Published in final edited form as:

Int J Obes (Lond). 2010 April ; 34(4): 781–787. doi:10.1038/ijo.2009.279.

Association between single-slice measurements of visceral and abdominal subcutaneous adipose tissue with volumetric measurements: the Framingham Heart Study

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Abstract

Objective—Volumetric visceral abdominal adipose tissue (VAT) and subcutaneous abdominal adipose tissue (SAT) as measured by computed tomography (CT) are associated with metabolic risk factors. We sought to identify the correlations of VAT and SAT between area-based measures at different anatomic locations with volumetric measurements to identify the optimal anatomic site, and to relate measurements at this site with metabolic risk factors.

Methods—We measured SAT and VAT volumes across the total imaging volume, whereas we measured SAT and VAT area at seven predefined anatomic landmarks in 200 participants from the Framingham Heart Study (mean age 54 years, 50% women) who underwent abdominal multi-detector CT. Correlation coefficients were used to assess the association between area measurements and volumes as well as metabolic risk factors stratified by gender.

Results—Area-based measurements of SAT and VAT obtained at all anatomic landmarks were strongly associated with SAT and VAT volumes (all $r > 0.93$, $P < 0.0001$ and $r > 0.87$, $P < 0.0001$, for women and men; respectively). Consistently, area-based measurements of SAT and VAT obtained at L_{3/4} were most strongly associated with volumetric measured VAT and SAT independent of age (both $r = 0.99$ in men, $r = 0.96$ for SAT and $r = 0.99$ for VAT in women, all P -value < 0.0001) and were similarly correlated with risk factors compared with SAT and VAT volumes (all $P < 0.05$ for fasting plasma glucose, triglycerides, high-density lipoprotein, systolic blood pressure).

Conclusion—Among area-based measurements of SAT and VAT, those obtained at the level of L_{3/4} were strongly associated with SAT and VAT volumes and cardio-metabolic risk factors in both men and women.

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Conflict of interest

The authors declare no conflict of interest.

Keywords

abdominal adiposity; adipose tissue quantification; single-slice measurement; computed tomography; cardiovascular risk factors

Introduction

Obesity is a major health-care issue in the United States and other industrialized countries with about 140 million Americans being overweight (body mass index (BMI)>25), and 66 million individuals classified as obese (BMI>30).¹ The prevalence of overweight and obesity has increased considerably over the last decade and is associated with insulin resistance, cardiovascular disease, sleep-disordered breathing and some types of cancer.² Large epidemiological studies have established generalized obesity as an independent risk factor for increased morbidity and all-cause mortality.³ However, several studies indicate that in addition to the total amount of body fat, the distribution of fat is important for cardiovascular disease risk assessment.³ Furthermore, it has been demonstrated that distinct fat depots, such as visceral abdominal adipose tissue (VAT), may have an important role in mediating cardiovascular risk.^{3,4}

Controversy exists whether volumetric measures of VAT better capture the distribution of fat in the abdomen as compared with single-slice measurements. In particular, a recent study suggested that single-slice measurements may actually be more strongly correlated with cardio-metabolic risk factors as compared with volumetric measures of VAT.⁵ However, this study was limited to men, and consisted of a relatively small sample size. Furthermore, studies that have sought to compare single-slice with volumetric measures of VAT have not consistently reported which measurement site is the best correlate to not only VAT and subcutaneous abdominal adipose tissue (SAT) volumes but also cardio-metabolic risk factors. Recent studies, using magnetic resonance imaging (MRI) technique, report that a single image 5–10 cm above L_{4/5} is a better approximation of total VAT mass than a single image taken at the level of L_{4/5}.^{6–9} Thus, further research is warranted to show that a single-slice image at a certain level may serve as both the best correlate to total VAT and SAT volume and to cardio-metabolic risk factors regardless of age and gender.

Thus, the aim of this study is to determine the correlation between single-slice computed tomography (CT) measurements of SAT and VAT obtained at different anatomical sites with the overall VAT and SAT volumes, and then assess the correlations with cardio-metabolic risk factors in 200 participants from the Framingham Heart Study.

Materials and methods

Study participants

Study participants were recruited from the Framingham Heart Study, a population-based study that began in 1948 with the recruitment of 5209 men and women, 28–62 years of age. In 1971, the offspring and spouses of the offspring of the original cohort were enrolled. In 2002, 4095 Third Generation Study participants, who had at least one parent in the offspring cohort, were enrolled. The exclusion criteria and the design of the study have been described elsewhere.^{10,11}

In 2002, the Framingham Heart Study Multi-Detector Computed Tomography (MDCT) study began. Among the Offspring and Third Generation participants, 3529 participants underwent coronary and abdominal aortic scanning by multidetector CT. From this cohort, we randomly selected 100 participants from the Offspring cohort and 100 participants from the Third

Generation cohort. The random sample was taken to ensure approximately equal number of men and women, and an approximately equal number of participants in each of the age groups of 35–44, 45–54, 55–64, 65–74 and 75–84 years were represented.

The study was approved by the institutional review boards of the Boston University Medical Center and Massachusetts General Hospital. All subjects provided written consent.³

MDCT scan protocol

Subjects underwent eight-slice multidetector CT imaging of the abdomen in a supine position as previously described (LightSpeed Ultra, General Electric, Milwaukee, WI, USA). Briefly, 25 contiguous 5 mm thick slices (120 kVp; 400 mA; gantry rotation time, 500 ms; table feed, 3:1) were acquired covering 125 mm above the level of S1. Details have been described elsewhere.¹²

Area-based adipose tissue measurements

Single CT slices were loaded onto the dedicated workstation (Aquarius 3D Workstation, TeraRecon, San Mateo, CA, USA). The subcutaneous adipose tissue area (cm²) and visceral adipose tissue area (cm²) were measured using a single slice (5 mm thickness, 50 cm field view) at different anatomical locations.^{13,14} We defined the following anatomic locations as measurement sites: (1) inferior surface of the vertebral body of L1; (2) inferior surface of L₂; (3) inferior surface of L₃; (4) inferior surface of L₄; (5) inferior surface of L₅; (6) at the level of the umbilicus; and (7) the superior portion of the right iliac crest ((1)–(6): Figure 1a, (7): Figure 1b).

For each anatomic location, SAT and VAT were measured by applying predefined image display settings (window width: –195 to –45 HU; center: –120 HU). In order to separate VAT from SAT, the abdominal muscular wall separating the two compartments was automatically traced and subsequently adjusted manually. We have previously shown that area-based measurements are highly reproducible.¹²

Volumetric adipose tissue measurements

Consistent with the area-based approach, SAT and VAT volumes were determined on an offline workstation (Aquarius 3D Workstation, TeraRecon). Using similar display settings, total amount of SAT and total amount of VAT were measured across the total imaging volume and were calculated in cm³. The abdominal muscular wall separating the two adipose tissue compartments was manually traced; inter-reader reproducibility for this measurement was excellent.¹²

Risk factors and covariates

Risk factors were ascertained during the seventh examination of the Offspring or the first examination of the Third Generation cohort. BMI was defined as weight (in kilograms) divided by the square of height (in meters). Waist circumference was measured at the level of the umbilicus to the nearest quarter inch. Plasma glucose, high-density lipoprotein cholesterol and triglycerides were measured in patients who were fasting in the morning. Systolic blood pressure was measured in the seated position and represents an average of two physician measurements. The median time from the cardiovascular risk factor evaluation to the scan acquisitions was 1.5 years.

Statistical analysis

Means and s.d. of all area and volumetric SAT and VAT measurements were calculated. Pearson's correlation coefficients were generated sex-specifically after adjusting for age

between the area and volumetric measures for SAT and VAT separately. All analyses were sex-specific because of the known difference in fat distribution between women and men. The sample was secondarily age-stratified above and below the median (50.5 years). We also generated Pearson's correlation coefficients between the different SAT and VAT area measurements as compared with the SAT and VAT volumetric measurements.

All analyses were conducted using SAS v9.1.3 (Cary, NC, USA). A P -value <0.05 was considered to be statistically significant.

Results

Study sample characteristics are presented in Table 1. Results of the single-slice area measurements of SAT and VAT and the volumetric measurements are provided in Table 2. On average, VAT areas were highest at L_{1/2} in men and at L_{4/5} in women, whereas SAT areas were highest at the anatomic landmarks L_{4/5} and the superior iliac crest in women and men.

At every anatomical landmark, VAT was higher in men as compared with women (all $P<0.0001$, except P -value at L_{5/S1}, $P=0.157$). Similarly for SAT, areas were higher in women at every level examined with the exception of L_{1/2}.

Correlations between area-based and volumetric VAT and SAT measurements

Overall, area-based measurements from all anatomic locations were strongly correlated with SAT and VAT volumes after adjustment for age in both women and men (r^2 -range (SAT) =0.85–0.98, r^2 -range (VAT)=0.76–0.98; all $P<0.0001$; Table 3). Area-based measurements of VAT performed at L_{3/4} had the strongest correlation with VAT volume for both sexes (both $r^2 = 0.98$, $P<0.0001$), whereas area-based measurements obtained at L_{1/2} had the weakest correlation with VAT volume in women and men ($r^2=0.90$, $P<0.0001$ and $r^2 = 0.76$, $P<0.0001$, respectively).

Area-based measurements of SAT performed at L_{3/4} in both sexes had a strong correlation with SAT volume ($r^2 = 0.92$, $P<0.0001$ and $r^2 = 0.98$, $P<0.0001$, respectively), although this association was slightly stronger for the area-based measurements obtained at the superior iliac crest in women and men ($r^2 = 0.94$, $P<0.0001$ and $r^2 = 0.98$, $P<0.0001$, respectively). In contrast, measurements of SAT obtained at L_{1/2} and L_{2/3} had the weakest correlation with SAT volumes in men and women (women: $r^2=0.86$, $P<0.0001$ and men: $r^2 = 0.85$, $P<0.0001$, Table 3).

Correlations between area-based and volumetric VAT and SAT measurements stratified by age in women and men

In older subjects (>50.5 years), area-based measurements of VAT at the level of L_{3/4} remained most strongly correlated with VAT volume ($r=0.98$, $P<0.0001$ and $r=0.99$, $P<0.0001$ for men and women, respectively; Table 4). Similarly, in younger subjects L_{3/4} remained the anatomical landmark most strongly correlated with both sexes (both $r=0.99$, $P<0.0001$).

On an average, L_{3/4} was also most strongly correlated to the overall amount of SAT when stratified above as well as below the median age for both genders (above median age $r=0.94$, $P<0.0001$ in women, $r=0.99$, $P<0.0001$ in men and below median age $r=0.97$, $P<0.0001$ in women, $r=0.99$, $P<0.0001$ in men).

Association between area measurements of SAT and VAT and metabolic risk factors

Overall, there was a strong correlation between area and volume measures of SAT and VAT with cardiovascular risk factors included in the analysis (Table 5). For VAT, L_{3/4} was generally

the strongest correlate with metabolic risk factors, particularly for triglycerides, high-density lipoprotein cholesterol and systolic blood pressure. However, for fasting plasma glucose, the correlation between the L_{3/4} area measures was 0.40 ($P<0.001$) in women, whereas the strongest correlation was observed at L_{1/2} ($r = 0.49$, $P<0.001$), although the sample size was relatively small ($n = 46$).

More variability was observed for SAT in terms of relations with cardio-metabolic risk factors. For triglycerides and high-density lipoprotein cholesterol, the correlation between L_{3/4} was 0.39 and -0.26 , respectively (both $P<0.01$) in women, whereas the strongest correlation was observed at L_{2/3} ($r=0.47$, $r=-0.34$; both $P<0.001$). For systolic blood pressure, the correlation between the L_{3/4} area measures was 0.37 in women ($P<0.001$) and for fasting plasma glucose was 0.28 in men ($P<0.01$), whereas the strongest correlation for both fasting plasma glucose and systolic blood pressure was observed at L_{1/2} ($r=0.68$ and 0.59 , respectively; both $P<0.05$).

Discussion

In our community-based sample, we investigated which single-slice CT measurement of SAT and VAT area was the strongest correlate of overall SAT and VAT volumes. Our results indicate that while all measurements obtained at L_{1/2}, L_{2/3}, L_{3/4}, the umbilicus, L_{4/5}, L_{5/S1} were highly correlated with SAT and VAT volumes, SAT and VAT area measured at L_{3/4} provided the best proxy of SAT and VAT burden for both sexes and different ages. Furthermore, we found that the area-based measurements of SAT and VAT at L_{3/4} were similarly correlated with cardiovascular risk factors when compared with the volumetric measurements.

Both CT and MRI have been used to estimate SAT and VAT in order to quantify abdominal adiposity. While initially MRI was used to estimate adipose tissue burden, recently due to newer and quicker imaging protocols, the widespread availability and its much lower cost, CT has emerged as a new modality to accurately assess SAT and VAT volume on consecutive axial slices of the abdomen.^{15,16} Our results support the finding that area-based measurements by CT provide a very good estimate of SAT and VAT volumes.

Our findings are in agreement with those of Demerath *et al.*,¹⁷ who reported that in 820 subjects, single MRI images located approximately at the L₃ vertebra accurately estimate total VAT volume in blacks and whites of both sexes, aged 18–88 years ($r=0.98$). We also confirm recent findings by the same group of investigators, indicating that MRI-based area measurement of VAT obtained at the inter-vertebral space of L_{3/4} provided the strongest association between the relationship between VAT and cardio-metabolic risk factors in women.¹⁸ Among men, however, they found the strongest association with cardio-metabolic risk factors for VAT measured at L_{2/3}. Although our observed associations are generally similar in strength, the observed difference may be attributed to different definitions of the measurement sites due to specification of the MRI data acquisition. Specifically, Demerath *et al.* determined their measurement site, 6 cm superior to L_{4/5}, which crossed the L₃ vertebra in 85% of the subjects and the L₂ vertebra and L_{2/3} intervertebral space in the remaining 15% of the subjects. Moreover, Demerath *et al.* used a slice thickness of 1 cm, whereas we used 0.5 cm, which enabled more precise adjustment to the inferior surface of L₃ as one of the anatomic landmarks.

Abate *et al.*⁷ found in their study, including 49 men, that single images at L_{2/3} and L_{3/4} have equivalent correlations between area measured VAT and total VAT. The results of this study suggest that the same imaging protocol can be used in both women and men, making data collection easier.

Using CT, Kuk *et al.*⁵ determined the association between single-slice area measurements and volume measurements in asymptomatic 85 male outpatients. In contrast to the findings in the

present paper, their results indicate that VAT area at L_{1/2} and L_{2/3} were more strongly correlated with VAT volume than measurements obtained at all other anatomical landmarks ($r=0.98$ for L_{1/2} and L_{2/3}).⁵ Nonetheless, the magnitude of the correlations in this study is similar, suggesting that most single-slice measurements will comparably rank individuals as compared with volumetric measurements.

Finally, we determined whether single-slice measurements of SAT and VAT were correlated with a limited set of cardiovascular risk factors. We found that measurements obtained at L_{3/4} were correlated with all of the predefined risk factors. This is important because not only does L_{3/4} represent the best proxy of volumetric measurements, but it also correlates strongly with indices of cardio-metabolic risk. Consequently, by performing single-slice measurements of SAT and VAT at the level of L_{3/4} in both men and women, radiation exposure to the participant could be reduced as compared with a volumetric protocol.

Strengths and limitations

Strengths of this study include the randomly selected community-based sample with participants not enriched for adiposity-related traits. All measurements were performed by a single blinded reader. Our limitations include the primarily white sample, which limits the generalizability to other ethnic groups. Furthermore, we cannot rule out that the lower sample size at L_{1/2} in men might account for the lower correlation between VAT volumes and single-slice measurements at this anatomic level. Thus, the interpretation of our results is limited by the small sample size of VAT and SAT measurements at the level of L_{1/2}.

Implications

VAT has been shown to be a stronger correlate of cardiovascular risk than systemic adiposity indexes, waist circumference or subcutaneous abdominal fat.³ In this study, we demonstrate that visceral and subcutaneous fat areas, especially obtained at L_{3/4}, are well correlated to metabolic risk factors as well as to visceral and subcutaneous fat volumes. Further research is needed to determine whether our findings can be applied to other ethnic groups.

Conclusion

A single area-based measurement of SAT and VAT obtained at L_{3/4} is a strong proxy for the overall SAT and VAT volume for both men and women and similarly associated with a selected set of cardiovascular risk factors. Further investigation is warranted regarding associations of single-slice adipose tissue with other body fat deposits, cardiovascular risk factors and clinical outcomes.

Acknowledgments

This study was supported by the National Heart, Lung and Blood Institute's Framingham Heart Study (N01-HC-25195).

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Figure 1.

(a) CT-Scout image illustrating the defined anatomical landmarks at the sagittal lumbar spine (1–5) and the umbilicus (6). L_{1/2} represents the inferior surface of the vertebral body of L1 (1); L_{2/3} represents the inferior surface of L2 (2); L_{3/4} represents the inferior surface of L3 (3); L_{4/5} represents the inferior surface of L4 (4); L_{5/S1} represents the inferior surface of L5. (b) CT-Scout image illustrating the defined anatomical landmark at the pelvis so called ‘right iliac crest’.

Table 1

Subject characteristics of 200 participants of the Framingham Offspring and Third Generation cohort included in the analysis

	Mean (s.d.) or % (n)	
	Men (n=100)	Women (n= 100)
Age (years)	54 ± 14	55 ±14
BMI (kg m ⁻²)	29 ± 4	27 ± 5
Waist from exam (cm)	101 ± 11	93± 14
Glucose (mg per 100 ml)	104± 19	94 ± 13
Triglycerides (25/50/75th)	76/111/175	67/94/150
HDL (mg per 100 ml)	47 ± 12	61 ± 16
Systolic blood pressure (mm Hg)	126 ± 14	120± 18
Current smoker	18% (18)	26% (26)
Subjects of the Offspring	51% (51)	49% (49)
Subjects of the Third Generation	49% (49)	51% (51)

Abbreviations: BMI, body mass index; HDL, high-density lipoprotein.

Table 2

Average volumetric and planimetric measures of VAT and SAT (mean $\pm$ s.d.) at the level of predefined anatomic landmarks

Variable	N	Men	N	Women	P-value
<i>VAT</i>					
Volume L ₂ -S ₁ (cm ³)	99	2170 $\pm$ 963.78	99	1395 $\pm$ 877.58	<0.001
L _{1/2} (cm ²)	14	233.77 $\pm$ 99.11	46	104.12 $\pm$ 71.92	<0.001
L _{2/3} (cm ²)	99	207.64 $\pm$ 100.94	99	103.81 $\pm$ 77.04	<0.001
L _{3/4} (cm ²)	99	197.85 $\pm$ 94.00	99	115.14 $\pm$ 80.60	<0.001
Umbilicus (cm ²)	99	180.56 $\pm$ 78.36	99	118.44 $\pm$ 77.75	<0.001
L _{4/5} (cm ²)	99	157.52 $\pm$ 71.60	99	121.74 $\pm$ 72.8	<0.001
Iliac crest (cm ²)	99	155.80 $\pm$ 70.85	99	118.78 $\pm$ 68.73	<0.001
L ₅ /S ₁ (cm ²)	99	107.62 $\pm$ 43.99	99	99.94 $\pm$ 56.49	0.157
<i>SAT</i>					
Volume L ₂ -S ₁ (cm ³)	99	2633 $\pm$ 1158	99	3091 $\pm$ 1410	0.014
L _{1/2} (cm ²)	14	171.94 $\pm$ 88.27	46	170.2 $\pm$ 86.0	0.880
L _{2/3} (cm ²)	99	168.36 $\pm$ 79.01	99	191.8 $\pm$ 107.3	0.084
L _{3/4} (cm ²)	99	215.38 $\pm$ 92.53	99	234.5 $\pm$ 118.8	0.219
Umbilicus (cm ²)	99	229.17 $\pm$ 104.51	99	258.3 $\pm$ 137.4	0.101
L _{4/5} (cm ²)	99	240.46 $\pm$ 115.40	99	296.5 $\pm$ 125.8	0.001
Iliac crest (cm ²)	99	240.56 $\pm$ 108.97	99	306.8 $\pm$ 131.6	<0.001
L ₅ /S ₁ (cm ²)	99	207.38 $\pm$ 110.23	99	320.1 $\pm$ 134.5	<0.001

Abbreviations: SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.

Table 3

Age-adjusted correlation by sex between total volume of SAT/VAT to two-dimensional measurements of SAT and VAT at different positions

	Women			Men		
	N	SAT Volume (r)	VAT Volume (r)	N	SAT Volume (r)	VAT Volume (r)
L _{1/2}	46	0.95	0.95	14	0.92	0.87
L _{2/3}	100	0.93	0.98	100	0.97	0.96
L _{3/4}	100	0.96	0.99	100	0.99	0.99
Umbilicus	100	0.94	0.98	100	0.99	0.92
L _{4/5}	100	0.96	0.97	100	0.99	0.95
Iliac crest	100	0.97	0.96	100	0.99	0.89
L ₅ /S ₁	100	0.97	0.96	100	0.94	0.90

Abbreviations: SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue. All correlations were significant with a *P*-value of <0.0001.

Age and sex stratified correlations of VAT and SAT volume with slice area at different anatomical sites; all *P*-values were <0.0001

Table 4

	Women			Men		
	<i>N</i>	SAT Volume (<i>r</i>)	VAT Volume (<i>r</i>)	<i>N</i>	SAT Volume (<i>r</i>)	VAT Volume (<i>r</i>)
<i>Age>median</i>						
L _{1/2}	33	0.94	0.94	12	0.93	0.83
L _{2/3}	50	0.88	0.97	50	0.98	0.95
L _{3/4}	50	0.94	0.99	50	0.99	0.98
Umbilicus	50	0.92	0.96	50	0.98	0.89
L _{4/5}	50	0.96	0.96	50	0.99	0.94
Iliac crest	50	0.96	0.96	50	0.99	0.86
L ₅ /S ₁	50	0.96	0.96	50	0.95	0.87
<i>Age<median</i>						
L _{1/2}	13	0.96	0.99	2	—	—
L _{2/3}	50	0.96	0.99	50	0.97	0.97
L _{3/4}	50	0.97	0.99	50	0.99	0.99
Umbilicus	50	0.97	0.99	50	0.99	0.96
L _{4/5}	50	0.97	0.98	50	0.99	0.96
Iliac crest	50	0.97	0.97	50	0.99	0.95
L ₅ /S ₁	50	0.97	0.96	50	0.94	0.93

Abbreviations: SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.

Table 5

Sex stratified and age-adjusted correlation between total volume and single slices of VAT/SAT to cardiovascular risk factors

	Women				Men			
	Glucose <i>r</i>	TRIG <i>r</i>	HDL <i>r</i>	SBP <i>r</i>	Glucose <i>r</i>	TRIG <i>r</i>	HDL <i>r</i>	SBP <i>r</i>
SAT Volume	0.27**	0.40***	-0.27**	0.35***	0.30**	0.15	-0.19	0.17
L _{1/2}	0.31*	0.24	-0.23	0.48***	0.68**	0.33	-0.09	0.59*
L _{2/3}	0.25*	0.47***	-0.34***	0.31**	0.27**	0.15	-0.12	0.14
L _{3/4}	0.22*	0.39***	-0.26**	0.37***	0.28**	0.13	-0.15	0.13
Umbilicus	0.35***	0.33***	-0.25**	0.30**	0.32***	0.15	-0.19	0.15
L _{4/5}	0.31**	0.35***	-0.26**	0.36***	0.30**	0.18	-0.22*	0.18
Iliac crest	0.29**	0.38***	-0.28**	0.34***	0.30**	0.16	-0.21*	0.16
L _{5/S1}	0.32**	0.41***	-0.30**	0.34***	0.33***	0.09	-0.28**	0.07
VAT volume	0.41***	0.51***	-0.36***	0.36***	0.31**	0.32***	-0.35***	0.29**
L _{1/2}	0.49***	0.34*	-0.31*	0.36**	0.26	0.17	0.11	0.61*
L _{2/3}	0.40***	0.50***	-0.37***	0.36***	0.30**	0.29**	-0.35***	0.28**
L _{3/4}	0.40***	0.51***	-0.38***	0.38***	0.32**	0.32***	-0.37***	0.28**
Umbilicus	0.36***	0.47***	-0.35***	0.33***	0.24*	0.28**	-0.29**	0.31**
L _{4/5}	0.45***	0.49***	-0.34***	0.37***	0.31**	0.28**	-0.31**	0.28**
Iliac crest	0.47***	0.50***	-0.35***	0.34***	0.27**	0.19	-0.23*	0.20*
L _{5/S1}	0.44***	0.48***	-0.32***	0.30**	0.21*	0.29**	-0.31**	0.28**

Abbreviations: HDL, high-density lipoprotein; SAT, subcutaneous adipose tissue; SBP, systolic blood pressure; TRIG, triglycerides; VAT, visceral adipose tissue.

All *P*-values

* *P*<0.05;

** *P*<0.01;

*** *P*<0.001.