Supplementary Material

Sex-specific estrogen levels and reference intervals from infancy to late adulthood determined by LC-MS/MS

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Content:

Supplemental Materials and Methods

Reagents and standards
Preparation of calibration, quality control and validation materials
Sample preparation
Analytical method
Operation procedure and method validation

Supplementary Table 1. Estrogens and their respective internal (labelled) standards
Supplementary Table 2. TurboFlow-LC system parameters
Supplementary Table 3. Optimized MS/MS interphase settings
Supplementary Table 4. Compound-specific mass spectrometry settings in negative ion mode
Supplementary Table 5. Method validation: Limit of detection and quantification, linear range and matrix effects
Supplementary Table 6. Method validation: Intra-day accuracy and precision
Supplementary Table 7. Method validation: Inter-day precision
Supplementary Table 8. Median concentrations of estrogens and androgens in women separated in early follicular phase, late follicular phase and luteal phase of menstrual cycle
Supplementary Figure 1. LC-MS/MS extracted ion chromatograms
Supplementary Figure 2. Estrogen standards spiked in serum pool from boys and in milli-Q water
Supplementary Figure 3. Logarithmic plots of female serum concentrations of estradiol and estrone as a function of age
Supplementary Figure 4. Logarithmic plots of male serum concentrations of estradiol and estrone as a function of age
Supplementary Figure 5. Female serum concentrations of free estradiol and free estrone by age and stratified by Tanner breast stage
Supplementary Figure 6. Male serum concentrations of free estradiol and free estrone by age and stratified by Tanner genital stage
Supplementary Figure 7. Female serum concentrations of free estradiol, free estrone, 17-hydroxyprogesterone, androstenedione, testosterone and DHEAS as a function of menstrual cycle days
Supplemental Materials and Methods

Reagents and standards

Certified reference material and native standards; estrone (E1), 17β-estradiol (E2) and estriol (E3) and the isotope labelled standards; estrone-2,3,4-13C3 (E1-is) and 17β-estradiol-2,3,4-13C3 (E2-is) were purchased from Cerilliant - Analytical Reference Standards - a Sigma-Aldrich company. Estriol-2,3,4-13C3 (E3-is) was purchased from Sigma-Aldrich (Brøndby, Denmark); see specification in Supplementary Table 1 and chemical structures in main paper Figure 1. Acetonitrile (AcN) and methanol (MeOH) were obtained from Fisher Scientific (Kamstrup, Danmark). Acetone, ethyl acetate, isopropanol, and ammonium acetate (NH4Ac) were obtained from Merck, and heptane was obtained from Honeywell Research Chemicals (both distributed by VWR international). Ammonium fluoride (NH4F) was obtained from Sigma-Aldrich (Brøndby, Denmark). MeOH was LC-MS Optima Grade, and all other chemicals were of analytical, HPLC, or MS grade. Milli-Q water was cleaned in a Millipore system (Milli-Q® Integral 5 with a Q-POD), and ethanol 96% v/v of technical grade was purchased from the hospital’s local pharmacy.

Preparation of calibration, quality control and validation materials

All native standards from suppliers were sonicated for 15 min. and diluted in 100% MeOH followed by sonication for 10 min. to native stock solutions (E1-stock, E2-stock and E3-stock) containing 10 µg/mL of each of the estrogen standards. From these stock solutions, a native standard mixture (E-mix-1) diluted in 40% MeOH containing E1 (0.2 µg/mL), E2 (0.2 µg/mL), and E3 (0.6 µg/mL) was prepared and further diluted to an E-mix-2 containing 2 ng/mL E1, 2 ng/mL E2, and 6 ng/mL E3 and an E-mix-3 containing 0.2 ng/mL E1, 0.2 ng/mL E2, and 0.6 ng/mL E3. For calibration curves, eight solutions of the native E-mix-2 and E-mix-3 diluted in 0.6 mM NH4F/40% MeOH were prepared in the concentration ranges: E1, 7.4-2589 pmol/L; E2, 7.3-2569 pmol/L; and E3, 21-7282 pmol/L. Furthermore, for determination of limits of detection (LOD), limits of quantifications (LOQ), and intra-day variation, the E-mix-2 and E-mix-3 were diluted to nine solutions (concentration ranges: E1, 3.7-355 pmol/L; E2, 3.7-352 pmol/L; E3, 34-3328 pmol/L) by spiking a randomly collected serum pool from prepubertal children (beforehand tested for low or no content of estrogens). Intra-day variation and inter-day variation were determined based on measurements of serum pool without spiking as well as serum pool spiked in three concentrations (Q low, Q middle, and Q high). All calibration and quality control materials were stored at -20°C until use. Solvent blank samples (Milli-Q water), control materials and standard solutions for calibration curves were further treated as the real samples.

E3-is (100 µg) was dissolved in 1.0 mL MeOH. Internal standards from supplier (E1-is and E2-is) and dissolved E3-is (100 µg/mL) were sonicated for 15 min. and diluted with 100% MeOH for preparation of internal stock solutions (E1-is-stock, E2-is-stock and E3-is-stock) containing 10 µg/mL of each the isotope labelled estrogen standards, after which the stocks were sonicated for 10 min. An internal standard mixture (E-is-mix-1) diluted in 40% MeOH containing E1-is (0.1 µg/mL), E2-is (0.1 µg/mL) and E3-is (0.2 µg/mL) were prepared from the stock solutions and diluted further to the final internal standard mixture, E-is-mix-2 containing 1 ng/mL E1-is, 1 ng/mL E2-is, and 2 ng/mL E3-is.

Sample preparation

After thawing, estrogens from all serum samples, including calibration and control materials, were purified by liquid-liquid extraction. Each sample was mixed by brief vortexing and left for 10 min. at room temperature. Two hundred µL of each sample was aliquoted to 1.5 mL Eppendorf tubes and added 40 µL of internal standard solution (E-is-mix-2) and 160 µL 0.6 mM NH4F/40% MeOH. The samples were vortexed on
a shaking table (IKA® VIBRAX VXR basic) briefly at 2500 rpm and at 1500-2000 rpm for 10 min. at room temperature. Next, 400 µL 50% heptane/ethyl acetate were added to all samples, and the samples were vortexed on the shaking table for 30 min. at room temperature following the same procedure as described above. Samples were then centrifuged (Eppendorf centrifuge 5430k) for 10 min. at 25200 rcf at 4°C and thereafter placed in an ethanol bath that was kept below 0°C using dry-ice pills. The organic and aqueous phases were separated by pouring the fluid organic phase (upper layer) into glass tubes. The organic extracts were evaporated to dryness (at 45°C and 5-15 psi N₂), and the residues containing the estrogens were re-suspended in 120 µL 0.6 mM NH₄F/40% MeOH (freshly prepared). The solution was briefly mixed and finally transferred to HPLC vials.

Analytical method

The content of E1, E2 and E3 in serum samples, controls and calibration materials were measured using a newly developed method for simultaneous quantitative determination of estrogens in human serum by isotope dilution TurboFlow-LC-MS/MS.

Analyses were performed on a Dionex UltiMate 3000 UHPLC system (Thermo Scientific, San Jose, CA, USA) with the integrated Transcend TLX TurboFlow sample preparation system (Thermo Scientific, San Jose, CA, USA) coupled with a triple quadrupole mass spectrometer (TSQ Quantiva, Thermo Scientific, San Jose, CA, USA) controlled by Aria MX 2.2 and Xcalibur 4.0 software (ThermoFinnigan, Bellefonte, PA, USA). Samples were introduced with a HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) and kept at 10°C. For sample extraction and chromatographic separation of the estrogens the TurboFlow-LC system was equipped with a loading Cyclone-P TurboFlow column (0.5 x 50mm) (Thermo Scientific, Franklin, MA, USA) followed by an analytical Kinetex® Phenyl-Hexyl column (100Å, 2.1 x 50 mm, 2.6 µm particle size) equipped with a Drop-in SecurityGuard Ultra cartridge (UHPCL Phenyl, 2.1 mm) in front of the analytical column. Both pre- and analytical column were purchased from Phenomenex. The loading column was operated at 22°C (controlled room temperature) and the analytical column was kept at 30°C with a Multisleeve column heater from Thermo Scientific. The MS/MS-system was equipped with a heated electrospray ionization source (HESI) running in negative mode with short shift to positive mode at the end of the time period duration. Solvents used on the TurboFlow (loading) system were: A, 5 mM NH₄Ac in 2% MeOH; B, MeOH; and C, acetone/isopropanol/acetonitril (10:45:45 v/v). Solvents used on the eluting system were: A, 0.4 mM NH₄F in H₂O adjusted to pH 7.5 by adding 200 µL 0.25% NH₄/L solvent (freshly prepared), and B, MeOH. A bypass valve was used for directing the flow to waste for the first two minutes and the last minute of the analysis time, respectively. The total duration time was 5.50 min. The injection volume was 80 µL with a flow rate and solvent programming as shown in Supplementary Table 2. The optimized MS/MS interphase settings used are shown in Supplementary Table 3, and the MS transitions, retention times, collision energies and S-lens settings optimized for each single analyte are shown in Supplementary Table 4. LC-MS/MS extracted ion chromatograms of internal standards (is), qualifier (q) and Quantifier (Q) ions of E1, E2 and E3 in the lowest calibration standard are shown Supplementary Figure 3.

Operation procedure and method validation

For calibration curves, the ratio between the area of native standard and internal standard was plotted as a function of concentrations of native standards. Through linear regression based on area ratios (sample area/internal standard area), the concentration of unknown samples and the control material were determined.

For method validation and all other analyses, two calibration curves in Milli-Q water were included at the beginning and the end of all sample batches.
Matrix effect and ion suppression were investigated in duplicate calibration curves in Milli-Q water and serum pool at 9 different concentration levels as described above for each compound. The responses from standards prepared in the serum pool were plotted as functions of the responses from standards prepared in Milli-Q water. Subsequently, 95% confidence intervals (CI) were calculated for slopes and intercepts of the linear regression (Supplementary Table 5) using the regression function in Analysis Toolpak for Microsoft Excel 2007. If the 95% CI included 1 for the slopes and 0 for the intercepts, no matrix effect was present. In cases in which the slope- and/or intercept constants in the equation for estrogen/serum calibration curves differed from the equation for estrogen/water calibration curves, matrix effects occurred. Sample results might then be corrected for this matrix effect by dividing with the slope coefficient or by deducting the intercept value estimated for the estrogen/serum calibration curve equation from all sample results. However, in this case in which the 95% CI’s of the slope and intercept of the calibration curve in serum was almost 1 and 0, further adjustment was not performed.

The intra-day variability was estimated based on five repeated calibration curves made in the serum pool: Accuracy (% recovery) and precision (relative standard deviation (RSD)) were calculated for a low, mean, and high concentration levels (Q low, Q middle and Q high) from the five repeated serum calibration curves. These five repeated estrogen/serum calibration curves were further used for determination of linearity and limit of detection (LOD) and quantification (Supplementary Table 6 and Supplementary Figure 2A-C). For this, the approach described by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) (1) on validation of analytical procedures was used: 3.3 times the standard error of the intercept of the calibration curve with the y-axis divided by the slope of the calibration curve using the five lowest calibration levels for each analyte (1,2). The standard error of the intercept and the slope of the calibration curve were calculated using the regression function in Analysis Toolpak for Microsoft Excel 2007 (Microsoft Corp., Redmond, WA). The inter-day variation (precision) was estimated from analysis of control material (Q low, Q middle, and Q high) examined in duplicates in 18 batches over a period of 2 months (Supplementary Table 7).

Supplemental References

1. ICH. ICH Validation of Analytical Procedures :Text and Methodology Q2(R1) 2005. [http://wwwichorg/products/guidelines/quality/article/quality-guidelineshtml](http://wwwichorg/products/guidelines/quality/article/quality-guidelineshtml). 2005:1-17.
2. Soeborg T, Frederiksen H, Fruekilde P, Johannsen TH, Juul A, Andersson AM. Serum concentrations of DHEA, DHEAS, 17alpha-hydroxyprogesterone, Delta4-androstenedione and testosterone in children determined by TurboFlow-LC-MS/MS. *ClinChimActa*. 2013;419:95-101.
Supplementary tables

**Supplementary Table 1.** Estrogens and their respective internal standards

|                | CAS     | Mw (g/mol) | Supplier           | Solution            | Catalog no.    |
|----------------|---------|------------|--------------------|---------------------|----------------|
| Estrone        | E1      | 53_16_7    | 270.37             | Cerilliant\(^a\)    | 1mg/mL in MeOH | E-075-1ML     |
| 17β-Estradiol  | E2      | 50_28_2    | 272.38             | Cerilliant\(^a\)    | 1mg/mL in ACN  | E-060-1ML     |
| Estriol        | E3      | 50_27_1    | 288.39             | Cerilliant\(^a\)    | 1mg/mL in MeOH | E-074-1ML     |
| Estrone-2,3,4-\(^{13}\)C\(_3\) | E1-is  | 1241684-29-6 | 273.3             | Cerilliant\(^a\)    | 100 µg/mL in MeOH | E-108-1ML     |
| 17β-Estradiol-2,3,4-\(^{13}\)C\(_3\) | E2-is  | 1261254-48-1 | 275.3             | Cerilliant\(^a\)    | 100 µg/mL in ACN | E-073-1ML     |
| Estriol-2,3,4-\(^{13}\)C\(_3\) | E3-is  | 1255639-55-5 | 291.3             | Sigma-Aldrich       | 100 µg\(^b\)  | 731668-100UG  |

\(^a\) Standards from Cerilliant were certified as control material

\(^b\) Solved *in-house* in 1.0 mL MeOH

**Supplementary Table 2.** TurboFlow-LC system parameters: mobile phases, solvent gradients, and flow-rate

| Step | Time (min) | Flow (mL/min) | % A | % B | % C | Tee | Loop | Flow (mL/min) | Gradient | % A | % B |
|------|------------|---------------|-----|-----|-----|-----|------|---------------|----------|-----|-----|
| 1    | 0.0        | 1.5           | 100 |     |     |     | out  | 0.4           | step     | 100 |     |
| 2    | 2.0        | 0.1           | 100 |     |     | T   | in   | 0.3           | ramp     | 47  | 53  |
| 3    | 3.0        | 1.5           | 100 |     |     |     | in   | 0.4           | ramp     | 47  | 53  |
| 4    | 4.0        | 1.5           | 15  | 85  |     |     | in   | 0.4           | ramp     | 1   | 99  |
| 5    | 6.0        | 1.5           | 100 |     |     |     | out  | 0.4           | ramp     | 1   | 99  |
| 6    | 8.0        | 1.5           | 100 |     |     |     | out  | 0.4           | ramp     | 100 |     |
| 7    | 10.5       | 1.5           | 100 |     |     |     | out  | 0.4           | step     | 100 |     |

\(^a\) Mobile phases for loading pump: A, 5 mM NH\(_4\)AC in 2% MeOH; B, MeOH; and C, acetone/isopropanol/acetonitrile (10:45:45)

\(^b\) Mobile phases for eluting pump: A, 0.4 mM NH\(_4\)F in H\(_2\)O + 200 µl 0.25% NH\(_3\)/L solvent; and B, MeOH
**Supplementary table 3.** Optimized MS/MS interphase settings

| Divert valve settings (min) | 1. injection to waste | 2. injection to MS/MS | 3. injection to waste |
|-----------------------------|-----------------------|-----------------------|-----------------------|
| Ion source type             | H-ESI                 |                       |                       |
| Spray Voltage\(^b\)         | Static                |                       |                       |
| Sheath gas (N\(_2\)) pressure (units) | 66                  |                       |                       |
| Auxiliary gas (N\(_2\)) pressure (units) | 21                  |                       |                       |
| Ion sweep gas (N\(_2\)) pressure (units) | 1                   |                       |                       |
| Ion transfer tube temperature (°C) | 350                 |                       |                       |
| Vaporizer temperature (°C)  | 415                   |                       |                       |
| Scan type                   | SRM                   |                       |                       |
| Cycle time (s)              | 0.2                   |                       |                       |
| Resolution (FWHM (Da))      |                       | Q1 0.7                | Q3 0.7                |
| Collision (CID) gas (Ar) pressure (mTorr) | 2                   |                       |                       |
| Chromatographic peak width (s) | 12                  |                       |                       |
| Chrom filter peak width (s) | 3                     |                       |                       |
| Skimmer Offset (V)          | 5                     |                       |                       |

\(^a\) Method duration time: 5.5 min
\(^b\) Method was running in negative mode with a short shift to positive mode at duration time from 4.5-5.0 min

**Supplementary Table 4.** Compound-specific mass spectrometry settings in negative ion mode

|              | Precursor (m/z) | Product (m/z) | Collision energy (v) | RF lenz (v) | Retention time (min) | Quantifier (Q), qualifier (q) and internal standard (is) ions |
|--------------|----------------|---------------|----------------------|-------------|----------------------|------------------------------------------------------------|
| E1           | 269.23         | 143.12        | 54                   | 76          | 3.80                 | q                                                          |
| E1\(^{13}C_3\) | 272.3          | 145.15        | 41                   | 76          | 3.80                 | Q                                                          |
| E2           | 271.23         | 148.14        | 41                   | 88          | 3.80                 | q                                                          |
| E2\(^{13}C_3\) | 274.3          | 183.13        | 43                   | 94          | 3.62                 | Q                                                          |
| E3           | 287.25         | 143.12        | 53                   | 99          | 2.42                 | Q                                                          |
| E3\(^{13}C_3\) | 290.3          | 172.2         | 38                   | 95          | 2.41                 | is                                                         |
| E3           | 287.25         | 171.12        | 42                   | 99          | 2.42                 | q                                                          |
**Supplementary Table 5.** Method validation: Limit of detection (LOD) and quantification (LOQ), linear range and matrix effects

|       | LOD   | LOQ   | Linear range | Matrix effects |
|-------|-------|-------|--------------|----------------|
|       | pmol/L| pmol/L| pmol/L       | Slope mean     |
|       |       |       |              | 95% CI lower  |
|       |       |       |              | 95% CI upper  |
|       |       |       |              | Intercept mean|
|       |       |       |              | 95% CI lower  |
|       |       |       |              | 95% CI upper  |
| E1    | 2.93  | 8.79  | 3.7-355      | 1.052          |
|       |       |       | 7.4-2655     | 1.023          |
|       |       |       | 0.9959       | 1.081          |
|       |       |       | 7.4-2655     | 0.131          |
|       |       |       | 0.9989       | 0.126          |
| E2    | 4.04  | 12.1  | 3.6-352      | 0.957          |
|       |       |       | 7.3-2569     | 0.920          |
|       |       |       | 0.9942       | 0.994          |
|       |       |       | 7.3-2569     | 0.083          |
|       |       |       | 0.9994       | 0.078          |
|       |       |       | 0.9994       | 0.089          |
| E3    | 12.3  | 37.0  | 34-3328      | 1.026          |
|       |       |       | 21-7282      | 1.006          |
|       |       |       | 0.9993       | 1.046          |
|       |       |       | 21-7282      | -0.009         |
|       |       |       | 0.9991       | -0.029         |
|       |       |       | 0.9991       | 0.011          |

a LOD, LOQ, linear range, and matrix effect were determined according to the ICH algorithm

b LOD, LOQ, and linear range were based on five repeated calibration curves prepared in spiked human serum pool

c Matrix effects were evaluated by slope and intercept with corresponding 95% confidence intervals (CI) when the responses at nine concentration levels of estrogen standards spiked in serum pool were plotted as functions of the responses at same concentration levels for standards prepared in Milli-Q water.

R2: Correlations coefficient, mean of five R2-values

in cursive: linear range for standards prepared in Milli-Q water for daily calibration curves

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**Supplementary Table 6.** Method validation: Intra-daya accuracy and precision of quality control materials in low, mean, and high concentrations

|       | Q low | Q middle | Q high |
|-------|-------|----------|--------|
| Meanb | Recovery | RSD | Meanb | Recovery | RSD | Meanb | Recovery | RSD |
| pmol/L | % | % | pmol/L | % | % | pmol/L | % | % |
| E1    | 14.6  | 98.9     | 17.1   | 43.2     | 97.3 | 5.5   | 367    | 103 | 2.6 |
| E2    | 15.0  | 102      | 19.3   | 46.6     | 106  | 7.5   | 356    | 101 | 2.4 |
| E3    | 138   | 99.3     | 5.3    | 430      | 103  | 2.3   | 3424   | 103 | 2.1 |

a Intra-day: five repeats of quality control materials prepared as serum pool spiked in low, mean and high concentration and analyzed within the same day.

RSD: relative standard deviation; R2: mean of five R2-values

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**Supplementary Table 7.** Method validation: Inter-day precisiona of quality control material in low, mean, and high concentrations

|       | Q low | Q middle | Q high |
|-------|-------|----------|--------|
| Mean  | RSD   | Mean     | RSD    | Mean  | RSD    |
| pmol/L| %     | pmol/L   | %      | pmol/L| %      |
| E1    | 38.5  | 5.2      | 84.0   | 5.4   | 353    | 4.2    |
| E2    | 31.2  | 12.3     | 61.6   | 8.4   | 188    | 4.9    |
| E3    | 111   | 6.0      | 69.1   | 8.9   | 143    | 6.0    |

a Inter-day based on serum pool spiked in low, mean and high level of standards, n=108 analyzed in 18 batches over an period of 2 month.

RSD: relative standard deviation
**Supplementary Table 8.** Median concentration and selected percentiles estrogens and androgens in 188 women from 24.7 to 43.9 years of age separated in early follicular phase from day 1-7, late follicular phase from day 8-14, the lutheral phase post ovulation from day 15 (15+ (all)), and from day 15, where ovulation was confirmed by a progesterone level > 10 nmol/L (15+ (ovul))

| Menstrual cycle day | 1-7 | 8-14 | 15+ (all) | 15+ (ovul) |
|---------------------|-----|------|-----------|------------|
| Percentile          |     |      |           |            |
| Estradiol (E2), pmol/L | 82.2 | 111 | 156 | 184 | 277 | 115 | 193 | 254 | 378 | 1016 | 71.6 | 186 | 264 | 355 | 934 | 149 | 229 | 303 | 392 | 1096 |
| Estrone (E1), pmol/L  | 66.6 | 142 | 181 | 294 | 524 | 132 | 263 | 437 | 675 | 1745 | 87.3 | 251 | 393 | 592 | 1266 | 222 | 357 | 499 | 674 | 1537 |
| Progesterone, nmol/L  | 0.23 | 0.31 | 0.43 | 0.69 | 7.78 | 0.24 | 0.35 | 0.54 | 4.03 | 51.9 | 0.28 | 5.52 | 27.9 | 42.1 | 83.1 | 10.8 | 28.1 | 35.5 | 51.7 | 83.2 |
| Estrone sulfate (E1-S), nmol/L | 0.66 | 1.50 | 2.25 | 3.08 | 7.35 | 1.37 | 2.60 | 4.35 | 7.20 | 35.7 | 0.34 | 2.06 | 3.68 | 5.74 | 16.9 | 1.35 | 2.58 | 4.07 | 7.10 | 24.9 |
| 17-hydroxyprogesterone, nmol/L | 0.52 | 0.72 | 1.01 | 1.19 | 2.93 | 0.65 | 1.06 | 1.44 | 3.23 | 7.26 | 0.76 | 1.39 | 3.94 | 6.08 | 15.9 | 1.71 | 4.04 | 5.20 | 6.98 | 16.5 |
| Androstenedione, nmol/L | 1.55 | 2.40 | 2.81 | 3.59 | 5.77 | 1.51 | 2.95 | 3.68 | 5.27 | 9.13 | 1.59 | 2.77 | 3.41 | 4.52 | 9.02 | 1.66 | 2.89 | 3.72 | 5.28 | 9.12 |
| Testosterone, nmol/L  | 0.44 | 0.68 | 0.84 | 1.13 | 2.21 | 0.51 | 0.92 | 1.10 | 1.34 | 2.79 | 0.44 | 0.82 | 0.96 | 1.26 | 2.69 | 0.45 | 0.88 | 1.06 | 1.40 | 2.78 |
| DHEAS, nmol/L         | 1052 | 2251 | 3284 | 4503 | 6746 | 1514 | 2739 | 4111 | 5543 | 7696 | 1816 | 2853 | 4167 | 5374 | 10176 | 1843 | 3081 | 4472 | 5755 | 10586 |

N (E1 and E2) | 47 | 53 | 89 | 48 |
N (all other steroids) | 42 | 48 | 75 | 48 |
Supplementary Figures

Supplementary Figure 1. LC-MS/MS extracted ion chromatograms of lowest calibration standard (std 1): Internal standards (is), qualifier (q), and Quantifier (Q) ions of E1 (7.4pM), E2 (7.3pM), and E3 (21pM).
Supplementary Figure 2. Estrogen standards spiked in serum a pool from boys and in milli-Q water.
Supplementary Figure 3. Logarithmic plots of female serum concentrations of free estradiol (E2) and free estrone (E1) as a function of age, from 0.25-61 years, n=1055 (a,b) and calculated free E2 and free E1 from 0.25-61 years, n=985 (c,d). Solid lines represent medians and dashed lines represent ± 1 and ± 2 standard deviations, respectively.
Supplementary Figure 4. Logarithmic plots of male serum concentrations of estradiol (E2) and estrone (E1) as a function of age, from 0.2-60 years, n=772 (a,b) and calculated free E2 and free E1 from 0.25-61 years, n=706 (c,d). Solid lines represent medians and dashed lines represent ±1 and ±2 standard deviations, respectively.
Supplementary Figure 5. Female serum concentrations (n=496) of free estradiol (free E2) and free estrone (free E1) by age, stratified by Tanner breast stages B1-B5: B1 (1-7 years), n=73; B1 (>7 years), n=152; B2, n=69; B3, n=69; B4, n=95 and B5, n=43 and number of adolescents postmenarche, n=112.
Supplementary Figure 6. Male serum concentrations (n=487) of free estradiol (free E2) and free estrone (free E1) by age, stratified by Tanner genital stages G1-G6: G1 (4-8 years), n=46; G1 (>8 years), n=180; G2, n=69; G3, n=23; G4, n=23 and G5-6, n=146.
Supplementary Figure 7. Female serum concentrations of (a) free estradiol (free E2), (b) free estrone (free E1), (c) 17-hydroxyprogesterone, (d) androstenedione, (e) testosterone, and (f) dehydroepiandrosterone sulfate (DHEAS) as a function of menstrual cycle days in 188 women from 24.7 to 43.9 years; early follicular phase from day 1-7, n=46 and n=41 (c,f); late follicular phase from day 8-14, n=53 (a,b); and n=48 (c,f), the luteal phase post ovulation from day 15 (15+ (all)), n=89 (a,b) and n=75 (c,f) and from day 15, at which ovulation was confirmed by a progesterone level > 10 nmol/L (15+ (ovul)), n=48 (a-f). Bars represent median, 25 and 75 percentiles. Significant differences are considered as p < 0.05, where ** = p < 0.05, *** = p < 0.005 and ¤ = p < 0.1 (border significant).