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Abstract

Objective: To evaluate the independent impact of age, obesity and metabolic risk factors on 13 circulating steroid levels; to generate reference intervals for adult men.
Design: Cross-sectional study.
Methods: Three hundred and fifteen adults, drug-free and apparently healthy men underwent clinical and biochemical evaluation. Thirteen steroids were measured by LC-MS/MS and compared among men with increasing BMI. Moreover, the independent impact of age, BMI and metabolic parameters on steroid levels was estimated. Upper and lower reference limits were generated in steroid-specific reference sub-cohorts and compared with dysmetabolic sub-cohorts.
Results: We observed lower steroid precursors and testosterone and increase in estrone levels in men with higher BMI ranges. By multivariate analysis, 17-hydroxyprogesterone and dihydrotestosterone decreased with BMI, while cortisol decreased with waist circumference. Estrone increased with BMI and systolic blood pressure. Testosterone decreased with worsening insulin resistance. 17-hydroxypregnenolone and corticosterone decreased with increasing total/HDL-cholesterol ratio. Age-related reference intervals were estimated for 17-hydroxypregnenolone, DHEA, 17-hydroxyprogesterone, corticosterone, 11-deoxycortisol, cortisol and androstenedione, while age-independent reference intervals were estimated for progesterone, 11-deoxycorticosterone, testosterone, dihydrotestosterone, estrone and estradiol. Testosterone lower limit was 2.29 nmol/L lower ($P = 0.007$) in insulin resistant vs insulin sensitive men. Furthermore, the upper limits for dihydrotestosterone (−0.34 nmol/L, $P = 0.045$), cortisol (−87 nmol/L, $P = 0.045$–0.002) and corticosterone (−10.1 nmol/L, $P = 0.048$–0.016) were lower in overweight/obese, in abdominal obese and in dyslipidaemic subjects compared to reference sub-cohorts, respectively.
Conclusions: Obesity and mild unmedicated metabolic risk factors alter the circulating steroid profile and bias the estimation of reference limits for testosterone, dihydrotestosterone, cortisol and corticosterone. Applying age-dependent reference intervals is mandatory for steroid precursors and corticosteroids.

Introduction

Steroid hormone determination in humans is undergoing a profound technological upgrade, as represented by the introduction of liquid chromatography tandem mass spectrometry (LC-MS/MS) multi-analyte quantitation (1, 2). In the last 15 years, severe disagreements among immunoassays from various manufacturers and between several immunoassays and LC-MS/MS were documented, mainly on the measurement of testosterone (T), estradiol (E2), dihydrotestosterone (DHT) and cortisol (F) (3, 4, 5, 6, 7). LC-MS/MS assays based on isotopic dilution

quantitation benefit from inherent elevated specificity and accuracy. Therefore, this technique usually provides good comparability with other LC-MS/MS assays as well as with gas chromatography–MS methods and reference procedures (2). A second major LC-MS/MS benefit consists in the possibility to evaluate challenging steroids, such as DHT and steroid metabolites as 11-deoxycortisol (11S) and corticosterone (B), whose immunometric measurement is usually not available on routine basis. Evidences on the role of these steroids in the pathogenesis, diagnosis and management of various endocrine diseases are being generated, thereby strongly supporting the extensive use of LC-MS/MS in the clinical practice (1, 8).

One major limitation in this path relies in the limited availability of steroid normative values. Indeed, the current knowledge about steroid circulating levels has mainly been generated by routine immunoassays (9). Therefore, an urgent need exists for updating reference intervals (RI) by means of reliable and traceable LC-MS/MS assays. In addition, in order to achieve the utmost diagnostic efficacy, it is necessary to refine reference limits according to gender, age and pathologic conditions, such as overweight/obesity and related metabolic alterations, affecting large part of the population (10).

Male ageing has been associated with a reduction in androgen and pro-androgen secretion (11). Moreover, a mutual negative impact exists between obesity, visceral fat, insulin resistance, dyslipidaemia, hypertension and the hypothalamus-pituitary-gonadal (HPG) axis, mostly resulting in low androgen and high estrogen levels (11). Ageing and cardiovascular risk factors are also strictly related with the hypothalamus-pituitary-adrenal (HPA) axis (12). However, the modulation of adrenal steroid secretion throughout the lifespan has poorly been documented so far, as well as little is known about corticosteroid imbalance due to obesity and comorbidities.

Large heterogeneity exists in literature about the health criteria used to define the reference cohorts. Studies involving the general population often did not apply an adequate subjects' characterization by means of specialized examination and laboratory assessment. In particular, subclinical and unmedicated conditions, such as overweight, abdominal obesity, dyslipidaemia, reduced insulin sensitivity and impaired blood pressure control, are too often disregarded (9). The poor standardization of blood sampling represents a further issue, as time of the day, the nutritional status and the venepuncture stress overall affect steroid secretion to a non-negligible extent (13, 14).

By focusing on a cohort of adult men selected from the general population and by applying standardized sampling conditions, the present study sought to: (1.) characterize the impact of obesity and metabolic risk factors on the circulating steroid profile and (2.) provide age-specific RI for a large panel of steroids, further demonstrating how non-restrictive selection criteria could alter the estimation of reference limits.

Materials and methods

Subjects

Men aged 18–74 years were recruited from the local general population (15). The study was approved by the S.Orsola-Malpighi Hospital ethical committee (85/2008/O/Tess). All subjects signed the informed consent before being interviewed and examined by a trained endocrinologist, between 0800 am and 1000 am after an overnight fasting. Waist circumference and body mass index (BMI) were registered. Systolic (SBP) and diastolic (DBP) blood pressures were measured in supine position after a 3 min rest. Inclusion criteria were: BMI ≥18.5 kg/m^2, weight stability in the last 3 months and complete sexual development. Steroidal and non-steroidal drug assumption in the previous 3 months (except antipyretic or non-steroidal anti-inflammatory compounds, tolerated up to 1 month before the study, and thyroxine replacement in compensated hypothyroidism), present or past endocrine, hepatic, renal, oncologic, autoimmune, cardiovascular, hematologic, neurologic or psychiatric diseases, sleep disorders, shift working, frequent flying or allergies requiring treatment caused exclusion from the study. Among 590 men examined, 213 reported assumption of one or more drugs (glucose lowering: $n=6$, cholesterol-lowering: $n=111$, anti-hypertensive: $n=83$, antidepressant: $n=13$), whereas, among drug-free subjects, 46 reported present or previous diseases and 10 men reported sleep disturbance. Therefore, 321 subjects were included in the present study.

Biochemical and hormonal evaluation

To avoid venepuncture stress, blood was withdrawn after 10 min saline infusion in Vacuette Z serum beads clot activator tubes (Greiner Bio-One, Kremsmunster, Austria). After 20 min settling, tubes were centrifuged (2000***g***, 10 min, room temperature) and serum for LC-MS/MS was stored at –80°C. Routine analyses on fresh blood

(intra- and inter-assay CV%) were performed: insulin (1.5 and 4.9%), total cholesterol (<1.0 and 2.7%), HDL cholesterol (<0.95 and 1.3%) and triglycerides (<1.5 and 1.8%) were measured by Modular Analytics E170 (Roche Diagnostics). Glucose was measured by Breeze-2 glucometer (Bayer <4.5%). The homeostatic model assessment-insulin resistance (HOMA-IR) and the total/HDL-cholesterol ratio were computed (16, 17).

Steroid measurement by LC-MS/MS

Thirteen steroid hormones were measured by two validated in-house LC-MS/MS assays, verified by certified reference materials and by multicentre ring trials (18). The first included F, 11S, B, 11-deoxycorticosterone (DOC), progesterone (P4), 17-hydroxyprogesterone (17OHP4), dehydroepiandrosterone (DHEA), androstenedione (A4) and T (15), and the second included 17-hydroxypregnenolone (17OHP5), DHT, estrone (E1) and E2 (19). Assays main information are reported in Supplementary Table 1 (see section on supplementary materials given at the end of this article).

Study design

After the removal of six subjects showing far outlier data (two 17OHP5, three 17OHP4 and one DOC values), the cohort was stratified according to the BMI level in normal weight (NW, $18.5 < \text{BMI} \leq 25.0$ kg/m^2; $n=175$), overweight (OW, $25.0 < \text{BMI} \leq 30.0$; $n=106$), class 1 obese (OB1, $30.0 < \text{BMI} \leq 35.0$; $n=24$) and class 2 plus class 3 obese (OB2, $35.0 < \text{BMI} \leq 45.0$; $n=10$). Anthropometric and metabolic features as well as steroid levels were compared among those BMI classes. Afterward, we assessed the independent impact of age, BMI, waist circumference, SBP, DBP, HOMA-IR, total/HDL-cholesterol and triglycerides on each steroid by multiple regression. Steroids were therefore classified in four sub-panels, as their levels resulted impacted by (1.) ageing, (2.) age plus one or more metabolic parameters, (3.) one or more metabolic parameters but not age or (4.) by none of the tested parameters. RI were defined as the central 95% of hormone distribution. For sub-panels 1 and 4, lower (LRL) and upper (URL) reference limits were estimated in the whole cohort. For sub-panels 2 and 3, limits were estimated in reference sub-cohorts of subjects displaying normal values of the parameter(s) identified for influencing the particular steroid levels according to the multiple regression result. To this aim, we identified subjects with normal (≤102 cm, norWC; $n=268$) or elevated (>102 cm, dysWC; $n=47$) waist circumference, with normal (<2.5, norHOMA-IR; $n=132$) or altered (≥2.5, dysHOMA-IR; $n=35$) HOMA-IR and with normal (<5, norChol; $n=146$) or altered (≥5, dysChol; $n=46$) total/HDL-cholesterol. Additionally, we defined a sub-cohort of NW subjects having normal SBP (≤130 mmHg) (NW and norSBP; $n=63$) and a sub-cohort of subjects who were OW and/or had altered SBP (≥145 mmHg) (OW and/or dysSBP; $n=150$).

Age-specific RI were estimated for steroids in sub-panels 1 and 2. Unique age-independent RI were estimated for steroids in sub-panels 3 and 4. Finally, to evaluate whether alterations in metabolic parameters influencing the steroid values could also influence the RI estimation, LRL and URL estimated in the reference sub-cohorts were compared with lower and upper limits calculated in sub-cohorts displaying the defined dysmetabolic features, respectively.

Statistical analysis

Source variables were not normally distributed at the Kolmogorov–Smirnov test and significantly skewed, therefore, Box Cox transformation was applied (20). Far outliers data were detected by the Tukey's test (21). Descriptive and steroid variables were compared among classes with increasing BMI range by the ANOVA trend test available on IBM SPSS package v. 20, IBM Co. Comparison between pairs of BMI classes was performed by Tuckey's HSD method for linear contrast analysis. Homogeneity of variance was not assumed for variables showing significant P value at the Levene's test. The stepwise multiple regression included age, BMI, waist circumference, SBP, DBP, HOMA-IR, total/HDL-cholesterol and triglyceride levels as covariates and each steroid as the dependent variable. The effect size (f^2) was estimated by Cohen's method for multiple regression according to the formula: $f^2 = \frac{sr^2}{\left(1 - R^2 \text{ full}\right)}$, where sr is the semipartial correlation coefficient for the predictor of interest and R is the full correlation coefficient of the multiple regression model (22). LRL and URL were estimated as the mean $-1.96 \times$ S.D. and mean $+1.96 \times$ S.D. of the transformed variables, respectively, and the values were back-transformed to the original unit (23). As 88 and 23% of the subjects displayed DOC and P4 levels below the LC-MS/MS sensitivity limits (0.236 and 0.156 nmol/L, respectively), respectively, their distribution could not be normalized. Therefore, the comparison of these steroids among BMI classes was performed by Kruskal-Wallis analysis, the URL was calculated as the 97.5 centile of

distribution and multiple stepwise regression was not performed.

Age-specific RI were estimated by modeling the transformed steroid variable on age distribution, according to the fractional polynomial regression by Royston and Wright (24). Age (X) was transformed in order to stabilize the steroid variable (Y) for large values of X according to the formula: $eX = \exp\left(\frac{(\log(0.01)\times(X-\min(X)))}{(\max(X)-\min(X))}\right)$. Then, the optimal model was selected for providing the lowest polynomial degree (parsimony) with maximum decrease in deviance (goodness of fit). Best-fit polynomial coefficients were selected by fp syntax in STATA version 13.0 (StataCorp LLC). RI were visually inspected using xrigls syntax in STATA.

Lower and upper limits were compared between reference and dysmetabolic sub-cohorts by the z distribution. Two-tailed P values <0.05 were considered significant. Data were analysed by MedCalc v.18.2.1 (MedCalc Software, Mariakerke, Belgium) except when specified.

Results

Anthropometric, metabolic and hormonal features of the cohort

The final cohort included 315 subjects. Table 1 reports the descriptive and hormonal features observed in NW (n=175), OW (n=106), OB1 (n=24) and OB2 (n=10) men. Men in classes at increasing BMI displayed increasing age (P=0.042), waist circumference, SBP, DBP, insulin, HOMA-IR, total/HDL-cholesterol, triglycerides (all P<0.001), glucose (P=0.007) and total cholesterol (P=0.003) levels, as well as lowering HDL levels (P<0.001).

Among steroid hormones, we observed a significant decrease in 17OHP5 (P=0.029), P4,d T (both P<0.001) and 17OHP4 (P=0.001) and a significant increase in E1 (P<0.001) in classes at increasing BMI. In particular, paired comparisons revealed that, with respect to NW, OW showed lower P4 (P=0.002), OB1 showed lower P4 (P=0.009) and T (P<0.001), whereas OB2 displayed lower P4 (P=0.010), 17OHP4 (P=0.015) and T (P<0.001). OB1 and OB2 also showed lower T levels compared to OW (both P<0.001). In addition, E1 levels in OB2 were significantly higher compared to NW (P=0.002) and OW (P=0.030). Though the trend analysis among BMI classes did not achieve statistical significance (P=0.060), we found lower DHT levels in OB1 compared with NW (P=0.004).

Independent impact of age, anthropometric and metabolic parameters on steroid levels

Results from the multiple stepwise regression are shown in Table 2 and Supplementary Fig. 1. Age associated with decreasing 17OHP5, DHEA, B and A4 (all P<0.001), 17OHP4 (P=0.007), 11S and F (both P=0.010), but not with T, DHT, E1 and E2 levels. A large effect size was observed for DHEA (f^2=0.86) and 17OHP5 (f^2=0.51), whereas for other steroids it accounted for less than 0.20. BMI negatively influenced 17OHP4 (f^2=0.06, P=0.003) and DHT (f^2=0.06, P=0.005), while positively affecting E1 (f^2=0.05, P=0.007). E1 also directly associated with SBP (f^2=0.03, P=0.046). F decreased with increasing waist circumference (f^2=0.05, P=0.005), while T decreased with increasing HOMA-IR (f^2=0.11, P<0.001). Total/HDL-cholesterol inversely associated with 17OHP5 (f^2=0.04, P=0.039) and B (f^2=0.09, P<0.001). No steroid associations were observed with DBP and triglycerides.

According to these results, DHEA, A4 and 11S were classified in sub-panel 1 (only influenced by age), 17OHP5, 17OHP4, B and F in sub-panel 2 (influenced by age plus metabolic parameters), T, DHT and E1 in sub-panel 3 (influenced by metabolic parameters) and E2 in sub-panel 4 (not influenced by the tested parameters).

Steroid reference intervals

Age-specific RI were generated within the whole cohort (n=315) for DHEA, 11S and A4 (sub-panel 1), within NW (n=175) for 17OHP4, within norWC (n=268) for F and within norChol men (n=146) for 17OHP5 and B (sub-panel 2) (Fig. 1). The largest decline from age 20 to 60 years in LRL and URL was displayed by DHEA (−68.9 and −71.0%, respectively) and 17OHP5 (−61.9 and −72.5%, respectively), followed by B (−38.4 and −47.5%, respectively) and A4 (−23.0 and −42.5%, respectively). Moreover, 17OHP4, 11S and F showed an URL decline of −29.0%, −25.0% and −18.9%, respectively. Detailed RI at every fifth year are listed in Table 3.

Age-independent RI were generated in the reference sub-cohort including norHOMA-IR (n=132) for T, NW (n=175) for DHT, NW and norSBP men (n=63) for E1 (all sub-panel 3) and in the whole cohort (n=315) for E2 (sub-panel 4) (Table 4). DOC and P4 URL calculated in the whole cohort (n=315) were 0.292 and 0.493 nmol/L, respectively.

Table 1 Anthropometric, metabolic and hormonal features of the cohort.

	Normal weight (NW, *n* = 175)	**Overweight** (OW, *n* = 106)	**Class 1 Obese** (OB1, *n* = 24)	**Class 2 and 3 Obese** (OB2, *n* = 10)	**ANOVA trend *P* value**
Age, years					
Mean ± S.D.	40.4 ± 13.7	49.0 ± 12.1[a]	50.8 ± 12.6[b]	48.7 ± 11.5	0.042
Min–max	18–74	21–74	24–71	30–64	
Body mass index, kg/m^2					
Mean ± S.D.	22.7 ± 1.5	26.6 ± 1.4[a]	31.7 ± 1.5[c,d]	38.6 ± 2.7[e,f,g]	< 0.001
Min–max	18.6–24.9	25.0–29.9	30.0–34.8	35.1–44.2	
Waist circumference, cm					
Mean ± S.D.	83.8 ± 7.0	95.6 ± 6.8[a]	109.3 ± 5.6[c,d]	123.1 ± 7.8[e,f,g]	< 0.001
Min–max	66–99	80–115	100–119	114–135	
Systolic blood pressure, mmHg					
Mean ± S.D.	124.1 ± 15.4	130.2 ± 15.9[h]	143.3 ± 17.9[c,i]	143.1 ± 17.9	< 0.001
Min–max	100–180	105–190	120–180	110–170	
Diastolic blood pressure, mmHg					
Mean ± S.D.	81.3 ± 7.6	85.4 ± 7.9[l]	89.0 ± 10.2[c]	92.0 ± 10.3[m]	< 0.001
Min–max	65–100	65–105	70–110	80–110	
Glucose, mg/dL					
Mean ± S.D.	81.1 ± 10.7	86.2 ± 13.0[l]	90.9 ± 15.8[b]	91.8 ± 16.1	0.007
Min–max	52–107	56–126	61–123	71–126	
Insulin, µU/mL					
Mean ± S.D.	5.2 ± 2.9	7.8 ± 4.0[a]	12.0 ± 5.5[c,d]	21.6 ± 5.6[n]	< 0.001
Min–max	1.6–21.8	2.6–23.0	6.0–29.6	11.0–33.5	
HOMA-IR					
Mean ± S.D.	1.14 ± 0.63	1.66 ± 1.03[a]	2.56 ± 1.32[c,i]	4.78 ± 1.79[e,f,n]	< 0.001
Min–max	0.33–4.58	0.60–6.08	1.22–7.02	2.44–7.69	
Total cholesterol, mg/dL					
Mean ± S.D.	180.3 ± 37.5	199.8 ± 31.8[a]	203.1 ± 48.0	210.6 ± 22.7[o]	0.003
Min–max	99–270	107–320	123–305	182–243	
HDL, mg/dL					
Mean ± S.D.	52.8 ± 13.2	48.3 ± 11.2	41.6 ± 10.3[c,p]	39.8 ± 4.0[m]	< 0.001
Min–max	33–96	23–88	27–70	35–48	
Total/HDL-cholesterol ratio					
Mean ± S.D.	3.47 ± 0.98	4.21 ± 1.20[a]	4.77 ± 1.44[c]	5.27 ± 0.66[e]	< 0.001
Min–max	1.72–6.62	1.75–8.30	2.82–8.03	4.67–6.75	
Triglycerides, mg/dL					
Mean ± S.D.	69.5 ± 38.3	94.2 ± 60.8[a]	121.6 ± 53.3[c]	143.3 ± 64.3[e]	< 0.001
Min–max	16–274	48–379	52–251	94–287	
17Hydroxypregnenolone (17OHP5), nmol/L					
Mean ± S.D.	8.75 ± 7.46	7.39 ± 6.51	6.87 ± 5.92	5.53 ± 2.86	0.029
Min–max	2.06–39.79	1.68–42.46	1.93–24.22	2.66–10.80	
DHEA, nmol/L					
Mean ± S.D.	16.7 ± 12.5	13.9 ± 11.0	14.0 ± 15.3	13.3 ± 6.1	0.141
Min–max	3.4–77.2	2.7–63.1	4.4–60.3	6.9–29.0	
Progesterone (P4), nmol/L					
Median (IQR)	0.251 (0.177–0.318)	0.211 (0.156–0.267)[l]	0.200 (0.156–0.235)[b]	0.156 (0.156–0.242)[m]	< 0.001[#]
Min–max	0.156–0.665	0.156–0.528	0.156–0.436	0.156–0.270	
17Hydroxyprogesterone (17OHP4), nmol/L					
Mean ± S.D.	3.34 ± 1.33	3.09 ± 1.15	2.73 ± 1.30	2.27 ± 0.84[o]	0.001
Min–max	0.94–7.19	1.20–6.86	0.82–6.41	1.24–4.17	
Corticosterone (B), nmol/L					
Mean ± S.D.	9.89 ± 8.88	9.63 ± 8.16	10.53 ± 8.24	6.99 ± 2.97	0.180
Min–max	1.20–49.39	2.25–45.81	2.11–37.41	3.15–11.95	
11Deoxycorticosterone (DOC), nmol/L					
Median (IQR)	0.236 (0.236–0.236)	0.236 (0.236–0.236)	0.236 (0.236–0.236)	0.236 (0.236–0.236)	0.330[#]
Min–max	0.236–0.443	0.236–0.265	0.236–0.236	0.236–0.236	
11Deoxycortisol (11S), nmol/L					
Mean ± S.D.	0.978 ± 0.732	0.975 ± 0.731	1.205 ± 0.927	0.945 ± 0.528	0.855
Min–max	0.225–4.312	0.225–3.764	0.263–4.130	0.358–1.804	

Table 1 Continued.

	Normal weight (NW, *n* = 175)	Overweight (OW, *n* = 106)	Class 1 Obese (OB1, *n* = 24)	Class 2 and 3 Obese (OB2, *n* = 10)	ANOVA trend *P* value
Cortisol (F), nmol/L					
Mean ± S.D.	342.7 ± 98.1	328.3 ± 89.1	359.3 ± 110.2	291.3 ± 58.0	0.186
Min–max	89.4–594.8	144.8–563.1	192.0–628.5	170.2–392.9	
Androstenedione (A4), nmol/L					
Mean ± S.D.	2.08 ± 0.82	1.94 ± 0.70	1.87 ± 0.81	1.87 ± 0.93	0.316
Min–max	0.71–5.07	0.85–4.00	0.89–4.60	0.84–3.13	
Testosterone (T), nmol/L					
Mean ± S.D.	17.5 ± 5.0	16.1 ± 4.9	11.8 ± 3.9[c,d]	9.5 ± 3.8[e,f]	< 0.001
Min–max	5.5–33.3	7.9–34.5	5.6–18.6	5.5–17.9	
Dihydrotestosterone (DHT), nmol/L					
Mean ± S.D.	1.149 ± 0.457	1.028 ± 0.425	0.825 ± 0.251[b]	0.858 ± 0.660	0.060
Min–max	0.512–2.730	0.487–2.515	0.505–1.475	0.531–1.950	
Estrone (E1), nmol/L					
Mean ± S.D.	0.114 ± 0.035	0.124 ± 0.037	0.126 ± 0.040	0.163 ± 0.060[m,q]	< 0.001
Min–max	0.055–0.242	0.057–0.210	0.044–0.206	0.091–0.261	
Estradiol (E2), nmol/L					
Mean ± S.D.	0.080 ± 0.028	0.087 ± 0.025	0.080 ± 0.034	0.088 ± 0.029	0.590
Min–max	0.036–0.198	0.039–0.162	0.040–0.186	0.057–0.137	

Blood was withdrawn between 0800 am and 1000 am after an overnight fasting. Mean values are reported as the back transformed means of the transformed variables, except for 11deoxycorticosterone and progesterone where the median and the interquartilic range (IQR) of the source variable are reported.

[#]Kruskal–Wallis test; [a]NW vs OW, $P < 0.001$; [b]NW vs OB1, $P < 0.010$; [c]NW vs OB1, $P < 0.001$; [d]OW vs OB1, $P < 0.001$; [e]NW vs OB2, $P < 0.001$; [f]OW vs OB2, $P < 0.001$; [g]OB1 vs OB2, $P < 0.001$; [h]NW vs OW, $P < 0.050$; [i]OW vs OB1, $P < 0.010$; [l]NW vs OW, $P < 0.010$; [m]NW vs OB2, $P < 0.010$; [n]OB1 vs OB2, $P < 0.010$; [o]NW vs OB2, $P < 0.050$; [p]OW vs OB1, $P < 0.050$; [q]OW vs OB2, $P < 0.050$.

HDL: high density lipoprotein; HOMA-IR: homeostatic model assessment insulin resistance.

Impact of metabolic risk factors on the estimation of reference limits

LRL and URL from the reference sub-cohorts were compared with lower and upper limits calculated in OW ($n = 140$) for 17OHP4 and DHT, in dysWC ($n = 47$) for F, in dysChol ($n = 46$) for 17OHP5 and B, in dysHOMA-IR ($n = 35$) for T and in OW and/or dysSBP ($n = 150$) subjects for E1, respectively. For 17OHP5, 17OHP4, B and F, comparisons were performed at every year of age, whereas for T, DHT and E1, the age-independent limits were compared (Fig. 2 and Table 4).

Upper limits for B in dysChol at ages 45–59 years were significantly lower compared with URL in norChol, with differences ranging from −9.6 to −10.4 nmol/L (average −10.1 nmol/L, −30.7%, *P*: 0.016–0.048). Moreover, F upper limits in dysWC at ages 46–65 years were significantly lower compared to URL in norWC, with differences ranging from −45 to 127 nmol/L (average −87 nmol/L, −17.6%, *P*: 0.002–0.045). No differences were detected in 17OHP5 and 17OHP4 limits between the reference and dysmetabolic sub-cohorts (Fig. 2).

T lower limit estimated in dysHOMA-IR was significantly lower than the LRL found in norHOMA-IR (−2.29 nmol/L, −26.9%, $P = 0.007$); however, significance was not achieved for the upper limit. At variance, DHT upper limit in OW was significantly lower than the URL calculated in NW (−0.34 nmol/L, −14.5%, $P = 0.045$); however, significance was not achieved for the lower limit. E1 limits calculated in the sub-cohorts of OW and/or dysSBP subjects were not different from reference limits in NW and norSBP sub-cohort (Table 4).

Discussion

The current knowledge about steroid circulating levels has predominantly been generated by immunoassays, often criticized for lacking accuracy, sensitivity and reproducibility. Moreover, steroid precursors and metabolites not measured on a routine basis have largely been neglected (25). Besides improving the quality of the measurement, LC-MS/MS enlarges the panel of measurable steroids, thereby prompting its application in several research areas (1, 10). Therefore, there is a need to re-define normal values and major confounding factors influencing the multitude of steroids now easily measurable in the bloodstream.

Table 2 Impact of age and metabolic parameters on steroid circulating levels.

Steroid variable	Age		BMI		Waist circumference		Systolic blood pressure		HOMA-IR		Total/HDL cholesterol	
	f^2	*P*	f^2	*P*	f^2	*P*	f^2	*P*	f^2	*P*	f^2	*P*
17-Hydroxypregnenolone (17OHP5)	0.51	<0.001 (–)		NS		NS		NS		NS	0.04	0.039 (–)
DHEA	0.86	<0.001 (–)		NS		NS		NS		NS		NS
17-Hydroxyprogesterone (17OHP4)	0.05	0.007 (–)	0.06	0.003 (–)		NS		NS		NS		NS
Corticosterone (B)	0.10	<0.001 (–)		NS		NS		NS		NS	0.09	<0.001 (–)
11-Deoxycortisol (11S)	0.04	0.010 (–)		NS		NS		NS		NS		NS
Cortisol (F)	0.05	0.010 (–)		NS	0.05	0.005 (–)		NS		NS		NS
Androstenedione (A4)	0.19	<0.001 (–)		NS		NS		NS		NS		NS
Testosterone (T)		NS		NS		NS		NS	0.11	<0.001 (–)		NS
Dihydrotestosterone (DHT)		NS	0.06	0.005 (–)		NS		NS		NS		NS
Estrone (E1)		NS	0.05	0.007 (+)		NS	0.03	0.046 (+)		NS		NS
Estradiol (E2)		NS		NS		NS		NS		NS		NS

Data are shown as Cohen's effect size (f^2) and *P* values of the multiple linear regression analysis. The negative (–) or positive (+) nature of the relationship is also reported.
NS: non-significant.

To limit the influence of confounding factors, our subjects selected from the general population were visited by a trained endocrinologist to exclude those affected by any disease or taking drugs. Moreover, we standardized procedural aspects such as the fasting condition, the early morning withdrawal and the avoidance of venepuncture stress. By monitoring easily accessible anthropometric and metabolic parameters, we found that more than half of our apparently healthy individuals displayed variable degrees of adiposity, hypertension, insulin resistance and dyslipidaemia. In the present study, we therefore provide novel data on the impact of excess weight, metabolic impairment and ageing on 13 circulating steroids.

In our cohort, the increasing severity of the obese status was paralleled by a linear decrease in delta-4 and delta-5 steroid precursors and by an imbalance in the

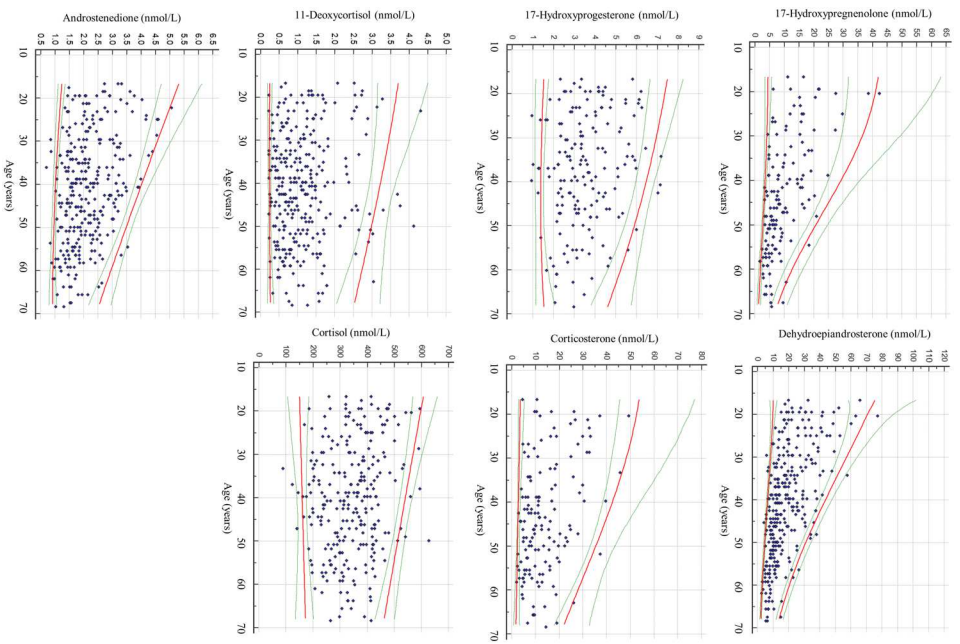

Figure 1
Distribution of steroid hormone levels in serum by age in the reference sub-cohorts. Continuous lines: lower and upper reference limits; dashed lines: 90% confidence intervals.

Table 3 Lower (LRL) and upper (URL) reference limits of age-dependent steroid hormones. Data are reported as the mean - 1.96 × SD. and mean + 1.96 × SD. of hormone distribution for LRL and URL, respectively, as calculated at every 5 years of adult age.

	Age (year)									
	20	25	30	35	40	45	50	55	60	65
17OHP5										
LRL	5.1 (41–6.3)	4.8 (3.9–5.8)	4.4 (3.6–5.2)	4.0 (3.4–4.6)	3.6 (3.0–4.0)	3.2 (2.7–3.4)	2.8 (2.3–3.0)	2.4 (1.8–2.7)	2.1 (1.4–2.4)	1.8 (1.1–2.2)
URL	46.4 (35.1–68.6)	42.4 (32.6–59.4)	38.1 (30.0–50.1)	33.5 (27.1–41.6)	33.5 (27.1–41.6)	33.5 (27.1–41.6)	33.5 (27.1–41.6)	33.5 (27.1–41.6)	33.5 (27.1–41.6)	33.5 (27.1–41.6)
DHEA										
LRL	9.9 (8.4–11.4)	9.9 (8.4–11.4)	9.9 (8.4–11.4)	9.9 (8.4–11.4)	9.9 (8.4–11.4)	9.9 (8.4–11.4)	9.9 (8.4–11.4)	9.9 (8.4–11.4)	9.9 (8.4–11.4)	9.9 (8.4–11.4)
URL	70.8 (58.9–87.4)	70.8 (58.9–87.4)	70.8 (58.9–87.4)	70.8 (58.9–87.4)	70.8 (58.9–87.4)	70.8 (58.9–87.4)	70.8 (58.9–87.4)	70.8 (58.9–87.4)	70.8 (58.9–87.4)	70.8 (58.9–87.4)
17OHP4										
LRL	1.50 (1.13–1.71)	1.50 (1.13–1.71)	1.50 (1.13–1.71)	1.50 (1.13–1.71)	1.50 (1.13–1.71)	1.50 (1.13–1.71)	1.50 (1.13–1.71)	1.50 (1.13–1.71)	1.50 (1.13–1.71)	1.50 (1.13–1.71)
URL	7.36 (6.57–8.05)	7.36 (6.57–8.05)	7.36 (6.57–8.05)	7.36 (6.57–8.05)	7.36 (6.57–8.05)	7.36 (6.57–8.05)	7.36 (6.57–8.05)	7.36 (6.57–8.05)	7.36 (6.57–8.05)	7.36 (6.57–8.05)
Cort. (B)										
LRL	3.5 (2.8–5.0)	3.5 (2.8–5.0)	3.5 (2.8–5.0)	3.5 (2.8–5.0)	3.5 (2.8–5.0)	3.5 (2.8–5.0)	3.5 (2.8–5.0)	3.5 (2.8–5.0)	3.5 (2.8–5.0)	3.5 (2.8–5.0)
URL	53.0 (46.0–77.3)	53.0 (46.0–77.3)	53.0 (46.0–77.3)	53.0 (46.0–77.3)	53.0 (46.0–77.3)	53.0 (46.0–77.3)	53.0 (46.0–77.3)	53.0 (46.0–77.3)	53.0 (46.0–77.3)	53.0 (46.0–77.3)
11S										
LRL	0.24 (0.19–0.31)	0.24 (0.19–0.31)	0.24 (0.19–0.31)	0.24 (0.19–0.31)	0.24 (0.19–0.31)	0.24 (0.19–0.31)	0.24 (0.19–0.31)	0.24 (0.19–0.31)	0.24 (0.19–0.31)	0.24 (0.19–0.31)
URL	3.65 (3.15–4.40)	3.65 (3.15–4.40)	3.65 (3.15–4.40)	3.65 (3.15–4.40)	3.65 (3.15–4.40)	3.65 (3.15–4.40)	3.65 (3.15–4.40)	3.65 (3.15–4.40)	3.65 (3.15–4.40)	3.65 (3.15–4.40)
Cortisol										
LRL	152 (111–182)	152 (111–182)	152 (111–182)	152 (111–182)	152 (111–182)	152 (111–182)	152 (111–182)	152 (111–182)	152 (111–182)	152 (111–182)
URL	598 (564–645)	598 (564–645)	598 (564–645)	598 (564–645)	598 (564–645)	598 (564–645)	598 (564–645)	598 (564–645)	598 (564–645)	598 (564–645)
A4										
LRL	1.20 (1.08–1.30)	1.20 (1.08–1.30)	1.20 (1.08–1.30)	1.20 (1.08–1.30)	1.20 (1.08–1.30)	1.20 (1.08–1.30)	1.20 (1.08–1.30)	1.20 (1.08–1.30)	1.20 (1.08–1.30)	1.20 (1.08–1.30)
URL	5.13 (4.58–5.85)	5.13 (4.58–5.85)	5.13 (4.58–5.85)	5.13 (4.58–5.85)	5.13 (4.58–5.85)	5.13 (4.58–5.85)	5.13 (4.58–5.85)	5.13 (4.58–5.85)	5.13 (4.58–5.85)	5.13 (4.58–5.85)

17OHP5, 17–hydroxypregnenolone; DHEA, dehydroepiandrosterone; 17OHP4, 17–hydroxyprogesterone; Cort. (B), cortisosterone; A4, androstenedione

sex steroids T, DHT and E1. Besides, corticosteroids, pro-androgens and E2 levels were unvaried. As excess weight and ageing are most often clustering with insulin resistance, dyslipidaemia and hypertension, we tried to dissect which specific alteration was independently influencing steroid levels. By this approach, we found that ageing has no independent effect on T, DHT, E1 and E2 levels. Conversely, BMI is independently related with lowering DHT and increasing E1. Such a finding is in line with the known androgen/estrogen imbalance resulting by the increased pro-androgens aromatization and by the elevated 3α-reduction of DHT in the hypertrophic adipose tissue (26). Among the several studies focused on the relationship between ageing, obesity, metabolic alterations and the male HPG, only a few used MS-based steroid assays. Recently, Travison *et al.* showed a 30% decrease in T LRL from age 40 to 80 years in non-obese men, also reporting that, when obese individuals where included in the reference population, the LRL was further reduced down to 53% (27). In another study in which 40–80 years old men were reporting very good or excellent health, obesity had an age-independent relationship with lowering T and DHT and increasing E1 and E2 (28). In the same study (28) and in others (29, 30), sex steroids were not or only minimally associated with ageing. Finally, Kelsey *et al.* derived from multiple studies that T levels peak around age 19 years and that no decline occurred after age 40 years (31). Taken together, these data suggest that age-related changes in sex steroids may rather be due to comorbidities accumulating with ageing than to ageing *per se* (28, 29, 30, 31). In partial agreement with these findings, we found HOMA-IR as the sole independent determinant of T levels. However, our study lacks sex hormone binding globulin and free T measurements, which could have helped in better characterizing the apparent hypotestosteronemia in men with reduced insulin sensitivity. In line with recent data (32), E2 levels in our cohort were not influenced by any of the tested parameters.

At variance from sex steroids, in our hands, steroid precursors and corticosteroids showed a steep decrease with ageing. The extent of the decrease was particularly relevant for DHEA and 17OHP5, and moderate for A4 and B, overall showing a reduction from age 20 to 60 years ranging between 20 and 70%. A 30% decline or less was also detected in 17OHP4, 11S and F URL across the same age range. Interestingly, when adjusting for age and metabolic parameters, the effect of BMI was only maintained on 17OHP4. As 17OHP4 is a crucial crossway precursor for both the gonadal and the adrenal steroidogenesis, we

Table 4 Upper and lower reference limits of age-independent serum steroids calculated in the reference sub-cohorts, and comparison with dysmetabolic sub-cohorts.

Steroid hormone	Reference sub-cohort				Dysmetabolic sub-cohort					
	Features	*n*	LRL (90% CI) nmol/L	URL (90% CI) nmol/L	Features	*n*	LL (90% CI) nmol/L	*P* value*	UL (90% CI) nmol/L	*P* value#
Testosterone	norHOMA-IR	132	8.51 (7.86–9.21)	27.80 (25.89–29.84)	dysHOMA-IR	35	6.22 (5.21–7.40)	***0.007***	23.46 (20.08–27.36)	*0.099*
Dihydrotestosterone	NW	175	0.54 (0.49–0.59)	2.35 (2.16–2.54)	OW	140	0.46 (0.42–0.52)	*0.074*	2.01 (1.83–2.22)	***0.045***
Estrone	NW and norSBP	63	0.059 (0.052–0.067)	0.202 (0.182–0.224)	OW and/or dysSBP	150	0.066 (0.061–0.071)	*0.207*	0.223 (0.209–0.239)	*0.186*
Estradiol	all	315	0.043 (0.041–0.046)	0.151 (0.143–0.159)		NA	NA	NA	NA	NA

P values refer to Z-test comparison of LRL (*) and URL (#) between dysmetabolic and reference sub-cohorts.
CI, confidence interval; dysChol, total/HDL-cholesterol ratio ≥5; dysHOMA-IR, homeostatic model assessment – insulin resistance ≥2.5; dysSBP, systolic blood pressure ≥145 mmHg; dysTG, triglycerides ≥150mg/dL; LL, lower limits; LRL, lower reference limit; NA, not applicable; norChol, total/HDL-cholesterol ratio <5; norHOMA-IR, homeostatic model assessment – insulin resistance <2.5; norSBP, systolic blood pressure ≤130 mmHg; norTG, triglycerides < 150mg/dL; NW, normal weight (BMI <25.0 kg/m2 and waist circumference <102 cm); OW, overweight-obese (BMI ≥25.0 kg/m2 and/ or waist circumference ≥102 cm); UL, upper limits; URL: upper reference limit.

cannot postulate whether this association affects the HPA or the HPG. Furthermore, we showed an inverse association between F and abdominal obesity. Such a finding is in apparent contrast with the known mutual

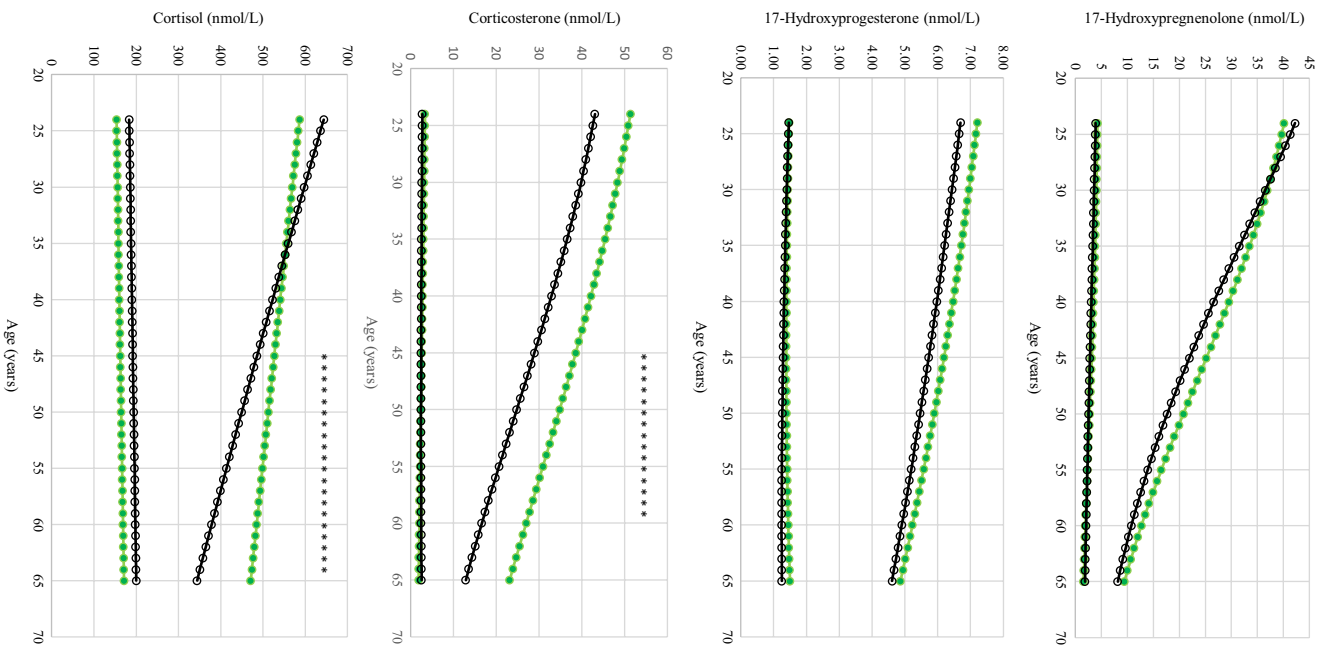

Figure 2
Steroid lower and upper limits per year of age in reference and dysmetabolic sub-cohorts. Black lines and white dots: lower and upper limits calculated in the reference sub-cohorts. Green lines and dots: lower and upper limits calculated in the dysmetabolic sub-cohorts. *$P < 0.05$.

association of chronic hypercortisolism with visceral fat accumulation and metabolic impairment. Nonetheless, in agreement with our finding, low early morning F levels were previously described in obese individuals, and the paradox was explained by the loss of HPA circadian rhythmicity, inducing low F secretion in the morning and high in the evening, and by the increased F clearance (33, 34). At variance from previous studies, we did not observe any association between F and adverse metabolic features, possibly because our cohort did not overall exhibit severe metabolic impairments (33).

We do not have a clear explanation for the inverse relationship of the total/HDL-cholesterol ratio with 17OHP5 and B. However, according to Cherradi and coauthors, HDL represents a major source for angiotensin II-mediated mineralocorticoid biosynthesis in adrenal cells, as assessed by measuring aldosterone and pregnenolone (35). We can therefore speculate that the positive association of 17OHP5, the direct product of pregnenolone, and B, the direct precursor of aldosterone, with HDL relative abundance in the cholesterol pool, could possibly be dependent on biochemical rather than dysmetabolic mechanisms.

No full consensus exists among the few recent studies analysing the impact of ageing, obesity and comorbidities on large circulating LC-MS/MS steroid profiles. Similar to our results, Eisenhofer *et al*. described an age decline in men from 22 to 70 years for DHEA, 17OHP4, B, 11S, F and A4 and an inverse association between BMI and P4, 17OHP4, DOC, B, F and T, but not 11S and A4. They also found an inverse association between hypertension and 17OHP4; however, they did not evaluate the effect of insulin resistance and dyslipidemia (36). Damgaard-Olesen and co-authors reported an age decline in men aged 30–60 years in 17OHP5, DHEA and A4, but not in 17OHP4 levels. They also described the negative impact of BMI on DHEA, 17OHP4, A4 and T and of metabolic syndrome on DHEA, 17OHP4 and T; however, relationships between steroids and individual metabolic parameters were not reported (37). Two other recent studies confirmed the age-decline in 17OHP5, DHEA and A4 in men of similar age (29, 30).

Analysing steroid relationships with various comorbidities allowed us to define specific reference sub-cohorts with the largest possible sample size, thus ensuring both unbiased and robust RI estimation. We also showed that, when calculated in dysmetabolic sub-cohorts, T, DHT, F and B limits were significantly different from reference limits, thereby demonstrating that tolerating reduced insulin sensitivity, overweight/obesity, excess visceral fat and dyslipidaemia may result in biased reference limit estimation. Hence, our study suggests that even mild metabolic imbalances are able to affect steroid secretion and metabolism, resulting in an altered circulating profile.

As reported in our recent review (9), consistent disagreements are found among RI reported so far, which are more severe for steroids usually not measured in the clinical routine (27, 29, 30, 36, 37, 38, 39, 40, 41). Reasons for such a disagreement in steroid levels as well as in their association with anthropometric and metabolic parameters could be found at different levels. As our data demonstrate, the clinical and biochemical characterization of the reference cohort influences steroid values. The metabolic parameters that we monitored are commonly available in routine clinical laboratories and should therefore be always considered. Most of the previous studies apparently did not exclude subjects taking drugs, some of them excluding steroidal drugs (29, 30, 36, 37, 38, 39, 40, 41). The exact time of blood withdrawal and the fasting status, both of which are known to impact steroid levels, are often not specified, whereas measures to avoid venepuncture stress were apparently not taken (9). The impact of the latter on cortisol secretion was repeatedly reported in literature (42). Conversely, to the best of our knowledge, no data are available on the impact of venepuncture stress on glucocorticoid metabolites and on androgen precursors. Such an aspect should therefore be investigated as a potential non-negligible confounding factor undermining the interpretation of information generated by novel large steroid-profiling techniques and their efficacy in clinical settings. In addition, the multivariate design may influence the associative observations. As to the elaboration of age-dependent data, our approach provided a continuous age modeling of RI based on a reasonable number of subjects, thus obviating data partitioning into arbitrary age categories (13, 23). Finally, even though LC-MS/MS is supposed to guarantee high accuracy, analytical performances may vary among different assays in terms of sensitivity, specificity and calibration. Limited data are available about the comparability among LC-MS/MS assays for serum steroids, mainly focusing on T and a few others. Though these data overall demonstrated the good inter-laboratory performance of LC-MS/MS (18, 43, 44, 45), no data are yet available on the majority of steroids included in modern large LC-MS/MS panels, and no certified materials or external quality assessment exist for many of them.

In conclusion, our study showed how excess weight and metabolic impairments frequently occurring in the

general unmedicated adult population can significantly influence the circulating steroid profile and affect the RI estimation. Moreover, we provided age-specific RIs for seven steroids and unique RI for six other steroids to be used in the clinical management of several endocrine diseases. Future studies will be needed to test the efficacy of our RI in clinical settings, both for steroids with known diagnostic relevance and for those hormones and metabolites still neglected by the current clinical paradigms. Partial agreement with previous data was highlighted, thereby witnessing the urgent need for an overall harmonization in preanalytical and analytical aspects of steroid measurement that will definitely allow the harmonization of steroid reference values.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/EJE-19-0928.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

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Author contribution statement

M M measured study samples, performed the statistical analysis and wrote the manuscript. G D D, M B, A G, V V and C P performed cohort recruitment and examination. A F measured study samples. U P designed the population study and wrote the manuscript. F F designed the study and wrote the manuscript.

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