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**Title:** Visceral and Subcutaneous Adipose Tissue Assessed by Magnetic Resonance Imaging in Relation to Circulating Androgens, SHBG, and LH in Young Men

**Short title:** Visceral and Subcutaneous Adipose Tissue in Relation to Circulating Androgens

**Authors:** Torben Leo Nielsen[1], Claus Hagen[1], Kristian Wraae[1], Kim Brixen[1], Per Hyltoft Petersen[2], Egil Haug[3], Rasmus Larsen[4], and Marianne Andersen[1]

**Affiliations:** [1] Department of Endocrinology, Odense University Hospital, Denmark;
[2] NOKLUS - Norwegian quality improvement of primary care laboratories, Division for General Practice, University of Bergen, Norway;
[3] Hormone Laboratory, Aker University Hospital, Oslo, Norway;
[4] Department of Informatics and Mathematical Modeling, Technical University of Denmark, Copenhagen, Denmark.

**Corresponding author and reprint requests:** Torben Leo Nielsen
**mail:** Torben Leo Nielsen, Dyrupgardvaenget 76, 5250 Odense SV, Denmark.
**E-mail:** Torben@dsa-net.dk **Phone, day:** +45 50947257 **evening:** +45 66171338

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**ABSTRACT**

**Context:** No large studies of young men have examined circulating sex hormones in relation to visceral and subcutaneous adipose tissues (VAT and SAT).

**Objective:** To investigate the role of VAT and SAT on circulating sex hormones and the impact of obesity on sex hormone reference-intervals.

**Design, Setting, and Participants:** Population-based study of 783 Danish, 20-29 year-old men employing dual-energy X-ray absorptiometry in all men and magnetic resonance imaging in 406 men.

**Main Outcome Measures:** Total, bioavailable, and free testosterone (TT, BT, FT), dihydrotestosterone (DHT), total and bioavailable estradiol ($E_2$ and $BE_2$), SHBG, and LH.

**Results:** In multiple regressions, VAT was an independent, inverse correlate of BT and FT. SAT correlated negatively with SHBG and positively with $BE_2$ adjusted for TT. Both VAT and SAT correlated inversely with TT and DHT: Adjusting for SHBG, only VAT remained significantly correlated. Low TT in viscerally obese men was not accompanied by increased LH. The androgen reference-intervals were significantly displaced towards lower limits in obese vs. non-obese men (TT: 8.5-29.3 nmol/l vs. 12.5-37.6 nmol/l; BT: 6.1-16.9 nmol/l vs. 7.6-20.7 nmol/l; FT: 0.29-0.78 nmol/l vs. 0.23-0.67 nmol/l; and DHT: 0.63-2.5 nmol/l vs. 0.85-3.2 nmol/l), while $E_2$ (36.5-166 pmol/l) and $BE_2$ (23.4-120 pmol/l) reference-intervals were not. In obese men, 22.9% had TT<12.5 nmol/l.

**Conclusions:** VAT correlate independently with BT and FT in young men. The inverse relationship between TT and SAT seems to be accounted for by variations in SHBG. The reference-intervals for TT, BT, FT, and DHT are displaced towards lower limits in obese men.

Inverse relations between serum total testosterone (TT) and fat mass have been shown in both observational and interventional studies(1-7). Some authors observed an inverse, linear relationship between free and bioavailable testosterone (FT and BT)(7), others reported reduced FT or BT in severely obese men(2), and some found no significant relationships(8;9). Some studies indicate a disruption of the hypothalamo-pituitary-gonadal axis with attenuated LH pulse amplitude in morbidly obese men(2;10). The few existing studies on the type of adiposity related with low androgen levels are inconsistent(1;4;11).

The diagnosis of androgen deficiency is uncomplicated in severe cases, but reference-intervals are necessary due to unspecific symptoms(12) and due to the growing use of androgen replacement therapy(13). Reference-intervals may lack statistical power and can be biased due to: 1) Unintentional inclusion of hypogonadal subjects, which can be limited by testicular examination and a medical history focusing on testicular, pituitary, and chronic diseases(14), medication(15-19), excessive alcohol intake(20), and anabolic steroid abuse. 2) Failure to recruit a population-based sample not mirroring the general population. 3) Inappropriate timing of blood sampling. 4) Use of inaccurate testosterone assays: Commercially available direct immunoassays for assessment of TT are widely used, but these assays suffer from lack of accuracy(21-24). An impact of obesity on androgen reference-intervals has not been reported, but the increasing prevalence of obesity and the variation in prevalence between regions may contribute to different reference-intervals.

We examined a population-based cohort of 783 Caucasian males, aged 20-29 years to investigate the impact of obesity on reference-intervals for serum TT, BT, FT, androstenedione (Δ4AD), dihydrotestosterone (DHT), estradiol ($E_2$), and bioavailable $E_2$ ($BE_2$). In addition, we addressed the following questions: 1) Are FT, BT, Δ4AD, DHT, $E_2$, and $BE_2$ related with adiposity in young men? 2) Are variations in sex hormones primarily attributable to central fat mass (CFM)? 3) If so,

are subcutaneous adipose tissue (SAT), visceral adipose tissue (VAT), or both involved? 4) Does LH correlate with specific types of adipose tissues?

## METHODS

### Subjects

Odense Androgen Study is a population-based study of 783 Caucasian men aged 20-29 years, living in Funen County, Denmark. The study population is described in detail elsewhere(25). In brief, questionnaires were mailed to 3,000 men, randomly drawn from the Danish Central Personal Registry (fig. 1). The 2,042 respondents were invited of whom 784 were included. One man dropped out. A medical history of hospitalization, chronic disease, medication, alcohol intake, and anabolic steroid abuse was obtained by questionnaire, by interview, and from electronic hospital records. A systematic physical examination was performed including testicular volume estimation using an orchidometer. Waist circumference (WC) was measured between the iliac crest and the lower ribs during expiration. A cut-off of 102 cm was used to define obesity(26). BMI was computed from body weight and height and a BMI$\geq$30 kg/m$^2$ was used as a second definition of obesity. The intraobserver CV's for BMI and WC were 1.2% and 1.7%, respectively. The 783 men matched the county population as regards body mass index (BMI), chronic disease, medication, physical activity, tobacco exposure, and sociodemography(25). The examinations started in March 2002 and terminated in May 2003. The local Ethic Review Board approved the study (#20010198), which was conducted according to the Declaration of Helsinki.

### Low-risk reference-population

We attempted to select a young, male reference-population at low risk for secondary androgen deficiency (n=685) by ruling out subjects meeting the following pre-specified criteria: history of

bilateral cryptorchidism or congenital hydrocele, small testes (both less than 9 ml), chronic disease, average alcohol intake≥6 units/day, anabolic steroid abuse, hypogonadotropism (serum LH<1.0 U/l), and subclinical hypothyroidism (serum TSH>6.0 mU/l). Chronic disease was subdivided into no medication or continuous systemic medication including high doses of inhaled corticosteroids (budesonide≥800 µg/day and fluticasone≥1,000 µg/day). The rule-out was performed prior to sex hormone assessments. The low-risk reference-population was partitioned into obese and non-obese men.

**Biologic within subject variation**

The biologic within subject variation was determined for all serum measurements in 20 men participating twice within 3-6 weeks. Their serum samples were analyzed in the same assay. Taking the analytical variation ($CV_{INTRAASSAY}$) into account, the biologic within subject variation ($CV_{BIOLOGIC}$) was derived from the total within subject variation ($CV_{TOTAL\text{-}WITHIN}$) by the formula: $(CV_{BIOLOGIC})^2=(CV_{TOTAL\text{-}WITHIN})^2-(CV_{INTRAASSAY})^2$.

**Biochemistry**

Fasting, venous blood drawn between 8 and 10 a.m. were centrifuged and serum aliquots were stored at -80ºC. TT, Δ4AD, and DHT were measured using an in-house RIA(27;28) employing extraction and chromatography (intraassay CV's: 8.2%, 9.4%, and 9.1%; interassay CV's: 13.8%, 11.4%, and 11.0%; $CV_{BIOLOGIC}$: 15.8%, 11.9%, and 12.1%, respectively). The accuracy of the TT assay is continuously monitored in an external quality assessment program (German Society of Clinical Chemistry). The mean bias estimated from 20 independent control samples was +5.4% (95% CI: +1.2 to +9.7 %. $E_2$ was measured using an in-house RIA(27;28) after extraction and chromatography (intraassay, interassay, and $CV_{BIOLOGIC}$: 7.4%, 10.5%, and 16.8%). In 48 men, $E_2$

assessments below the limit of detection (40 pmol/l) were substituted by values one unit below the detection-limit(13;29;30).

SHBG was measured by an immunoluminometric assay (Immulite 2000, DPC, Los Angeles, CA; intraassay, interassay, and $CV_{BIOLOGIC}$: 3.0%, 5.0%, and 8.8%, respectively) and albumin by the bromine-cresol-green method (Roche/Hitachi-917, Roche Diagnostics; intraassay, interassay, and $CV_{BIOLOGIC}$: 0.7%, 2.0%, and 2.6%, respectively). LH and TSH were assessed by immuno-fluorometric assays (DELFIA, Wallac, Finland). Intraassay, interassay, and $CV_{BIOLOGIC}$ were 5%, 6%, 26.4% and 3.0%, 5.0%, 27.9%, respectively.

BT, FT, and $BE_2$ were calculated from validated formulas(31).

$$FT = \frac{-B+SQRT(B^2-4 \bullet A \bullet C)}{2 \bullet A}$$

where:

$$A = K_{SHBG} \bullet (K_{ALBUMIN} \bullet albumin + 1)$$

$$B = K_{SHBG} \bullet (SHBG - TT) + K_{ALBUMIN} \bullet albumin + 1$$

$$C = -TT$$

BT was calculated by:

$$BT = \frac{FT + K_{ALBUMIN} \bullet albumin \bullet FT}{1 + K_{ALBUMIN} \bullet FT}$$

The association-constants were: $K_{SHBG}$: $1.0 \bullet 10^9$ l/mol, $K_{ALBUMIN}$: $3.6 \bullet 10^4$ l/mol(32).

$BE_2$ was calculated substituting TT, FT, and BT in the formulas with $E_2$, free $E_2$, and $BE_2$ and association-constants for $K_{SHBG,E2}$ of $0.68 \bullet 10^9$ l/mol and $K_{ALBUMIN,E2}$ of $6.0 \bullet 10^4$ l/mol(33).

**Clinical and paraclinical fat parameters**

CFM and lower extremity fat mass (LEFM) were measured by dual-energy X-ray absorptiometry (DXA) using a Hologic4500A densitometer (Waltham, MA). The $CV_{TOTAL-WITHIN}$ for CFM and

LEFM between two scans were 5.8% and 4.0%, respectively. Magnetic resonance imaging (MRI) was performed in the first 406 consecutively included subjects with an open, low field (0.2 Tesla) MR unit (Magnetom Open Viva, Siemens AG, Germany). Three abdominal slices (10 mm thick, 20 mm apart, lower slice at the dorsal, intervertebral space of L4/L5) were recorded using an axial, T1-weighted gradient-echo sequence (repetition time: 450 msec, echo time: 15 msec, acquisition matrix: 512 x 288, field of view: 400 mm). A bias correction algorithm was developed to ensure uniform pixel intensities of adipose tissue throughout all images(34). Following subtraction of perivertebral and bone marrow fat, the area of total abdominal adipose tissue (TAT) was assessed from bimodal histograms discriminating between adipose and non-adipose tissue (Adobe Photoshop 7.0). The visceral compartment was demarcated and the VAT area was quantified. SAT was computed subtracting VAT from TAT. Finally, the areas of the three slices were integrated into volumes. The measurements were initiated when the intraobserver CV for VAT in repeated pilot determinations of 51 images fell below 10%. The intraobserver CV's for TAT, SAT and VAT were 3.4%, 1.7% and 7.2%, respectively. The correlation coefficient between TAT and CFM was 0.964.

**Data analysis**

Natural logarithm transformations were performed to obtain Gaussian distributions of the serum parameters and the DXA- and MRI parameters. In bar charts, LEFM, CFM, VAT and SAT were categorized by means of SD scores: <-1.0, [-1.0;-0.5[, [-0.5;0.0[, [0.0;0.5[, [0.5;1.0[, and ≥1.0 (Table 1). Linear regression analysis was used to test for univariate, linear trends between the SD-score and hormonal concentrations in the low-risk reference-population. The variation in androgen levels (dependent variables) in relation to LEFM vs. CFM were analyzed including all men (except three anabolic steroid abusers) using multiple regression. These analyses were performed using continuous SD-scores with adjustment for chronic disease/medication and were repeated for SAT

vs. VAT. If both independents were significantly correlated with TT, DHT, or $E_2$, additional analyses were performed adjusting for SHBG.

The reference-intervals (defined by the 2.5 and 97.5 percentiles) were established by rankit plots(35): The ordinate of the cumulative standard normal distribution was transformed from percentiles to rankits. For each reference-interval, the method proposed by Lahti *et al*(36) was used to interpret, whether a partitioning of the reference-population into obese and non-obese men was advisable. The androgen levels of the ruled out men were compared to the low-risk reference-population using multiple regression analysis to adjust for WC. The odds-ratios of having a TT level below the lower limit of non-obese men were calculated for the obese men and the ruled out men by logistic regression. The level of significance was set at $p<0.05$. Data were analyzed using Stata Statistics/Data Analysis software version 8.2 (StataCorp, College Station, TX).

**RESULTS**

The low-risk reference-population comprised 685 men in whom the serum TT reference-interval was 11.7-37.9 nmol/l (95% CI's: 11.2-12.1 nmol/l and 36.3-39.2 nmol/l, respectively). The reference-intervals for TT, BT, FT, DHT, and Δ4AD are shown in Table 2. Defining obesity as WC≥102 cm and BMI≥30 kg/m$^2$, the prevalence of obesity in the low-risk reference-population was 10.2% (n=70) and 8.0% (n=55), respectively. The median WC and BMI in the 685 men were: 88.0 cm (range: 68.5-131.0 cm) and 24.2 kg/m$^2$ (range: 16.0-39.8 kg/m$^2$), respectively. Within the low-risk reference-population, the distributions of all androgens (except Δ4AD) were significantly displaced to the left in obese men (WC≥102 cm), while $E_2$ and $BE_2$ were not (Table 2). Partitioning was suggested for TT, BT, FT, and DHT (partitioning is reasonable, when the decision for one limit is definite and the other ambiguous)(36). Almost identical reference-intervals were established defining obesity by BMI (data not shown). Sixteen obese men in the low-risk reference-population

(22.9%) had TT levels below the lower limit (12.5 nmol/l) of non-obese men (odds ratio: 10.4, 95% CI: 4.8-22.5). Only seven of these sixteen men (43.8%), had BT levels below the lower limit for BT.

A total of 98 subjects were ruled out of the low-risk reference-population. Table 3 shows the serum concentrations of TT, BT, DHT, Δ4AD, LH, $E_2$, and $BE_2$ in the low-risk reference-population and the ruled out subjects. TT was significantly decreased in all ruled out groups, except for bilateral cryptorchidism, high alcohol intake, and subclinical hypothyroidism. The unaltered TT in cryptorchidism was accompanied by significantly elevated LH concentrations. TT concentrations below 12.5 nmol/l were seen in four men with small testes (odds ratio: 8.5, 95% CI: 2.3-31.5) and in six men treated with prednisolone, tramadol, valproic acid (2 men), lamotrigine, and cimetidine (odds ratio: 4.1, 95% CI: 1.4-11.7). Extremely low TT, BT, DHT, and LH were found in two abusers of anabolic steroids. Adjusting for WC, the 98 ruled out men had significantly reduced TT, BT, DHT, and $E_2$ compared to the low-risk reference-population and 15 of the 98 men (15.3%) had TT levels below 12.5 nmol/l (odds ratio: 3.6, 95% CI: 1.8-7.4).

Within the low-risk reference-population, SHBG, TT, BT, and DHT were inversely correlated with CFM and LEFM in univariate analyses (fig. 2). We found a non-significant inverse trend between $E_2$ and CFM and LEFM, but after adjustment for TT, both $E_2$ and $BE_2$ correlated positively with CFM and $BE_2$ also with LEFM (fig. 3). In multiple regression analyses, CFM was independently correlated with reduced SHBG, TT, BT, FT, and DHT, while the adjusted measure of $BE_2$ correlated positively with CFM. LEFM correlated positively with SHBG (Table 4).

The 406 men examined by MRI comprised 363 subjects within the low-risk reference-population. In univariate analyses, inverse relationships were found between SHBG, TT, BT, DHT, $E_2$, and the volumes of both VAT and SAT (fig. 2 and 3). Multiple regressions (Table 4) demonstrated that: VAT was independently related with reduced BT and FT, while SAT was not; SAT was

independently related with reduced SHBG, while VAT was not; both VAT and SAT were negatively correlated with TT and DHT, but only VAT attained significance when adjusted for SHBG. Finally, no significant relationships were observed with $E_2$ and $BE_2$, but SAT was an independent correlate of the adjusted measure of $BE_2$.

LH increased linearly with increasing VAT ($p=0.004$) when omitting the most viscerally obese men (fig. 3) and was significantly elevated in the group of men with the second largest VAT volumes compared to the leanest ($p=0.02$) and to the utmost viscerally obese men ($p=0.006$). There were no relations between LH and SAT, CFM or LEFM.

The study population was included over a year: TT and BT were 1.7 and 1.0 nmol/l higher from April-September vs. October-March ($p<0.01$). BT declined by an average 0.17 nmol/l/year in these 20-29 year old men ($p<0.0001$), while VAT and SAT increased by 2.4 and 3.5 $cm^2$/year (mean of the three slices, $p<10^6$ and $p=0.017$, respectively). These findings did not change any relationships reported above. The hour of blood sampling did not significantly influence the concentrations.

## DISCUSSION

Our population-based data obtained in healthy young men demonstrate a linear, inverse relationship between CFM and TT, and also between CFM and BT, FT, and DHT. The use of abdominal MRI in a large number of consecutively included subjects showed that the decreased BT and FT were attributable to VAT. This is in line with the observations by Seidell *et al* in 23 healthy 25-50 year old men(4), whereas no relation between FT and VAT was found in a study of seventeen obese men aged 25-69 years(11). We are not aware of any large, population-based studies of young men that examined these issues with actual measures of VAT and SAT. Couillard *et al*(1) used computerized tomography to examine the relationship between VAT, SAT, and androgens in 217 men aged 17-64

years. They did not include measures of FT or BT, but found that TT correlated inversely with SAT, but not with VAT.

Our data obtained in relatively lean young men (BMI≥35 kg/$m^2$: N=6; BMI≥40 kg/$m^2$: none) demonstrate that reduced BT and FT are not restricted to massively obese men. Thus, reduced TT in obese men is not uniquely secondary to declines in SHBG levels. However, we did find that part of the decline in TT could be accounted for by declining SHBG levels observed with increasing SAT. This is in agreement with the study by Couillard *et al*(1). In studies of 40-70 year-old men (37;38), testosterone as well as SHBG has been linked with the metabolic syndrome and excess VAT. The increase in VAT by age(1) may result in diverging relationships in younger vs. older cohorts. We demonstrated that this increase in VAT was highly significant and well under way from age 20 to 29 years. The positive correlations between CFM and the TT adjusted measures of $E_2$ and $BE_2$ are anticipated signs of increased aromatase activity in obese men. The independent relationship between SAT and BE2 adjusted for TT is in line with previous findings(39). The lack of relationship with the unadjusted measures may be explained by reduced levels of substrate (testosterone) in the obese men and a lower aromatase activity in young compared to older men(39), in whom $E_2$ increases with increasing adiposity(40;41). Besides variations in substrate availability and aromatase activity, the decline in SHBG in obesity may also subject a higher proportion of non-SHBG bound androgens to metabolism. Schneider *et al* found an increased hormonal clearance of androgens to estrogens in obese men(41). However, the extraglandular formation of $E_2$ by aromatization from TT constitute less than 1%(42) of the approximately 5-6 mg of TT produced daily by the testes in normal men. Therefore, it is less likely that increased metabolic clearance rates in the presence of normal production rates can account for the inverse relation between adiposity and androgen levels

Our observation of increased LH and concomitant declines in TT and BT with increasing VAT may suggest an intact function of the hypothalamic-pituitary feedback system in response to declining Leydig cell function. The abrupt drop in LH levels in the most viscerally obese subjects may reflect a sudden incapacity of the pituitary to keep up its LH secretion. Our observation is in line with previous studies reporting an attenuation of LH pulse amplitude in severely obese men(2;10). The identification of a possible link between visceral obesity and decreased LH levels would be a significant discovery. Free fatty acids have been linked with inhibition of another anterior pituitary hormone, growth hormone(43;44). The enrollment of more massively obese men could possibly have provided further evidence for a biphasic relationship, but the population-based design of the study opposed such a selection. At the other end, only three men were underweight (BMI<18.5 kg/m$^2$), so the parabolic relationship was not due to enrollment of underweight or anorectic subjects, in whom hypogonadotropic hypogonadism is common(45). In addition to attenuation of LH pulse amplitudes and aromatization of androgens to estrogens, Isidori *et al*(46) have proposed that increased leptin levels in obese men may inhibit Leydig cell function.

Our partitioning of the reference-population into obese and non-obese subjects produced significantly different reference-intervals for TT, BT, FT, and DHT. This has not been shown previously. We suggest that reference-intervals for these androgens should be established in non-obese men, as nearly one in every four obese men had TT levels below the reference-limit of non-obese men. However, the diagnosis of androgen deficiency in obese men necessitate additional measures of either FT or BT, as only every second obese man with low TT had BT levels below the lower limit of BT in non-obese men. This study is the first to present statistical reference-intervals for TT, BT, FT, and DHT in obese, but otherwise healthy, young men: We found that TT, BT, and FT levels as low as 8.5, 6.1, and 0.23 nmol/l, respectively, are within the statistically expected range in these men: These limits may be useful to discriminate between severely, hypogonadal, obese men

and men with subnormal levels secondary to the obesity itself. A reversibility of the latter condition has been documented by Niskanen *et al*(3), who showed that weight loss and subsequent long term weight maintenance in obese men normalized hypogonadal TT and BT levels in 70 and 50%, respectively. However, obese men with low androgen levels may benefit from androgen replacement therapy, as the relation between androgen levels and obesity is probably bidirectional(6;47): Woodhouse *et al* found a linear relationship between adiposity and the dose of testosterone administered to young subjects who had their endogenous androgen production blocked(6). The subjects receiving the lowest dose experienced a drop in TT from mid-normal to hypogonadal levels with a concomitant increase in total, central, and peripheral fat mass. A similar pattern was observed in a parallel study of older men(48). Moreover, testosterone and DHT inhibit the differentiation of mesenchymal pluripotent stem cells towards the adipogenic lineage and stimulate the commitment into the myogenic lineage via the androgen receptor. In the recently published clinical guidelines from The Endocrine Society, increased body fat are listed only amongst the less specific signs associated with androgen deficiency(12). So far, there is not sufficient evidence to support androgen replacement therapy in obese, but otherwise asymptomatic men with low TT.

Future studies combining clinical and paraclinical signs and symptoms of androgen deficiency with androgen measurements may evaluate whether the reference-limits of obese men in our study are applicable as decision limits for androgen replacement therapy in obese men.

Of the ruled out subjects, a high risk of low TT was seen in men with small testes, the medically treated men, and in anabolic steroid abusers. The difference in androgen levels between medically treated and untreated chronic diseases may be explained by more serious disease in men requiring medication, but may also be related to the treatment(15;19;49-51). The majority of the ruled out groups had significantly decreased TT, indicating that the rule out procedure was reasonable,

overall. The concept of establishing a reference-interval in a “low risk” population has previously been employed by Jorgensen *et al*(52).

The assays used in this study were optimized to ensure complete dissociation of the sex hormones from binding proteins (by extraction) and to avoid cross reactivity with other steroids (by celite chromatography). To focus on the relation between androgen levels and adiposity, a young population with a narrow age-interval was chosen to minimize the effects of age on androgen levels and binding proteins. Possible influences of co-morbidity are limited in younger men and efforts were taken to rule out conditions that could possibly influence both androgens and body composition (testicular pathology, chronic diseases, abuse of anabolic steroids, etc.). We have previously shown(25) that our cohort matched the background population of Young, Danish men. Several imaging techniques and anthropometric measures are used to study the apparent negative and positive metabolic consequences of CFM and LEFM, respectively(53;54). We used DXA to measure CFM and LEFM and found that reduced SHBG and androgen levels with increasing adiposity were attributable to CFM. When analyzed for independent relations with the fat types actually constituting the CFM, namely VAT and SAT, we found that SHBG was related with SAT, while BT and FT were related with VAT. Accordingly, assessment of CFM using DXA (and probably anthropometrics) is not necessarily valuable as a measure of visceral adiposity.

In conclusion, TT, BT, FT, DHT, and SHBG decline linearly with increasing CFM in young men: VAT independently account for the decline in BT and FT, while the decline in TT and DHT with increased SAT was secondary to decreased SHBG levels. The $E_2$/TT-ratio is increased with increasing CFM, but increased metabolic clearance can hardly explain the inverse relation between androgen levels and adiposity, which is possibly related with decreased secretion of androgens of either primary or secondary origin. The reference-intervals for TT, BT, FT, and DHT were

displaced towards lower limits in obese men suggesting that reference-intervals for these androgens should be established in healthy, non-obese men.

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**Table 1.** Categorization of central fat mass, lower extremity fat mass, visceral adipose tissue, and subcutaneous adipose tissue, respectively: Within the low-risk reference-population, these measures obtained by DXA (upper panel) and MRI (lower panel) were grouped by means of standard deviation scores. For comparison, the corresponding body mass indices and waist circumferences of the six groups are shown for each DXA/MRI measure [a].

| Standard deviation score | <–1.00 | –1.00 ; –0.51 | –0.51 ; –0.01 | 0.00 ; 0.49 | 0.50 ; 0.99 | ≥ 1.00 |
|---|---|---|---|---|---|---|
| Number of subjects | 108 | 103 | 131 | 131 | 103 | 109 |
| **Central fat mass, kg** | **2.87 (1.68-3.56)** | **4.00 (3.56-4.45)** | **5.16 (4.47-5.77)** | **6.54 (5.77-7.49)** | **8.44 (7.50-9.73)** | **12.8 (9.77-28.1)** |
| Body mass index, kg/m$^2$ | 21.3 (16.0-25.1) | 22.5 (18.8-25.7) | 23.6 (19.0-28.3) | 24.5 (20.2-28.5) | 26.4 (22.7-30.9) | 29.7 (24.3-39.8) |
| Waist circumference, cm | 78.1 (68.5-88.0) | 81.8 (74.0-98.0) | 85.4 (73.5-96.0) | 89.1 (78.5-99.5) | 94.0 (86.0-104) | 105.0 (89.5-131) |
| **Lower extremity fat mass, kg** | **2.72 (1.59-3.44)** | **3.87 (3.44-4.25)** | **4.74 (4.25-5.21)** | **5.69 (5.22-6.33)** | **6.79 (6.33-7.49)** | **9.17 (7.52-15.2)** |
| Body mass index, kg/m$^2$ | 21.3 (16.0-25.0) | 22.8 (18.8-28.0) | 23.8 (19.3-30.6) | 24.7 (20.2-28.5) | 25.9 (19.0-32.0) | 29.4 (23.0-39.8) |
| Waist circumference, cm | 78.5 (68.5-92.0) | 82.9 (72.0-99.5) | 85.5 (74.0-99.5) | 90.1 (78.5-104) | 92.6 (76.0-105) | 103.2 (78.5-131) |

| Standard deviation score | <–1.00 | –1.00 ; –0.51 | –0.51 ; –0.01 | 0.00 ; 0.49 | 0.50 ; 0.99 | ≥ 1.00 |
|---|---|---|---|---|---|---|
| Number of subjects | 58 | 54 | 69 | 69 | 54 | 59 |
| **Visceral adipose tissue, liter** [b] | **0.34 (0.23-0.40)** | **0.43 (0.40-0.47)** | **0.52 (0.47-0.57)** | **0.63 (0.57-0.71)** | **0.80 (0.72-0.87)** | **1.07 (0.87-1.88)** |
| Body mass index, kg/m$^2$ | 21.9 (16.0-26.9) | 23.0 (19.3-28.3) | 23.6 (18.3-28.4) | 24.4 (20.1-33.4) | 25.6 (20.2-33.6) | 28.2 (23.8-36.4) |
| Waist circumference, cm | 79.5 (68.5-98.0) | 82.7 (75.0-96.0) | 84.8 (72.5-102) | 88.4 (77.0-109) | 92.3 (79.0-118) | 99.5 (87.0-125) |
| **Subcut. adipose tissue, liter** [b] | **0.46 (0.27-0.62)** | **0.71 (0.62-0.83)** | **1.00 (0.84-1.16)** | **1.31 (1.17-1.46)** | **1.66 (1.46-1.92)** | **2.58 (1.93-4.61)** |
| Body mass index, kg/m$^2$ | 21.3 (16.0-25.1) | 22.4 (18.8-25.4) | 23.5 (20.2-28.3) | 24.6 (21.3-28.3) | 26.0 (23.7-30.3) | 29.2 (23.6-36.4) |
| Waist circumference, cm | 78.3 (68.5-98.0) | 80.7 (74.0-88.0) | 84.7 (77.0-96.0) | 88.8 (78.5-99.5) | 92.9 (85.0-103) | 102 (88.5-125) |

Geometric mean (min-max).

[a] Please note that the categorizations were performed separately for central fat mass, lower extremity fat mass, visceral adipose tissue, and subcutaneous adipose, respectively. Thus the intervals (min-max) of these measures of adiposity do not overlap, while the corresponding intervals of body mass index and waist circumference overlap considerably.

[b] To convert to the mean cross-sectional area ($cm^2$) of the MRI slices, divide by 0.009 liter/cm (9 cm x 0.001 liter/$cm^3$)

**Table 2.** Reference-intervals before and upon partitioning of the low-risk reference-population into obese and non-obese men.

| | | Concentration, nmol/l (95% CI) [a] | | | | | | Interpretation | |
|---|---|---|---|---|---|---|---|---|---|
| | Ref. limit | Ref.-pop., n=685 | | Non-obese, n=615 | | Obese, n=70 | | $\Delta^b$ / SD[c] | Decision |
| Total testosterone | Lower | 11.7 | (11.2–12.1) | 12.5 | (12.0–13.0) | 8.5 | (7.5–9.7) | 1.37 | Partition |
| | Upper | 37.9 | (36.3–39.2) | 37.6 | (36.2–39.0) | 29.3 | (25.9–33.3) | 0.91 | Partition |
| Bioavailable testosterone | Lower | 7.3 | (7.0–7.5) | 7.6 | (7.3–7.8) | 6.1 | (5.4–6.9) | 0.88 | Partition |
| | Upper | 20.4 | (19.8–21.2) | 20.7 | (20.1–21.4) | 16.9 | (15.3–18.8) | 0.81 | Partition |
| Free testosterone | Lower | 0.28 | (0.27–0.29) | 0.29 | (0.28–0.30) | 0.23 | (0.21–0.26) | 0.94 | Partition |
| | Upper | 0.78 | (0.75–0.81) | 0.78 | (0.76–0.81) | 0.67 | (0.60–0.74) | 0.62 | Ambiguous |
| Dihydrotestosterone | Lower | 0.81 | (0.77–0.84) | 0.85 | (0.81–0.89) | 0.63 | (0.55–0.73) | 0.90 | Partition |
| | Upper | 3.2 | (3.1–3.4) | 3.2 | (3.1–3.4) | 2.5 | (2.2–2.8) | 0.75 | Ambiguous |
| Androstenedione | Lower | 1.9 | (1.8–2.0) | 1.9 | (1.8–2.0) | 1.8 | (1.5–2.1) | 0.16 | No partitioning |
| | Upper | 7.6 | (7.2–7.9) | 7.5 | (7.2–7.9) | 7.3 | (6.4–8.5) | 0.08 | No partitioning |
| Total estradiol | Lower | 36.5 | (158–174) | 36.5 | (34.7–38.5) | 36.4 | (32.4–40.8) | 0.01 | No partitioning |
| | Upper | 166 | (158–174) | 168 | (160–177) | 144 | (124–165) | 0.40 | Ambiguous |
| Bioavailable estradiol | Lower | 23.4 | (22.2–24.7) | 23.3 | (22.0–24.7) | 23.9 | (20.4–28.0) | 0.06 | No partitioning |
| | Upper | 120 | (114–127) | 121 | (114–128) | 115 | (99–131) | 0.13 | No partitioning |

[a] The unit for total and bioavailable estradiol are pmol/l.

[b] After natural log transformation: $\Delta$ = The difference between the reference limit in healthy non-obese and obese men.

[c] After natural log transformation: SD = (Upper limit in non-obese – Lower limit in non-obese) / 4.

Lahti *et al*[32] recommend: $\geq$0.75: partitioning; 0.25–0.75: ambiguous decision; $<$0.25: partitioning irrelevant

**Table 3.** Androgen and LH levels in 20-29 year-old men of the low-risk reference-population, the healthy non-obese and obese, and in ruled out men.

| Rule-out criteria | N [b] | TT, nmol/l | BT, nmol/l | DHT, nmol/l | $E_2$, pmol/l | LH, U/l |
|---|---|---|---|---|---|---|
| Healthy ref-population | 685 (33) | 21.0 (20.5–21.4) | 12.2 (12.0–12.5) | 1.61 (1.57–1.66) | 77.6 (75.4–79.9) | 3.4 (3.3–3.5) |
| Healthy non-obese [c] | 615 (17) | 21.7 (21.2–22.1) | 12.5 (12.2–12.8) | 1.66 (1.62–1.71) | 78.4 (76.0–80.8) | 3.4 (3.3–3.5) |
| Healthy obese [c] | 70 (16) | 15.8 (14.7–17.1) $^{\dagger\dagger}$ | 10.1 (9.5–10.8) $^{\dagger\dagger}$ | 1.25 (1.15–1.36) $^{\dagger\dagger}$ | 71.1 (65.4–77.4) $^{\dagger}$ | 3.2 (3.0–3.5) |
| Bilateral cryptorchidism | 12 (0) | 20.9 (17.3–25.2) | 12.7 (10.9–15.0) | 1.47 (1.34–1.90) | 79.2 (64.5–97.2) | 4.9 (3.7–6.6) $^{**}$ |
| Congenital hydrocele | 5 (0) | 17.1 (15.4–19.0) $^{*}$ | 10.7 (8.4–13.6) | 1.38 (1.10–1.72) | 69.3 (39.9–120) | 2.8 (2.1–3.8) |
| Both testicles < 9 ml | 15 (4) | 14.0 (11.0–17.7) $^{****}$ | 8.4 (6.1–11.7) $^{****}$ | 1.37 (1.08–1.73) | 71.4 (61.2–83.3) | 3.1 (2.2–4.3) |
| Chronic disease | 44 (7) | 16.8 (15.5–18.2) $^{***}$ | 9.8 (9.1–10.6) $^{***}$ | 1.34 (1.11–1.62) $^{**}$ | 66.7 (60.3–73.7) $^{**}$ | 3.7 (3.3–4.2) |
| – medication | 12 (1) | 19.1 (15.4–23.7) | 10.8 (9.4–12.5) | 1.55 (1.17–2.05) | 64.7 (53.5–78.3) $^{*}$ | 3.9 (2.9–5.3) |
| + medication | 32 (6) | 16.0 (14.8–17.3) $^{***}$ | 9.5 (8.7–10.3) $^{***}$ | 1.26 (0.99–1.62) $^{**}$ | 67.4 (59.6–76.3) $^{*}$ | 3.6 (3.1–4.1) |
| Alcohol intake ≥ 6/day | 9 (1) | 17.0 (14.0–20.7) | 11.5 (9.7–13.5) | 1.34 (1.04–1.74) | 73.3 (58.2–92.4) | 3.6 (2.5–5.3) |
| Anabolic steroid abuse [a] | 3 (2) | 1.5 (1.2–16.1) | 1.2 (1.2–10.1) | 0.52 (0.05–1.28) | 42.0 (39.0–44.0) | 0.6 (0.0–3.2) |
| LH ≤ 1 U/l [a] | 3 (1) | 14.4 (12.4–21.1) | 7.9 (9.3–13.9) | 1.36 (1.33–1.79) | 98.0 (39.0–110) | 1.0 (0.6–1.0) |
| TSH > 6.0 mU/l | 7 (0) | 18.4 (14.2–24.0) | 10.9 (8.4–14.0) | 1.46 (1.20–1.78) | 69.4 (52.1–92.6) | 4.0 (2.5–6.4) |
| Sum of ruled out men | 98 (15) | 16.0 (14.6–17.6) $^{****}$ | 9.7 (8.9–10.7) $^{****}$ | 1.31 (1.17–1.48) $^{***}$ | 69.0 (64.5–73.8) $^{**}$ | 3.4 (3.1–3.8) |

Geometric mean (95% CI)

[a] in small subgroups the figures represent the median (min-max) and no statistical tests were performed.

[b] Number of men with TT<12.5 nmol/l in brackets.

[c] Obesity defined by a waist circumference (WC) ≥ 102 cm.

$^{\dagger}$ $p<0.05$ $^{\dagger\dagger}$ $p<10^{-6}$ (compared to the healthy non-obese in students t-test).

$^{*}$ $p<0.05$, $^{**}$ $p<0.01$, $^{***}$ $p<0.001$, $^{****}$ $p<10^{-6}$ (compared to the healthy reference-population and adjusted for WC in multiple regression).

**Table 4.** Central and lower extremity fat mass (upper panel) and visceral and subcutaneous adipose tissue (lower panel) as independent contributors to the variation in SHBG and sex hormone levels in multiple linear regression analyses.

| | | 1st independent: central fat mass | | | 2nd independent: lower extremity fat mass | | |
|---|---|---|---|---|---|---|---|
| Analysis [a] | Dependent variable | Coefficient nmol/l / SDS [b] | 95% CI nmol/l / SDS [b] | Trend p | Coefficient nmol/l / SDS [b] | 95% CI nmol/l / SDS [b] | Trend p |
| 1 | SHBG | –5.9 | –7.2 ; 4.7 | $< 10^{-6}$ | –2.0 | –0.8 ; –3.3 | 0.001 |
| 2 | Total testosterone (TT) | –2.8 | –3.6 ; –2.0 | $< 10^{-6}$ | 0.8 | –0.02 ; 1.5 | 0.057 |
| 3 | Bioavailable testosterone | –0.7 | –1.1 ; –0.2 | 0.002 | 0.1 | –0.3 ; 0.5 | 0.68 |
| 4 | Free testosterone | –0.025 | –0.041 ; –0.008 | 0.003 | 0.007 | –0.010 ; 0.023 | 0.43 |
| 5 | Dihydrotestosterone | –0.11 | –0.20 ; –0.02 | 0.021 | –0.04 | –0.13 ; 0.05 | 0.43 |
| 6 | Estradiol | –2.7 | –7.4 ; 2.0 | 0.26 | 1.4 | –3.4 ; 6.1 | 0.58 |
| 6.b | Adjusted for TT [c] | 3.5 | –0.9 ; 8.0 | 0.12 | –0.3 | –4.7 ; 4.1 | 0.89 |
| 7 | Bioavailable estradiol | 1.5 | –1.8 ; 4.7 | 0.38 | –0.2 | –3.5 ; 3.1 | 0.88 |
| 7.b | Adjusted for TT [c] | 4.4 | 1.1 ; 7.6 | 0.008 | –1.0 | –4.2 ; 2.2 | 0.53 |

| | | 1st independent: visceral adipose tissue | | | 2nd independent: subcutaneous adipose tissue | | |
|---|---|---|---|---|---|---|---|
| Analysis [a] | Dependent variable | Coefficient nmol/l / SDS [b] | 95% CI nmol/l / SDS [b] | Trend p | Coefficient nmol/l / SDS [b] | 95% CI nmol/l / SDS [b] | Trend p |
| 8 | SHBG | –0.7 | –1.9 ; 0.4 | 0.21 | –3.2 | –4.3 ; –2.1 | $< 10^{-6}$ |
| 9.a | Total testosterone (TT) | –1.2 | –1.9 ; –0.5 | 0.001 | –1.0 | –1.7 ; –0.3 | 0.005 |
| 9.b | Adjusted for SHBG [d] | –1.0 | –1.7 ; –0.4 | 0.002 | –0.01 | –0.6 ; 0.6 | 0.98 |
| 10 | Bioavailable testosterone | –0.7 | –1.1 ; –0.3 | 0.002 | 0.01 | –0.4 ; 0.5 | 0.97 |
| 11 | Free testosterone | –0.024 | –0.040 ; –0.008 | 0.003 | 0.002 | –0.015 ; 0.018 | 0.86 |
| 12.a | Dihydrotestosterone | –0.08 | –0.14 ; –0.01 | 0.024 | –0.08 | –0.14 ; –0.01 | 0.018 |
| 12.b | Adjusted for SHGB [d] | –0.06 | –0.12 ; –0.001 | 0.049 | 0.006 | –0.06 ; 0.07 | 0.84 |
| 13.a | Estradiol | –4.0 | –8.8 ; 0.8 | 0.10 | 0.09 | –4.4 ; 4.6 | 0.97 |
| 13.b | Adjusted for TT [c] | –1.1 | –5.5 ; 3.3 | 0.62 | 2.5 | –1.5 ; 6.6 | 0.22 |
| 14.a | Bioavailable estradiol | –2.5 | –5.9 ; 0.8 | 0.14 | 1.9 | –1.3 ; 5.1 | 0.23 |
| 14.b | Adjusted for TT [c] | –1.1 | –4.3 ; 2.1 | 0.51 | 3.2 | 0.1 ; 6.2 | 0.041 |

[a] All analyses performed with exclusion of three anabolic steroid abusers and adjusting for chronic disease and medication (data not shown)

[b] Coefficients expressed as nmol/l per standard deviation score (SDS) except for estradiol measures (pmol/l per SDS).

[c] Adjustment performed as the substrate of aromatization, testosterone, varies with the variation in the adipose tissues.

[d] In analyses where both independents were significant, inverse correlates of the dependent variable, additional analyses were performed with adjustment for SHBG.

**FIGURE LEGENDS**

**Fig. 1:** Inclusion of the study-population, establishment of the low-risk reference-population, and division into healthy non-obese and obese men.

**Fig. 2:** Serum SHBG (panel a), total testosterone (panel b), bioavailable testosterone (panel c), dihydrotestosterone (panel d), and luteinizing hormone (panel e) in 20-29 year-old healthy men in relation to four fat depots: central fat mass assessed by DXA (column 1, n=685), lower extremity fat mass assessed by DXA (column 2, n=685), visceral adipose tissue assessed by MRI (column 3, n=363), and subcutaneous adipose tissue assessed by MRI (column 4, n=363).
P values in bar charts are results from univariate linear regression analyses.
$^{\ddagger}$omitting men with SD-scores≥1.0 from analysis.
$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, and $^{****}p<0.0001$ *vs*. men with SD-scores<–1.0 (students t-test); $^{\S}p=0.006$ *vs*. the preceding group (students t-test). Whiskers: SEM.

**Fig. 3:** Serum total estradiol (panel a), total estradiol adjusted for total testosterone (panel b), bioavailable estradiol (panel c), and bioavailable estradiol adjusted for total testosterone (panel d) in 20-29 year-old healthy men in relation to four fat depots: central fat mass assessed by DXA (column 1, n=685), lower extremity fat mass assessed by DXA (column 2, n=685), visceral adipose tissue assessed by MRI (column 3, n=363), and subcutaneous adipose tissue assessed by MRI (column 4, n=363).
P values in bar charts are results from univariate linear regression analyses.
$^{*}p<0.05$ and $^{**}p<0.01$ *vs*. men with SD scores<–1.0 (students t-test). Whiskers: SEM.

**Fig. 1**

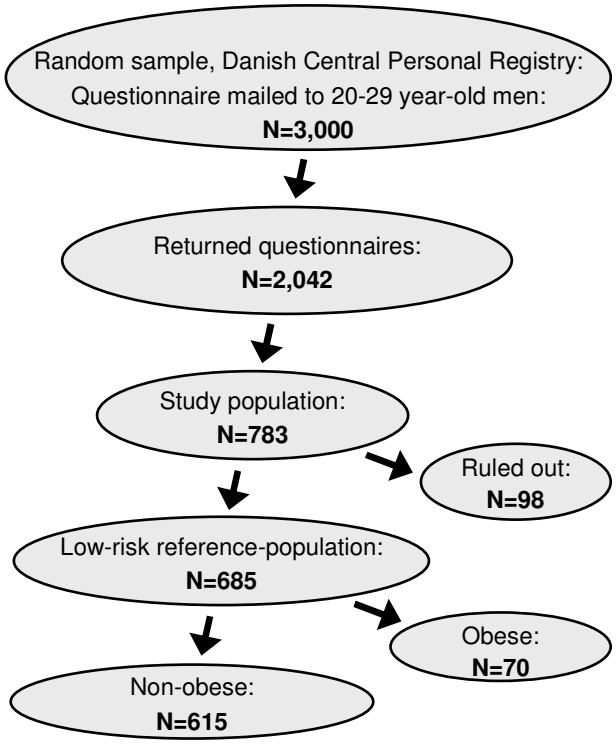

Random sample, Danish Central Personal Registry:
Questionnaire mailed to 20-29 year-old men:
N=3,000
Returned questionnaires:
N=2,042
Study population:
N=783
Ruled out:
N=98
Low-risk reference-population:
N=685
Obese:
N=70
Non-obese:
N=615

**Fig. 2**

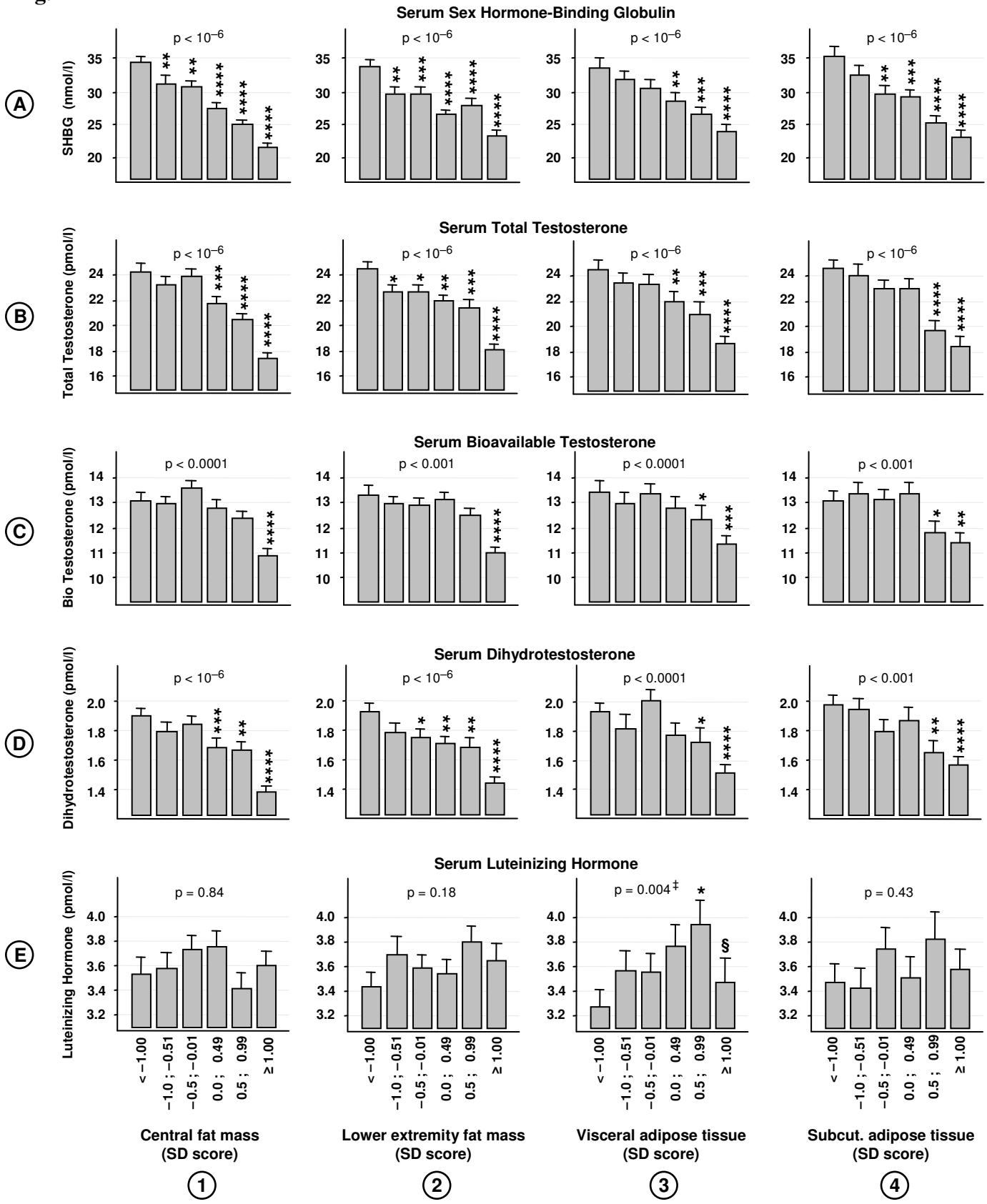
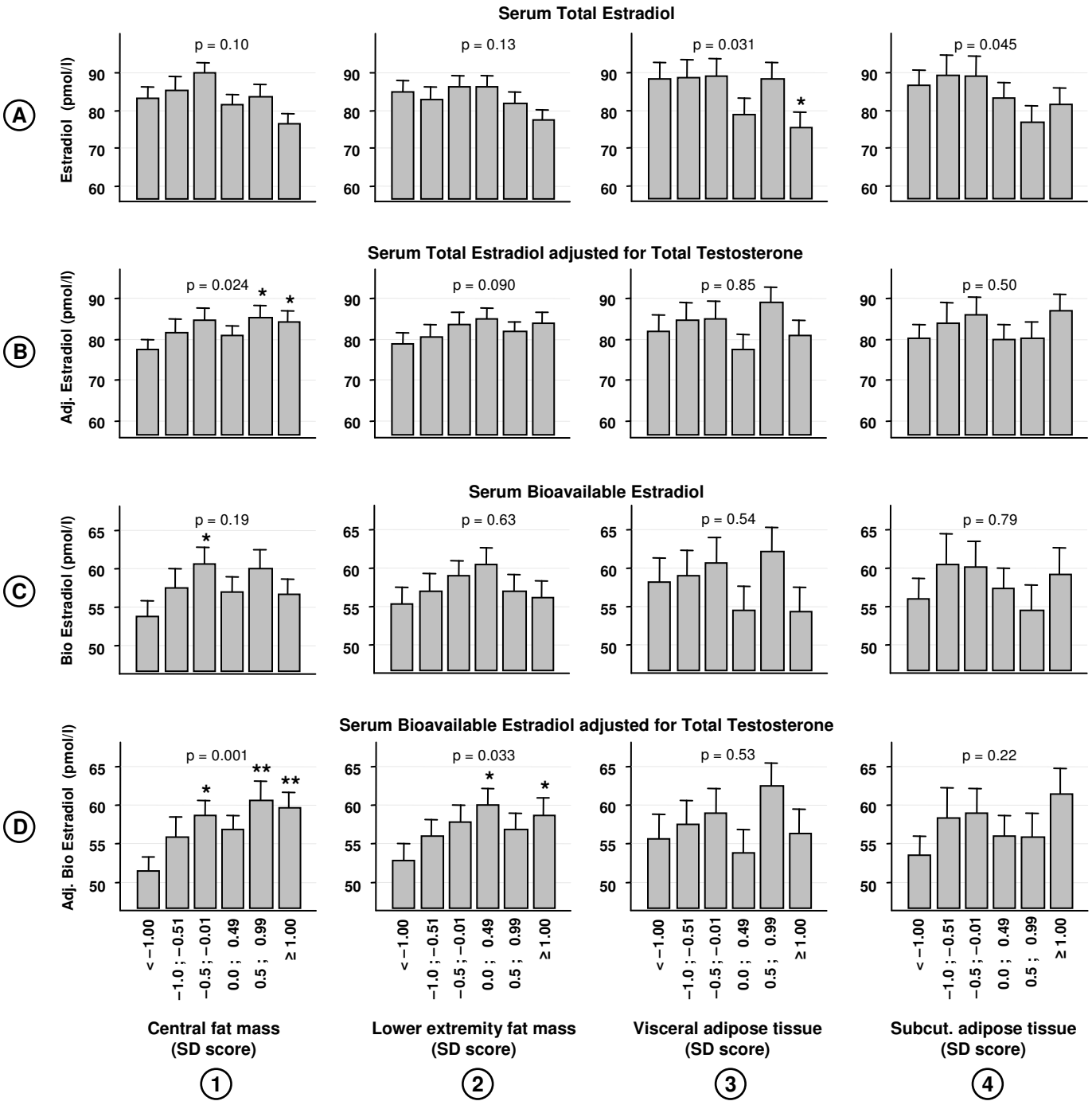

Serum Sex Hormone-Binding Globulin
SHBG (nmol/l)
p < 10−6
Serum Total Testosterone
Total Testosterone (pmol/l)
p < 10−6
Serum Bioavailable Testosterone
Bio Testosterone (pmol/l)
p < 0.0001
p < 0.001
p < 0.0001
p < 0.001
Serum Dihydrotestosterone
Dihydrotestosterone (pmol/l)
p < 10−6
p < 10−6
p < 0.0001
p < 0.001
Serum Luteinizing Hormone
Luteinizing Hormone (pmol/l)
p = 0.84
p = 0.18
p = 0.004‡
p = 0.43
< −1.00
−1.0 ; −0.51
−0.5 ; −0.01
0.0 ; 0.49
0.5 ; 0.99
≥ 1.00
Central fat mass (SD score)
Lower extremity fat mass (SD score)
Visceral adipose tissue (SD score)
Subcut. adipose tissue (SD score)
A
B
C
D
E
1
2
3
4

**Fig. 3**


Serum Total Estradiol
A
Estradiol (pmol/l)
p = 0.10
p = 0.13
p = 0.031
p = 0.045
Serum Total Estradiol adjusted for Total Testosterone
B
Adj. Estradiol (pmol/l)
p = 0.024
p = 0.090
p = 0.85
p = 0.50
Serum Bioavailable Estradiol
C
Bio Estradiol (pmol/l)
p = 0.19
p = 0.63
p = 0.54
p = 0.79
Serum Bioavailable Estradiol adjusted for Total Testosterone
D
Adj. Bio Estradiol (pmol/l)
p = 0.001
p = 0.033
p = 0.53
p = 0.22
< −1.00
−1.0 ; −0.51
−0.5 ; −0.01
0.0 ; 0.49
0.5 ; 0.99
≥ 1.00
Central fat mass (SD score)
Lower extremity fat mass (SD score)
Visceral adipose tissue (SD score)
Subcut. adipose tissue (SD score)
1
2
3
4