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Running title: Testosterone and adiposity in women

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Disclosure statement
The authors declare no conflict of interest.

Summary

Objective: Research findings on the relationship between serum androgens and adipose tissue in older females are inconsistent. We aimed to clarify the relationship using state-of-the art techniques to evaluate associations between body fat distribution and plasma testosterone (T) levels in older postmenopausal women.

Design: Observational, cross-sectional study of healthy, community dwelling postmenopausal women

Patients and Measurements: Postmenopausal women, (60-80 years old) were included in this study. Overall body composition was evaluated by dual-energy x-ray absorptiometry. Abdominal and thigh fat depots were measured by magnetic resonance imaging. Circulating T concentrations by liquid chromatography-tandem mass spectrometry.

Results: Thirty-five women (66.6 ± 0.8 years) participated in this study. T levels were positively associated with clinical proxy measure of adiposity including weight (ρ=0.39), BMI (ρ=0.43) and waist circumference (ρ=0.39) (all $p<0.05$). Fat mass and percent body fat were correlated with T levels (ρ=0.42 and 0.38 respectively, both $p<0.05$). T correlated with overall and superficial abdominal fat (ρ=0.34 and 0.37 respectively, both $p<0.05$) but not with visceral adipose tissue. T increased with greater thigh fat (ρ=0.49, $p<0.05$) in both superficial and deep depots (ρ=0.50 and 0.35 respectively, both $p<0.05$).

Conclusion: Our results suggest that postmenopausal women with higher circulating T levels have both higher regional and overall body adiposity. These findings underscore the sexual dimorphism in the relationship between serum androgen levels and adiposity.

Key words: body composition, regional adiposity, sexual dimorphism, superficial adipose tissue, visceral adipose tissue, IMAT

Introduction

Testosterone is produced by the gonads and to a lesser extend the adrenal glands. Testosterone (T) has important physiologic effects in both sexes. It promotes the formation of lean muscle mass [1], reduces percent body fat [1,2], affects energy levels [3], contributes to the development and maintenance of bone density [4] and has significant effects on libido, sexual function, mood and general well-being [5]. As the dominant sex steroid, circulating T concentrations are approximately 10 to 20-fold higher in men compared to women [6,7]. Circulating sex steroid levels decrease with aging [8-10]. After menopause, T levels in women fall to even lower levels due to diminished ovarian function [11]. In contrast, aging is associated with increased adiposity.

Adipose tissue has a central role in triglyceride storage [12] and is an endocrine organ. In addition of regulating the synthesis and release of adipokines [13,14], data suggest that adipose tissue represents an intracrine source of androgens synthesis [15] and recent mechanistic studies have begun to uncover site-specific intra-adipose mechanisms of androgen activation [16-18]. Adipose tissue distribution has long been acknowledged as sexually dimorphic [19,20]. Men typically accumulate fat in the abdominal region (i.e. “apple-shaped”, android pattern) whereas women tend to accumulate fat in the gluteal-femoral region (i.e. “pear-shaped”, gynoid pattern) [21,22]. Following menopause, visceral fat deposition increases with a parallel increase in metabolic risk [19]. The linkage between adiposity and metabolism has been well established [23,24] as is the sexually dimorphic relationship between sex steroids and insulin sensitivity [25]. For example, men with higher T levels have more lean muscle mass and are more insulin sensitive [26] while women with high androgen levels (e.g. polycystic ovarian syndrome, PCOS) exhibit increased insulin resistance [27,28]. Whereas an inverse relationship has been well established between T and adiposity in males [6,29,30], the exact relationship in women is unclear. Studies are conflicting.

Some report positive associations [31,32] while others have found either negative [33] or no association at all [6,34,35].

These inconsistent findings may result from methodological limitations in measuring T at low levels. Women have very low circulating T levels and traditional assays, such as radioimunnoassays (RIA), are less reliable near the level of detection [36]. However, advances in sex steroid measurement and imaging technologies now make it possible to investigate this methodologically challenging question with precision. The sensitivity and accuracy of liquid chromatography-tandem mass spectrometry (LC-MS/MS) make it the "gold standard" for measuring circulating sex steroids [37,38]. Similarly, quantifying body fat depots has been imprecise and approaches have been proxy in nature (i.e. BMI, waist circumference, skin-fold measurement). Dual energy absorptiometry (DXA) and magnetic resonance imaging (MRI) techniques provide precise measures of whole body and regional adiposity. Therefore, the aim of this study was to evaluate levels of circulating T in aging post-menopausal women using state of the art tools and to investigate its relationship with whole-body and regional adiposity.

Materials and Methods

Study design, participants and clinical assessment:

Community dwelling, stable-weight women aged 60-80 years were recruited for the study. Postmenopausal women (defined as no menstrual periods for more than one year), in good general health, not taking hormonal replacement therapy (or herbal formulations) to treat menopausal symptoms at least 6 months prior to enrollment were included. Those women who reported tobacco use (e.g. active smokers), steroid medications, prior diagnosis of diabetes or abnormal thyroid, liver or kidney function tests were excluded. The ethics committee of the

117 Canton (state) Vaud approved the study and all participants provided written informed consent
118 prior to the initiation of study procedures.
119
120 *Anthropometric and body composition assessment*
121 Participant height (cm) was measured using a wall-mounted stadiometer and body weight (kg)
122 using a standard calibrated medical scale (Seca GmBh, Hamburg, Germany) in the fasting state
123 and wearing a hospital gown. Body mass index was calculated as weight divided by height
124 squared (Kg/m^2). Lean and fat masses were determined by dual energy X-ray absorptiometry
125 (DXA) (Discovery A; Hologic Inc., Bedford, MA) under controlled conditions (i.e. proper
126 hydration and not exercising prior to the test). The DXA scanner was calibrated prior to
127 imaging. Each scan was performed by a trained technician and a study investigator supervised
128 the proper position and placement of markers.
129
130 *Regional adipose tissue measurements*
131 Abdominal and thigh images were obtained using a 3-tesla magnet (VERIO; SIEMENS,
132 Erlangen, Germany). Thirteen series of five images with 10 mm thickness and 10 mm gap
133 between images were taken for each subject in the supine position from the sternal notch to the
134 patella. One subject did not complete the imaging series due to claustrophobia. All images were
135 de-identified and analyzed in a blinded fashion.
136
137 Images used for the abdominal region spanned the femur heads (first image) to the heart (last
138 image). Abdominal fat volumes were determined using a point counting program (MATLAB
139 R2007a, MathWorks, Natick, MA, USA) as previously described [39]. Briefly, after contour lines
140 are drawn to separate visceral from subcutaneous tissues and a standardized grid of 15 mm is
141 superimposed on the image series. Grid crossings falling on adipose tissue are counted

142 providing an unbiased and accurate estimation of volume [40]. The intra- and inter-rater
143 coefficients of variations (CV) were 2.2 % and 4.3 % respectively for visceral adipose tissue
144 (VAT) and 2.7 % and 1.1 % respectively for abdominal subcutaneous adipose tissue (ASAT).
145
146 Thigh adipose tissue volumes were analyzed with the MIPAV software (Medical Image
147 Processing, Analysis, and Visualization, 7.2.0 version, NIH, Bethesda, USA). First the length
148 of the femur was estimated using all images spanning the greater trochanter to the patella. The
149 middle 5 images were selected with the central image corresponding to the center of the femur.
150 Muscle, subcutaneous and intermuscular adipose tissue (IMAT) were measured in each image
151 as described elsewhere [41]. In brief, images were first homogenized using an established
152 normalization (N3) algorithm to correct for varying shading caused by radiofrequency coil
153 uniformity or gradient driven eddy currents [42]. Pixels of bone, bone marrow and bone fat were
154 masked and excluded from fat analysis. Two concentric lines were drawn. The first line defined
155 the limit of the leg (including skin). The second line marked the fascia surrounding muscles
156 (excluding subfascial adipose tissue). The adipose tissue between the two lines was defined as
157 thigh subcutaneous adipose tissue (TSAT). To separate fat from muscle within the fascia, five
158 or more volumes of interest were placed over the cleanest muscular areas (without fat), in fat
159 and between both tissues. A histogram of pixel intensities within the volumes of interest
160 allowed defining each image specific threshold fat/muscle. IMAT was identified as all pixels
161 above this threshold. TSAT and IMAT in each image are expressed in cm^2. To obtain volume,
162 each area of interest was summed across the 5 images. To account for gaps between images,
163 each image is duplicated. Units were transformed from cm^3 to liters. Intra- and inter-rater CV
164 were 5.9 % and 6.3 % respectively for TSAT and 6.9 % and 9.0 % respectively for IMAT.
165
166 *Biochemical analysis*

Blood samples were collected following a 12-hour overnight fast, spun and plasma aliquots were frozen at -80C prior to measurement. Blood glucose, total cholesterol, triglycerides and HDL cholesterol were analyzed at the hospital laboratory of analytical biochemistries using a Cobas automated analyzer (Cobas 8000, Roche-Diagnostics, Basel, Switzerland). Glycated hemoglobin was measured using high performance liquid chromatography (HPLC D-100, Bio-Rad Laboratories, CA, USA). The limit of detection for glucose, triglycerides, total and HDL cholesterol are 0.11, 0.1, 0.1 and 0.08 (mmol/L) respectively and 3.5% for HbA1c. For all these analytes, the intra- and interassay coefficients of variation are <3%.

Liquid chromatography high-resolution tandem mass spectrometry

Details on sample preparation, calibrators and control materials have been described in details previously [36]. Briefly, frozen plasma samples were thawed at room temperature, vortex mixed, centrifuged (20,000x g, 4^{o}C, 5 min) and pipetted (100µl) into a 96 deep-well plate (Eppendorf, Hamburg, Germany). Calibrators, controls and internal standards were included in the well plate (100µl each). After top sealing, the plates were placed onto an Orbit P2 sample shaker (Labnet, Edison, NJ, USA) for 10 minutes followed by centrifugation (2,500 x g, 4^{o}C, 1 min). A solid phase extraction 96 well plate (Oasis MCX, Walters, MA, U.S.A) was prepared by passing 200µl methanol followed by 200µl of ultra-pure water through each well using a positive pressure processer (Walters, MA, U.S.A). The samples and the calibrators were added to individual wells along with internal standard mix to a total volume of 200µl per well. Following washing steps with 5%NH_4OH and 20% MeOH, final contents were evaporated under N_2 using a Turbo Vap 96 system (Biotage, Uppsala, Sweden). Each well content was reconstituted with 75µl (MeOH: H_2O / 2:3) prior to LC-HRMS/MS analysis. The LC-HRMS/MS [36] system uses an auto sampler (CTC-PAL Analytics, Zwingen, Switzerland), an ultra-high pressure pump unit and an Orbitrap Q-Exactive mass spectrometer system (Thermo Scientific, Waltham, MA, USA). Xcalibur software version 2.2 (Thermo Fisher Scientific,

193 Waltham, MA, USA) was used for data acquisition, processing and reporting. The level of
194 detection for T is 0.01 nmol/L [43] and inter-serial CV was <5%.
195
196 *Statistical Procedures*
197 Statistical analysis was performed using JMP v12 for windows (SAS, Cary, NC, USA). Values
198 are expressed as mean ± standard error of the mean and as 95% confidence interval.
199 Associations were assessed using the Spearman rank correlation coefficient. A p value < 0.05
200 was considered statistically significant.
201
202 **Results**
203 Thirty-five postmenopausal women participated in the study (66.6±0.8, range 61-76 years).
204 BMI and waist circumference (WC) were on average 25.5 ± 1.0 Kg/m^2 and 86.9 ± 2.8 cm
205 respectively. Fasting total cholesterol (5.62 ± 0.13 mmol/L), HDL cholesterol (1.89 ± 0.07
206 mmol/L), triglycerides (1.13 ± 0.11 mmol/L), glucose (5.53 ± 0.08 mmol/L), HbA1c (5.57 ±
207 0.04 %) and T (0.66 ± 0.06 nmol/L) were within age-appropriate normal ranges.
208
209 Whole body composition and regional adipose tissue depositions are presented in Table 1. We
210 were unable to acquire leg and abdominal MRI in one subject due to a previously unrecognized
211 claustrophobia - all other data were obtained and included in the analysis. Abdominal and thigh
212 regions had similar proportions of superficial to deep fat. In the abdomen, 2/3 of adipose tissue
213 was ASAT and 1/3 VAT. In the thigh, 2/3 of fat was TSAT and 1/3 IMAT.
214
215 T was positively associated with clinical proxy measures of adiposity such as weight (ρ=0.39,
216 p=0.02), BMI (ρ=0.43, p=0.01) and WC (ρ=0.39, p=0.03). Relationships between T and
217 adiposity parameters obtained by DXA (fat mass, % body fat) and those obtained from MRI

(abdominal and thigh depots) are shown in Figure 1. No significant associations were found between T and either lean mass, glucose, HbA1c, cholesterol or triglycerides.

The achieved power was computed using the absolute rho as the effect size. For an alpha level of 0.05, a sample size of 34 and a $|\rho|$ of 0.4 (average of all correlation coefficients between T and all measures of adiposity 0.397), the calculated post-hoc power was of 0.80.

Discussion

In men, there is a clear inverse association between T levels and adiposity [6,44,45] yet available evidence on the relationship between circulating androgens and fat depots in women is limited and conflicting [31-34,46,47]. Using state of the art measurement techniques in postmenopausal women, we identified positive associations between plasma T and several measures of adiposity, (*i.e.* fat mass, percent of body fat, abdominal and thigh fat depots) as well as with clinical surrogates such as waist circumference, weight and BMI.

It is well established that adiposity is associated with numerous metabolic disorders [48]. Notably, both the quantity and distribution of body fat is sexually dimorphic [21,22]. There are however, large metabolic differences between adipose tissue depots [23]. In both sexes, increased VAT is associated with metabolic risk including hyperinsulinemia, dyslipidemia and hypertension [48,49]. In older women, TSAT is associated with protective health benefits including greater insulin sensitivity [24] independent of abdominal fat [50]. Recently, the difference between TSAT and IMAT has received increasing attention as these depots are thought to have opposite relationships with insulin sensitivity [51-53]. Given the important differences in regional depositions and metabolism, we sought to explore the relationship between T and specific thigh and abdominal fat deposition by employing state of the art measurements of regional adipose

243 tissue. Using volumetric MRI measures [41], we found thigh adipose tissue, deep and superficial,
244 to be positively associated with circulating T levels in postmenopausal women. Previously, De
245 Pergola et al. found an inverse relationship with femoral superficial adipose tissue measured by
246 ultrasound [33]. Moreover, a recent study using a single mid-thigh computed tomography (CT)
247 image observed a positive relationship between T and overall thigh adipose tissue [46]. To our
248 knowledge, the relationship of IMAT and TSAT with T observed in our cohort has yet to be
249 described in either men or women.
250
251 Anthropometric measures such as BMI, WC and waist to hip ratio (WHR) are commonly used
252 in clinical and epidemiological settings as proxy measures of adiposity to predict metabolic
253 morbidity [3,19]. We identified positive associations between T and WC, weight and BMI
254 respectively. This is in agreement with large epidemiological studies in the same populations
255 of interest that have examined WC, or WHR, and T measured by RIA [31,54]. In contrast to proxy
256 methods, DXA and underwater weighing measure whole body adiposity, yet these techniques
257 require specialized equipment. Using DXA, we observed positive relationships between T, total
258 fat mass and percent body fat. Our results are in agreement with some studies that used DXA
259 to measure adiposity in women [7,32,47], but not with others. Indeed, several studies have failed to
260 show any relationship between T levels and body fatness assessed by either DXA [34], underwater
261 weighing [46] or bioimpedence [35].
262
263 Our volumetric measures of total abdominal adiposity were positively related to T. This was
264 also true for ASAT but not VAT. Our results are in contrast to studies measuring abdominal
265 adiposity in men [6]. In women, data are conflicting, particularly in postmenopausal females.
266 Indeed, in younger women, greater abdominal fat is associated with higher circulating
267 androgens [55]. In healthy middle-aged Australian white women, baseline T predicted the

accumulation of VAT five years later [56]. In a clinical trial, Lovejoy et al. [57] demonstrated administering a weak androgen (nandrolone decanoate) induced elevated VAT levels in obese white women. Among postmenopausal women, a negative association was found between T and VAT assessed by either ultrasound [33] or via single CT image [58]. Further, two recent studies using a single CT image to assess VAT did not find any associations between abdominal adipose tissue and T [6,46].

The inconsistent findings may result from methodological limitations in measuring T at very low levels. Both RIA and immunoassays suffer inadequate sensitivity and lack precision for measuring androgens in women at very low levels [59]. Such methods are problematic for studies of female population and particularly postmenopausal women. A relative strength of this study was that we employed LC-HRMS/MS [37,38]. This method is particularly appropriate for measuring T in postmenopausal women as T levels can be detected as low as 0.01nM [43]. It is worthwhile to note that in this population, several confounding variables can affect serum T levels (e.g. oophorectomy, hormone replacement therapy). While our sample size can be interpreted as relatively small, none of the participants had such confounding factors. Another strength of this study is that we used multiple measures of adiposity with different levels of granularity, ranging from anthropometric markers to overall body fatness and regional fat depots (superficial and deep). Importantly, when quantifying abdominal adipose tissue, we used whole abdomen volume which is more reproducible than partial abdomen measures [60] or a single CT image.

Among our limitations, we were unable to measure free T (*e.g.* by equilibrium dialysis) or compute indirectly free T levels through measures of sex-hormone binding globulin. Although we recognize that free T levels would be of interest regarding the biological effects of

testosterone, we believe that the study objective was realized without free T measurement or calculation given the accuracy and appropriateness of the employed LC-HRMS/MS methodology in the population of interest. Our data only provide observations of associations in older postmenopausal women thus findings should not be generalized to younger populations. In addition, the sample was entirely white European (Caucasian) and it is possible that racial differences exist. Lastly, given that adipose tissue biopsies were not performed in this study, we do not provide mechanistic insights. Indeed, mechanistic studies of androgen conversion within adipose tissue show evidence that specific enzymes capable of activating androstenedione to testosterone are higher in adipocytes from ASAT than VAT [15-17], while other androgen inactivating enzymes are detected in adipocytes from VAT but not ASAT [18,61]. Further research is needed to elucidate the mechanism of the observed associations, particularly those regarding regional superficial and deep adipose depots, and examine the clinical impact on heath in postmenopausal women.

Conclusion

In summary, among a cohort of healthy postmenopausal women, plasma T levels were positively associated with superficial and deep fat depositions in the thigh, superficial abdominal adipose tissue as well as with overall body adiposity. Our findings emphasize the clear gender differences with opposing relationships between androgens and adiposity.

318

319

Figure legend

Figure 1: Associations between testosterone and measures of adiposity in post-menopausal women

AT is adipose tissue, ASAT is abdominal subcutaneous AT, TSAT is thigh subcutaneous AT, VAT is visceral AT, IMAT is intermuscular AT. Solid lines are the tendency lines, dotted lines represent the 95% CI. N=35 for fat mass and body fat. N=34 for abdominal AT, thigh AT, ASAT, TSAT, VAT and IMAT.

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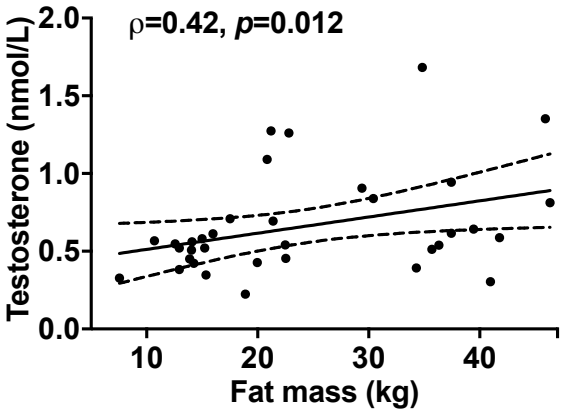
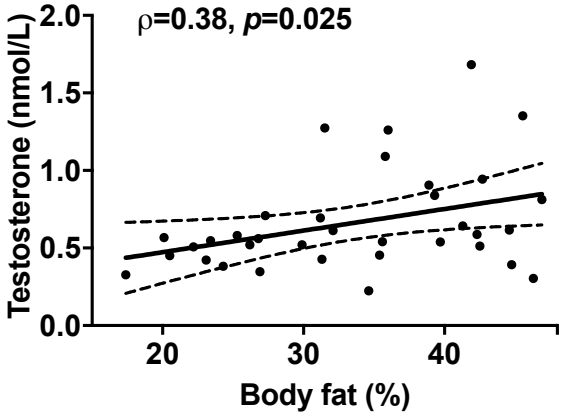
Tables

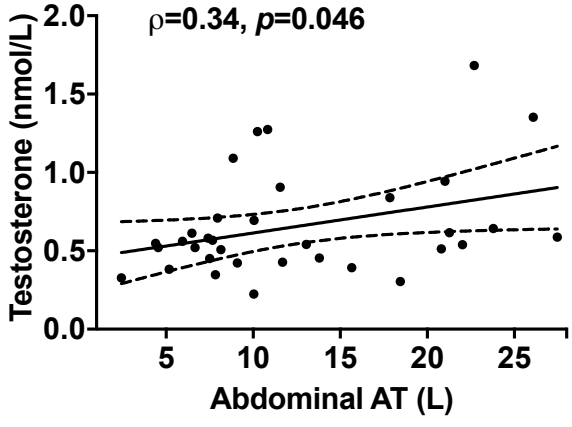
Table 1: Whole body composition and regional adipose tissue deposition

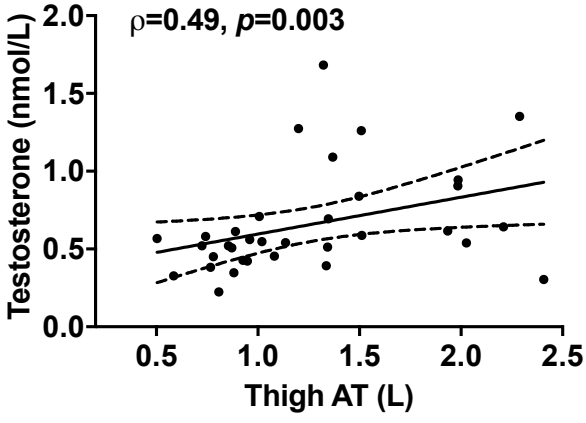
VARIABLES	Mean ± SEM	95% CI	Range (min – max)
DXA (n=35)			
Fat mass (Kg)	24.36 ± 1.92	(20.45 - 28.26)	(7.56 - 46.34)
Lean mass (Kg)	42.84 ± 1.07	(40.68 - 45.01)	(32.04 - 56.03)
Body fat (%)	33.53 ± 1.46	(30.56 - 36.50)	(17.40 - 46.90)
Abdominal MRI (n=34)			
Abdominal AT (L)	12.60 ± 1.20	(10.17 - 15.04)	(2.44 – 27.47)
VAT (L)	3.57 ± 0.30	(2.95 - 4.19)	(0.76 – 8.12)
ASAT (L)	9.03 ± 0.95	(7.10 - 10.95)	(1.68 – 22.14)
Thigh MRI (n=34)			
Thigh AT (L)	1.26 ± 0.09	(1.08 - 1.44)	(0.50 – 2.41)
TSAT (L)	0.98 ± 0.07	(0.85 - 1.12)	(0.39 – 1.69)
IMAT (L)	0.27 ± 0.02	(0.23 - 0.30)	(0.10 – 0.54)

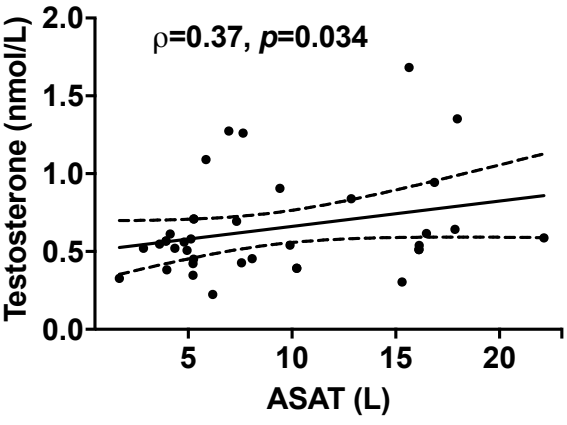
Kg= kilograms, L = liters, AT= adipose tissue, VAT=visceral AT, ASAT=abdominal subcutaneous AT, TSAT=thigh subcutaneous AT, IMAT=intermuscular AT

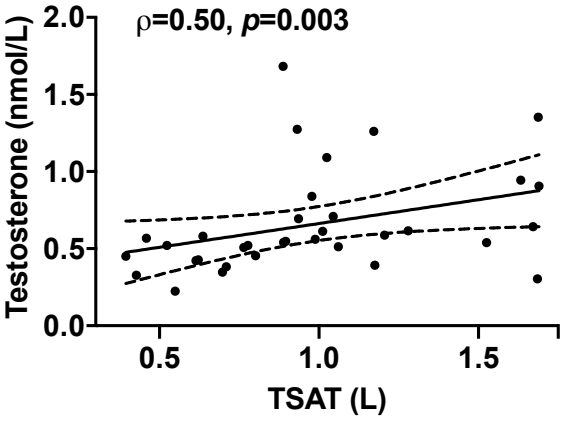

ρ=0.42, p=0.012
Testosterone (nmol/L)
Fat mass (kg)

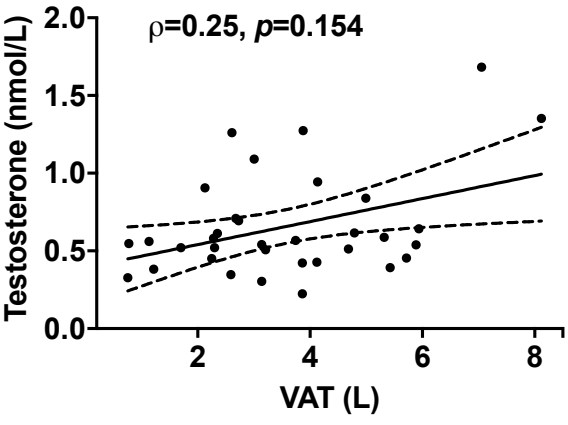

ρ=0.38, p=0.025
Testosterone (nmol/L)
Body fat (%)

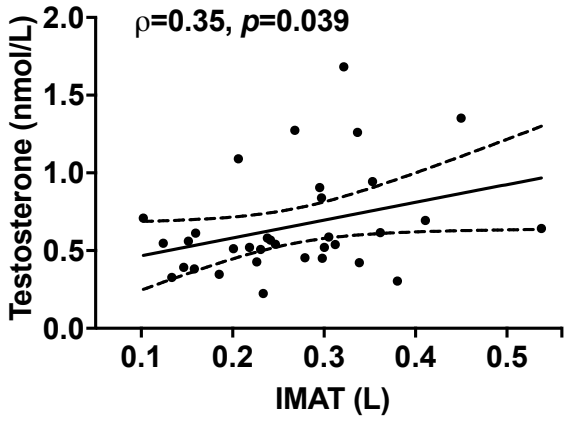

ρ=0.34, p=0.046
Testosterone (nmol/L)
Abdominal AT (L)


ρ=0.49, p=0.003
Testosterone (nmol/L)
Thigh AT (L)


ρ=0.37, p=0.034
Testosterone (nmol/L)
ASAT (L)


ρ=0.50, p=0.003
Testosterone (nmol/L)
TSAT (L)


ρ=0.25, p=0.154
Testosterone (nmol/L)
VAT (L)


ρ=0.35, p=0.039
Testosterone (nmol/L)
IMAT (L)