JH, Andersen AN, Vestergaard ET, Schorring ME, Linneberg A, Main KM, Andersson AM, Juul A. Sex-specific Estrogen Levels and Reference Intervals from Infancy to Late Adulthood Determined by LC-MS/MS. *J Clin Endocrinol Metab.* 2020;105.

45. Elmlinger MW, Kuhnel W, Ranke MB. Reference ranges for serum concentrations of lutropin (LH), follitropin (FSH), estradiol (E2), prolactin, progesterone, sex hormone-binding globulin (SHBG), dehydroepiandrosterone sulfate (DHEAS), cortisol and ferritin in neonates, children and young adults. *Clin Chem Lab Med.* 2002;40:1151-60.

46. Soldin OP, Hoffman EG, Waring MA, Soldin SJ. Pediatric reference intervals for FSH, LH, estradiol, T3, free T3, cortisol, and growth hormone on the DPC IMMULITE 1000. *Clin Chim Acta.* 2005;355:205-10.
FIGURE LEGENDS

Figure 1. Age reference curves for female puberty

Probit models for age of occurrence for indicated breast stages determined clinically (Tanner B; dashed lines) and by ultrasound (US B; solid lines). The US B1 stage was defined by prepubertal breast morphology, but it was radiologically distinct and more advanced than the baseline stage US B0.

Figure 2. Hormone references in relation to chronological age

Age references for (a) estradiol (E₂; No.=547), (b) estrone (E₁; No.=561), (c) luteinizing hormone (LH; No.=600), (d) follicle-stimulating hormone (FSH; No.=599), (e) sex hormone-binding globulin (SHBG; No.=601) and (f) LH/FSH ratio (No.=599). All healthy participants were included, and the variable number of observations for the individual hormones were due to insufficient serum volume to determine the respective analytes. Filled dots represent prepubertal girls (Tanner B1) and open dots represent pubertal girls (Tanner B2+). Continuous median, lower limit (2.5th percentile) and upper limit (97.5th percentile) centiles with 90% CIs were estimated by nonparametric method from a moving window of 120 observations.
**TABLE LEGENDS**

**Table 1. Reference intervals for Tanner and US breast stages**

Sample size (n) and analyte levels corresponding to the median and resampled 95% reference intervals are presented for indicated puberty breast stage partitions. Column “Z” summarizes Harris-Boyd standard deviate tests to determine justified partitioning of current and the previous partition (‘yes’ or not (n.s., not significant). Abbreviations: B, Tanner breast stage; E₁, estrone; E₂, estradiol; LH, luteinizing hormone; FSH, follicle-stimulating hormone; SHBG, sex hormone-binding globulin; US B, ultrasound breast stage.

**Table 2. Predictive markers of female puberty onset**

Baseline characteristics (median, 95% CI) for girls grouped by Tanner or US definitions of puberty onset in the thelarche age window 8 to 12 years were leveraged in receiver operating characteristics (ROC) analyses. Abbreviations: AU, arbitrary units; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value. Sens. (sensitivity) and Spec. (specificity) represent ROC curve coordinates for the optimal cutoff point.

**Table 3. Baseline characteristics for clinically prepubertal girls stratified by ultrasound breast stages**

Baseline characteristics (median, 95% CI) for 264 girls with a prepubertal clinical presentation, stratified by US breast stages. Statistically significant differences (Mann-Whitney U) between pairwise strata (US B0 vs US B1, or US B1 vs US B2) are annotated. Abbreviations: AU, arbitrary units; E₁, estrone; E₂, estradiol; LH, luteinizing hormone; FSH, follicle-stimulating hormone; SHBG, sex hormone-binding globulin. Significant differences obtained by Mann-Whitney U tests between columns US B0 and US B1, or between US B1 and US B2 were denoted *P<0.05, **P<0.01 or ***P<0.001.
| Reference          | Stage | No. | Median | 2.5<sup>th</sup> percentile (90% CI) | 97.5<sup>th</sup> percentile (90% CI) | Z     |
|--------------------|-------|-----|--------|---------------------------------------|---------------------------------------|-------|
| Estradiol (E<sub>2</sub>), pmol/L | LC-MS/MS | B1   | 260    | 14.0 | 4.4 (3.6 – 5.0) | 37.9 (34.0 – 45.0) | - |
|                    |       | B2   | 69     | 34.0 | 12.6 (12.0 – 14.5) | 119.1 (96.3 – 139) | a |
|                    |       | B3   | 66     | 78.5 | 12.3 (0.4 – 31.0) | 192.4 (151 – 214) | a |
|                    |       | B4   | 78     | 118   | 41.8 (27.0 – 53.0) | 398.9 (290 – 423) | a |
|                    |       | B5   | 81     | 135   | 55.2 (50.0 – 63.0) | 476.1 (385 – 573) | a |
|                    | Tanner stage | US B0 | 134   | 13.0 | 3.9 (2.7 – 4.5) | 38.8 (33.8 – 47.8) | a |
|                    |       | US B1 | 111   | 16.0 | 5.6 (3.1 – 6.3) | 41.9 (34.0 – 75.0) | a |
| Ultrasound stage   |       | US B2 | 78    | 32.5 | 11.2 (7.3 – 13.8) | 154.5 (100 – 214) | a |
|                    |       | US B3 | 62    | 80.5 | 17.1 (14.9 – 26.3) | 192.0 (177 – 199) | a |
|                    |       | US B4 | 97    | 115   | 33.4 (0.4 – 53) | 426.9 (337 – 573) | a |
|                    |       | US B5 | 67    | 135   | 43.4 (27.0 – 57.8) | 435.9 (287 – 463) | a |
| Estrone (E<sub>1</sub>), pmol/L | LC-MS/MS | B1   | 269   | 0.1 | ≤ 0.1 | 0.5 (0.4 – 0.6) | - |
|                    |       | B2   | 73    | 0.3 | ≤ 0.1 | 3.9 (2.3 – 5.4) | a |
|                    |       | B3   | 71    | 2.7 | ≤ 0.1 | 8.6 (6.8 – 9.5) | a |
|                    |       | B4   | 90    | 3.6 | 0.2 (0.1 – 0.6) | 39.6 (16.5 – 54.2) | a |
|                    |       | B5   | 89    | 4.6 | 0.7 (0.4 – 1.0) | 34.8 (17.3 – 47.1) | a |
|                    | Tanner stage | US B0 | 140   | 0.1 | ≤ 0.1 | 0.4 (0.3 – 0.5) | - |
|                    |       | US B1 | 114   | 0.1 | ≤ 0.1 | 0.4 (0.4 – 1.0) | n.s. |
| Ultrasound stage   |       | US B2 | 83    | 0.2 | ≤ 0.1 | 3.6 (2.2 – 5.4) | a |
|                    |       | US B3 | 65    | 2.8 | 0.1 (0.1 – 0.2) | 12.2 (8.7 – 16.9) | a |
|                    |       | US B4 | 111   | 4.3 | 0.2 (0.1 – 0.5) | 37.6 (15.2 – 54.2) | a |
|                    |       | US B5 | 74    | 4.1 | 0.4 (0.1 – 0.9) | 34.7 (18.3 – 47.1) | n.s. |
| FSH, IU/L          | IMMULITE 2000 XPi | B1   | 267   | 1.6 | 0.4 (0.4 – 0.5) | 5.3 (4.6 – 6.4) | - |
|                    |       | B2   | 73    | 3.3 | 1.3 (1.2 – 1.3) | 9.1 (7.4 – 12.1) | a |
|                    |       | B3   | 71    | 5.4 | 1.3 (1.1 – 1.6) | 10.6 (8.4 – 12.3) | a |
|                    |       | B4   | 90    | 5.5 | 0.4 (0.1 – 1.2) | 11.1 (9.5 – 13.3) | n.s. |
|                    |       | B5   | 90    | 6.1 | 1.4 (1.1 – 1.9) | 10.9 (10.0 – 11.5) | n.s. |
|                    | Tanner stage | US B0 | 138   | 1.6 | 0.4 (0.3 – 0.5) | 5.1 (3.5 – 7.2) | - |
|                    |       | US B1 | 114   | 1.5 | 0.4 (0.1 – 0.6) | 5.0 (4.5 – 5.6) | n.s. |
|                    |       | US B2 | 83    | 3.2 | 1.2 (1.1 – 1.3) | 7.8 (7.3 – 8.1) | a |
|                    |       | US B3 | 65    | 5.6 | 0.7 (0.1 – 1.5) | 10.6 (8.5 – 12.3) | a |
|                    |       | US B4 | 112   | 5.6 | 1.2 (0.1 – 2.1) | 11.4 (8.7 – 13.3) | n.s. |
|                    |       | US B5 | 74    | 6.1 | 1.0 (0.3 – 1.7) | 11.0 (10.1 – 11.5) | n.s. |
| SHBG, nmol/L       | IMMULITE 2000 XPi | B1   | 269   | 102 | 51.5 (46.0 – 56.8) | 177.7 (174) | - |
|                    |       | B2   | 73    | 78.0 | 35.6 (30.0 – 41.6) | 164.2 (137) | a |
|                    |       | B3   | 71    | 62.0 | 28.1 (26.0 – 32.8) | 126.3 (114 – 130) | a |
|                    |       | B4   | 90    | 60.5 | 27.9 (24.0 – 32.6) | 110.9 (101 – 121) | a |
|                    |       | B5   | 90    | 54.0 | 15.5 (12.0 – 20.3) | 99.8 (90.1 – 105) | a |
|                    | Tanner stage | US B0 | 140   | 103 | 56.0 (51.0 – 64.0) | 179.4 (176) | - |
|                    |       | US B1 | 114   | 99.5 | 50.4 (37.0 – 57.9) | 169.2 (155) | a |
|                    |       | US B2 | 83    | 83.0 | 28.5 (14.0 – 39.2) | 158.6 (130) | a |
|                    |       | US B3 | 65    | 62.0 | 27.0 (25.0 – 33.2) | 130.4 (102 – 158) | a |
|                    |       | US B4 | 112   | 57.0 | 19.3 (12.0 – 26.5) | 119.0 (104 – 126) | a |
|                    |       | US B5 | 74    | 56.5 | 19.8 (17.0 – 26.0) | 102 (92.5 – 112) | n.s. |

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Table 2

| Girls age 8-12 y | Tanner B1 | Tanner B2+ |
|------------------|-----------|-----------|
| Sample size, No. | 185       | 116       |
| Age, y           | 9.3 (8.0 – 11.8) | 10.5 (8.6 – 11.9) |
| Endocrine profile, AU | -1.0 (-1.6 – 0.5) | 0.1 (1.2 – 2.6) |
| Estradiol E₂, pmol/L | 6.2 (1.0 – 40.7) | 24.0 (6.4 – 126) |
| Estrone E₁, pmol/L | 17.0 (5.1 – 39.1) | 33.0 (13.5 – 98) |
| LH, IU/L         | 0.1 (≤ 0.5) | 0.2 (0.1 – 2.4) |
| FSH, IU/L        | 1.7 (0.6 – 5.1) | 3.3 (1.3 – 7.5) |
| SHBG, nmol/L     | 102 (52 – 178) | 75 (37 – 159) |

| Girls age 8-12 y | US B0/1 | US B2+ |
|------------------|---------|--------|
| Sample size, No. | 170     | 126    |
| Age, y           | 9.3 (8.0 – 11.9) | 10.8 (8.5 – 12.0) |
| Endocrine profile, AU | -1.0 (-1.6 – 0.45) | 0.4 (1.1 – 4.8) |
| Estradiol E₂, pmol/L | 6.3 (1.0 – 41.6) | 35.0 (1.3 – 242) |
| Estrone E₁, pmol/L | 17.0 (4.7 – 39.9) | 43.0 (12.0 – 139) |
| LH, IU/L         | 0.1 (≤ 0.4) | 0.4 (0.1 – 6.7) |
| FSH, IU/L        | 1.7 (0.5 – 5.0) | 3.8 (1.3 – 8.1) |
| SHBG, nmol/L     | 102 (54 – 180) | 70 (30 – 133) |

### Receiver operating characteristics (ROC) curve output

| AUC  | PPV (%) | NPV (%) | Cut-off | Sens. | Spec. |
|------|---------|---------|---------|-------|-------|
| Tanner B |         |         |         |       |       |
| 0.85 | 71.4 | 85.1 | 10.2 y | 0.78 | 0.81 |
| 0.91 | 75.0 | 88.4 | 0.5 AU | 0.80 | 0.85 |
| 0.90 | 84.8 | 84.1 | 19.5 pmol/L | 0.72 | 0.92 |
| 0.84 | 77.8 | 86.0 | 28.5 pmol/L | 0.77 | 0.87 |
| 0.82 | 67.9 | 84.0 | 2.6 IU/L | 0.76 | 0.77 |
| 0.80 | 66.9 | 82.2 | 86.5 nmol/L | 0.73 | 0.77 |

| Ultrasound |         |         |         |       |       |
|           |         |         |         |       |       |
| 0.81 | 68.2 | 83.1 | 9.9 y | 0.81 | 0.72 |
| 0.90 | 71.9 | 88.8 | 0.7 AU | 0.85 | 0.78 |
| 0.87 | 86.8 | 79.1 | 19.5 pmol/L | 0.66 | 0.92 |
| 0.84 | 79.4 | 80.6 | 28.5 pmol/L | 0.71 | 0.87 |
| 0.81 | 86.8 | 77.1 | 0.3 IU/L | 0.63 | 0.93 |
| 0.82 | 70.8 | 79.4 | 2.6 IU/L | 0.73 | 0.78 |
| 0.79 | 71.7 | 77.3 | 84.5 nmol/L | 0.68 | 0.80 |
Table 3

| Variable                        | US B0            | US B1            | US B2            |
|---------------------------------|------------------|------------------|------------------|
| Stratum size, No.               | 139              | 111              | 14               |
| Age, years                      | 8.2 (6.5 – 11.4) | 8.9* (6.8 – 11.9)| 9.7 (8.0 – 11.6)|
| Pubic hair (PH2+), %            | 6.6%             | 5.4%             | 40.0%            |
| Endocrine profile, AU           | -1.1 (-1.7 – 0.1)| -1.1 (-1.5 – 0.0)| -0.6*** (-1.2 – 1.8) |
| Estradiol E2, pmol/L            | 4.4 (0.8 – 30.6) | 4.90 (1.4 – 21.3)| 12.5* (2.3 – 112.0)|
| Estrone E1, pmol/L              | 13.0 (4.2 – 35.7) | 16.0** (5.4 – 34.0)| 20.5 (7.3 – 60.0)|
| LH, IU/L                        | 0.1 (0.1 – 0.4)  | 0.1 (0.1 – 0.4)  | 0.1 (0.1 – 2.1)  |
| FSH, IU/L                       | 1.6 (0.4 – 4.5)  | 1.5 (0.5 – 4.9)  | 2.4** (1.1 – 7.9) |
| SHBG, nmol/L                    | 103 (56 – 180)   | 101 (53 – 169)   | 87* (14 – 128)   |
Figure 1

Breast stage cumulative proportion

Age, y

Stage 1 2 3 4 5
