Comparative analysis of three human adipocyte size measurement methods and their relevance for cardiometabolic risk

Sofia Laforest[1,2,3], Andréanne Michaud[4], Gaétan Paris[5], Mélissa Pelletier[1,3], Hubert Vidal[6], Alain Géloën[6] and André Tchernof[1,2,3]

Author affiliations:
1: Endocrinology and Nephrology, CHU de Quebec-Laval University, Quebec City, Canada;
2: School of Nutrition, Laval University, Quebec City, Canada;
3: Quebec Heart Lung Institute, Quebec City, Canada;
4: Montreal Neurological Institute, McGill University, Montreal, Canada;
5: Gynecology Unit; Laval University Medical Center Quebec City, Canada;
6: University of Lyon, CARMEN INSERM U1060, INSA-Lyon, F-69621, Villeurbanne, France.

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***Address for correspondence*:** André Tchernof, Ph.D.
Endocrinology and Nephrology
CHU de Quebec-Laval University
2705 Laurier Blvd. (R-4779)
Québec, (Québec)
CANADA G1V 4G2

Tel: (418) 654-2296
Fax: (418) 654-2761
E-mail: andre.tchernof@crchudequebec.ulaval.ca

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Study importance

What is already known about this subject?

- Adipocyte size is a recognized marker of adipocyte function and cardiometabolic risk
- Various methods to assess fat cell size have been used

What does your study add?

- Comparative analysis of three adipocyte size measurement methods generated systematic differences in adipocyte median diameter
- Associations with adiposity were only slightly affected by the technique
- Osmium fixation generated stronger associations with cardiometabolic risk factors than collagenase digestion and histological analysis

Abstract

Objective: To determine whether adipocyte diameters from three measurement methods are similarly associated with adiposity measurements and cardiometabolic variables.

Methods: Surgical samples of omental (OM) and abdominal subcutaneous (SC) adipose tissue were obtained in a sample of 60 women (age 35-59 years; BMI 20.3-41.1 kg/m^2). Median adipocyte diameter of the main cell population was determined by collagenase digestion, osmium tetroxide fixation and histological analysis. Adiposity and cardiometabolic risk factors were assessed.

Results: Adipocyte diameter was consistently smaller with formalin fixation than with collagenase digestion, whereas osmium-fixed cells were larger ($p<0.0001$, for all). Median adipocyte diameters derived from all methods were intercorrelated ($r=0.46$ to 0.83, $p<0.001$ for all). Positive associations were found between adipocyte diameter from all techniques and regional or total adiposity measurements ($p<0.01$ for all). OM adipocyte diameter was positively associated with fasting glucose, insulin and HOMA-IR ($r=0.30$ to 0.52, $p<0.05$ for all), with osmium-fixed cell size as a stronger correlate. Osmium-fixed cell diameter was also a better correlate of plasma adiponectin and leptin.

Conclusions: Although measurement techniques generated systematic differences in adipocyte size, associations with adiposity were only slightly affected by the technique. Osmium fixation generated stronger associations with cardiometabolic risk factors than collagenase digestion and histological analysis.

Introduction

Adipocyte size has been studied in humans and rodents for more than 50 years. Collagenase digestion, osmium tetroxide fixation and histological analysis have been used to assess fat cell size, yet no particular technique has emerged as the gold standard (1). Our recent analysis of the literature in humans showed that cell size generally increases as a function of obesity level, but that the three techniques generate different results in comparable populations (1). Histological analysis apparently generates lower mean fat cell size across all BMI values compared to collagenase digestion and osmium fixation (1). However, our analysis also revealed that there is a large inter-individual variability in adipocyte size and no association with adiposity or metabolic abnormalities is observed at high BMI values, consistent with plateauing values in patients with severe obesity (1).

Collagenase digestion has been developed by Rodbell (2) and is the most widely used technique to assess adipocyte size in humans. Adipose tissue is digested with collagenase to separate mature adipocytes from the stroma-vascular fraction by floatation. This method generates live cells, which makes it useful for functional cell assays. Limitations of this technique include the fact that small cells do not float easily due to their low lipid content, and that large cells are very fragile (3). However, the introduction of adenosine in the solution possibly minimizes this bias (4). Histological analysis can be used to assess adipocyte cell size in a retrospective manner. This method is frequently used when the primary investigation outcomes are adipose tissue morphology and *in situ* markers. For example, this technique is useful for immunostaining experiments because adipocyte lipid droplets are easily seen with a perilipin antibody (5). Adipose tissue is rather fragile; therefore use of a fixating agent and necessity to cut samples may damage whole-tissue architecture. Furthermore, the amount of time needed for analysis may limit the number of patients examined with either collagenase digestion or histological analysis. Fixation of collagenase-digested cells either with formaldehyde or osmium tetroxide can be performed to postpone

analysis and increase the number of samples tested (6). Multisizer counter has also been used to measure size of collagenase-isolated, unfixed rat adipocytes (7).

If the investigation outcome is fat cell population distribution, osmium tetroxide fixation followed by Multisizer counter analysis is the best option. The osmium acid fixation technique for adipocytes was developed by Hirsch et Gallian (8). Osmium tetroxide fixes intracellular lipids and allows analysis of a substantially higher number of cells, limiting potential measurement bias. Originally, cell number was analyzed with a Coulter counter and mean cell size was obtained with triolein density and the weight of whole adipose tissue before fixation. More recent Multisizer counters allow direct measurement of adipocyte diameter following fixation (9-13). This method generates a bimodal distribution of adipocyte sizes, with subpopulations of very small and large adipocytes (14-16). Cell size distributions can be generated and analyzed by fitting an exponential-Gaussian formula to obtain parameters describing adipocyte subpopulations including very small adipocytes (13, 17-19).

To our knowledge, no study has ever compared these three most commonly used techniques to assess fat cell size for their association with total and regional adiposity as well as cardiometabolic risk factors. Our objective was to investigate if there are differences in fat cell size related to the technique used. We also aimed to determine whether adipocyte diameters from these methods were similarly associated with adiposity measurements and cardiometabolic variables as there are still discrepancies in the literature on these correlations. Based on our previous analysis of the literature (1), we tested the hypothesis that median fat cell size obtained from histological analysis is lower than that of collagenase-digested and osmium tetroxide fixation approaches, yet all measurements are similarly correlated to adiposity and body fat distribution measurements as well as cardiometabolic risk variables.

Methods

Subjects and ethics statement

The study included 60 women (lean to obese) scheduled for gynecological surgery (total or subtotal abdominal hysterectomies) at the Gynecology Unit of Laval University Medical Center. Ranges of age and BMI were 35.2 -59.4 years and 20.3-41.1 kg/m^2, respectively. Women were excluded if they had Cushing syndrome, hyperthyroidism, cancer, cardiovascular diseases, type 1 or 2 diabetes and if they reported weight loss or gain in the past year. The study was explained to each participant and a written consent was obtained. The study was approved by the Research Ethics Committees of Laval University Medical Center (C09-08-086).

Adiposity and body fat distribution measurements

On the morning of or a few days before surgery, body weight, height, body mass index (BMI) and waist circumference were measured. Participants also underwent a computed tomography (CT) exam (GE Light Speed 1.1 CT scanner, General Electric Medical Systems, Milwaukee, WI) at the L4L5 levels to assess visceral and abdominal subcutaneous (SC) adipose tissue areas as previously described (20). Total body fat mass and lean body fat mass were assessed by dual-energy X-ray absorptiometry (DXA) (Hologic QDR-4500A densitometer with whole-body fan beam software v8.26a:3-Hologic Inc., Bedford, MA).

Plasma lipid profile, glucose homeostasis and adipokine measurements

Fasting blood samples were collected on the morning of surgery after a 12h-overnight fast. From these samples, cholesterol and triglyceride levels in both plasma and lipoprotein fractions were measured (21). The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated from fasting glucose and insulin levels as previously described (22). Enzyme-linked immunosorbent assay was performed on these samples for the following adipokines: leptin (Human Leptin ELISA kit; EMD Millipore; Billerica, MA, USA), adiponectin (Human Adiponectin ELISA Kit; B-Bridge International Inc; Santa Clara, CA, USA), interleukin-6 (IL-6) (Human IL-6 Quantikine HS ELISA; R&D Systems; Minneapolis, MN, USA)

and tumor necrosis factor-α (TNF-α) (Human TNF-α Quantikine HS ELISA; R&D Systems; Minneapolis, MN, USA).

Adipose tissue sampling and fat cell size measurements

Abdominal subcutaneous (SC) and omental (OM) adipose tissues were collected at the site of the surgical incision and at the distal portion of the greater omentum, respectively. Samples were immediately separated into three subsamples for cell size analysis (~1g for collagenase digestion (CD), ~50-100 mg for formalin fixation and paraffin embedding H&E histological slides (HIS) and ~50 mg for osmium tetroxide fixation (OS)).

Collagenase digestion was performed on the first subsample of fresh tissue, as previously described (21). 0.1 µM adenosine was added in the Krebs-Ringer Henseleit (KRH) buffer to limit cell breakage. Fat cell diameter was measured on mature adipocyte suspension pictures captured using a phase contrast microscope attached to a camera and a computer interface (Scion Image Software, Scion Corporation, Frederick, MA, USA) as recommended in (23). The diameter of 250 adipocytes in each depot was measured. The second subsample of fresh tissue was fixed in a solution of osmium tetroxide, as described previously (9). Briefly, 50 mg of adipose tissue was incubated in osmium tetroxide collidine-HCL solution for at least 96 hours at room temperature. Samples were then rinsed with NaCl 0.9% for 24h and rinsed with 8 M urea for 48h. Isolated, fixed cells were resuspended in PBS 0.01% Triton X-100 and rinsed through a 250 µm nylon mesh. Cells were resuspended in glycerol and diluted into beakers containing Isoton II solution (Beckman Coulter, Villepinte, France) and analyzed using a Beckman Coulter Multisizer IV (Beckman Coulter, Villepinte, France) with a 400 µm aperture. The range of cell sizes analyzed was 25 to 240 µm. Cell size distributions were determined with at least 12 000 cells per sample and were analyzed by fitting an exponential (small cells)-Gaussian (large adipocytes) formula (non-linear least-squares function). Only the mode (center of the Gaussian peak), which is the median diameter of the large cells was used in the present analysis. Osmium tetroxide data were used in a previous publication on this topic (9). Finally, the last subsample of fresh adipose tissue was fixed in

formaldehyde and paraffin-embedded. Sections of 5 µm of OM and SC adipose tissues were mounted on the same slide and were stained with hematoxylin/eosin dyes. Whole slides were digitalized by scanning total area at 20X magnification and 0.24 µm/pixel resolution using a NanoZoomer Hamamatsu scanner (Hamamatsu Photonics KK, Systems Division). The smallest and largest diameters of each cell were manually measured on an average of 100 adipocytes per sample using CaloPix software (Tribvn, Chatillon, France). The mean of these two values was used in analyses.

The intra-observer coefficient of variation was 5.2% (95% confidence interval [CI], 1.6% to 8.8%) and 5.8% (95% confidence interval [CI], 3.2% to 8.4%) for n=8 OM and SC samples, respectively. The inter-observer coefficient of variation was 3.1% (95% CI, 1.5% to 4.8%) and 4.4% (95% CI, 0.1% to 8.9%) for n=8 OM and SC samples, respectively.

Statistical analyses

Student's paired t-tests were performed to examine depot differences for each measurement method. Differences among techniques and BMI categories were determined by mixed model analysis. Pearson correlation coefficients were computed to quantify associations between adipocyte median diameter for each technique and adiposity measurements as well as cardiometabolic risk variables. Because we focused on the main cell population, median cell size of the Gaussian curve was used for the osmium technique. For collagenase and histology, mean cell size and median cell size were not significantly different within each technique. For consistency, we used median cell size of all three measurements methods. Non-normally distributed variables were log- or boxcox-transformed. All data are presented as mean ± SEM. Statistical analyses were performed with JMP software or SAS (SAS Institute Inc, Cary, NC, USA).

Results

Characteristics of the sample

Table 1 shows the main characteristics of the study population. Women were covering a large range of obesity levels according to BMI values spanning from 20.3 to 41.1 kg/m^2. The study sample included pre- and postmenopausal women with an average age of 47 years. Women were mostly overweight with a mean BMI of 27.1±4.4 kg/m^2 and presented evidence of abdominal fat accumulation according to their mean waist circumference value of 92.4 cm. They displayed a normal plasma lipid profile and glucose homeostasis values on average.

Differences in adipocyte size according to the measurement method

Average cell size distribution curves according to the three techniques are shown in **Figure 1**. **Figure 1A**, **C** and **E** show the presence of a Gaussian distribution for all techniques in both depots (OM and SC). The three techniques also presented a significant proportion of small cells, which appeared left of the Gaussian distribution.

As expected in this population composed exclusively of women, SC median adipocyte diameter was significantly higher compared to OM median adipocyte diameter for all techniques ($p<0.0001$) (**Figure 1B**, **D**, and **F**). Despite small differences in adipocyte distributions (**Figure 1**), all techniques were strongly intercorrelated in both depots as shown in **Figure 2**, with correlation coefficients ranging between 0.43 and 0.83 ($p<0.002$ for all).

Adipocyte size and obesity level

To assess the variation in fat cell size as a function of BMI category, a mixed-model analysis was performed. In each BMI category (lean, overweight and obese), osmium-fixed median adipocyte diameter was consistently larger and formalin-fixed tissue median fat cell diameter was smaller than collagenase-digested median adipocyte diameter in both depots ($p<0.0001$, for all) (**Figure 3**). In OM adipose tissue,

median adipocyte diameters of lean and overweight women were significantly different from those of women with obesity ($p<0.01$), whereas the difference between lean and overweight women did not reach statistical significance ($p_{trend}=0.06$) (**Figure 3A**). Both, SC collagenase-digested and osmium-fixed median adipocyte diameters were increased through BMI categories ($p<0.05$, for all) (**Figure 3B**). Differences between lean and overweight women did not reach statistical significance for histological analysis of SC median adipocyte diameter ($p_{trend}=0.06$). Nevertheless, histological analysis of SC median adipocyte diameter was significantly higher in women with obesity compared to lean or overweight women ($p<0.05$). Using a correction factor for the formalin-fixed tissue cell diameters generated values similar to those of the collagenase-digested cells for each depot and each BMI category (**Figure 4**) (24, 25). These results suggest that various correction factors (24-26) could be used when comparing studies in which adipocyte size has been measured by collagenase digestion and histological analysis. Of note, osmium-fixed median adipocyte diameter was still larger than collagenase-digested and formalin-fixed tissue median cell diameter possibly reflecting the space occupied by the osmium following fixation.

Adipocyte size and cardiometabolic risk factors

We then investigated whether the three measurement methods had an impact on the relationship between adipocyte size and adiposity indices and/or metabolic risk variables. As expected, OM median adipocyte diameter derived from all techniques was strongly and positively correlated with BMI, waist circumference, total body fat mass, total body fat percentage, trunk fat mass and adipose tissue areas ($p\leq0.05$) (**Table 2**). SC median adipocyte diameter was also strongly associated with these parameters regardless of the technique (**Table 2**) ($p\leq0.05$). OM median adipocyte size was positively associated with fasting glucose, fasting insulin as well as HOMA-IR, for all techniques ($p\leq0.05$) (**Table 3**). The strongest correlation coefficients were observed with osmium-fixed median cell sizes (**Table 3**). SC median adipocyte diameters from the histological and osmium techniques were also related to fasting insulin and HOMA-IR, although correlation coefficients were of slightly lower magnitude ($p\leq0.05$) (**Table 3**). SC median adipocyte diameters from all techniques were not associated with fasting glucose. OM median fat

cell size from the three techniques was also correlated with parameters of the plasma lipid profile, particularly VLDL-cholesterol and VLDL-triglyceride concentrations ($p \leq 0.05$) (**Table 3**). Significant associations were only found between osmium- or formalin-fixed median adipocyte diameter in OM adipose tissue with levels of HDL-cholesterol, total triglycerides as well as the total cholesterol-to-HDL-cholesterol ratio. In SC adipose tissue, the total cholesterol-to-HDL-cholesterol ratio was associated with median adipocyte diameter from the three methods. Plasma HDL-cholesterol levels were negatively correlated with collagenase-digested and osmium-fixed SC median cell diameters, whereas concentrations of VLDL-cholesterol, VLDL-triglyceride and total triglycerides were only associated with formalin-fixed tissue SC median adipocyte diameter. Plasma leptin and adiponectin concentrations were both associated with OM and SC median fat cell size from all methods **(Table 3**). Although very stringent, Bonferroni correction showed that only associations between osmium-fixed median adipocyte diameter and cardiometabolic risk remained significant (data not shown). Associations between cell size measurements and adiposity indices were generally unaffected by Bonferroni correction (not shown).

Discussion

We investigated whether differences were found in fat cell size with three different techniques. We also examined how adipocyte diameter from these methods was associated with adiposity measurements and cardiometabolic risk variables. As expected, there was a difference in median fat cell size according to the method used. Histological median adipocyte diameter was lower and osmium tetroxide-measured median adipocyte diameter higher than that obtained by collagenase digestion in each BMI category in both the OM and SC depots. Yet, all techniques were strongly intercorrelated in both depots. Furthermore, median fat cell size from all techniques was, in general, similarly associated with adiposity values. However, osmium-fixed median adipocyte size generated stronger associations with cardiometabolic variables, especially glucose homeostasis parameters and plasma adipokine levels.

To our knowledge, this is the first study to clearly compare the three most widely used techniques to assess adipocyte size in two abdominal fat depots in a sample of healthy women well-characterized for body fat distribution and body composition (CT and DXA). Previous studies comparing fat cell measurement methods have been published (3, 14, 27, 28). However, these studies often compared a widely used method (for the time) with a novel method developed by the authors. For example, older literature often compared novel methods with ocular cell sizing of collagenase-digested cells, which has been replaced by computerized picture analysis of isolated cells. It is noted across the literature that semi-automatic methods are not yet widely used among research teams, as the quality of the image is often a limitation. Furthermore, these methods still need significant input from the observer (final choice of cells counted, correction for incomplete membranes, decisions about the cells on the edge of the image, etc.). Considering these steps, semi-automated techniques may not be as fast as expected, or faster than manual analysis, as initially proposed. These notions support the rationale of our study, i.e to analyze variation in adipocyte size related to the measurement technique using simple, manual techniques.

Each technique presented a Gaussian curve and all three techniques and measurements were strongly intercorrelated in each fat compartment. However, as shown with the exponential curve, a population of small cells was observed in osmium-fixed and to a lesser extent in collagenase-digested and formalin-fixed tissue. The explanation for higher small cell frequency in the osmium technique compared to other methods is uncertain, but may reflect differences in sensitivity. Some factors may explain the low abundance of small cells in histological analyses and collagenase digestion such the lower number of cells examined compared to other techniques and the difficulty to visualize small cells. Furthermore, the absence of blood vessels in histological fields examined may induce this bias, since perivascular regions are thought to be the niche of adipose precursor cells (29, 30). McLaughlin et al. (12, 13) proposed that these cells may represent cells undergoing differentiation or immature adipocytes that are unable to store the excess dietary lipids. Some studies reported that small cells were more abundant in adipose tissue

292 from subjects with obesity, which led to other hypotheses about their origin (9, 31, 32). They have been
293 proposed to originate from multilocular cells that are ruptured during the fixation process or during the
294 collagenase treatment, although there is no clear evidence of this phenomenon occurring (14, 33). Their
295 presence among collagenase-digested cells, found both in our sample and in another animal study (31),
296 provide convincing evidence that these small cells do represent lipid-containing adipocytes and not
297 artefacts of the osmium tetroxide fixation method. A threshold value of 25 μm was applied in the osmium
298 tetroxide method to discriminate cells from artefacts (9). The biological relevance of these small cells also
299 remains an open question.

300

301 As expected, median fat cell size in both depots varies as a function of obesity level regardless of the
302 method. Central obesity was more strongly associated with fat cell size in OM and SC adipose tissue than
303 overall adiposity. The chosen sampling sites may partly explain these results. SC adipocyte diameter was
304 larger than OM adipocyte diameter with every method, reflecting the depot-specific difference in fat cell
305 size reported in our previous studies in women (9, 34) and our detailed analysis of the literature (1). The
306 association between visceral adiposity and cardiometabolic risk factors is well known (35). Here we
307 showed for the first time in the same sample of women that adipocyte hypertrophy measured by three
308 different techniques is related to altered blood lipids and glucose homeostasis. OM and SC adipocyte
309 diameter assessed by osmium fixation methods were more closely correlated to some of the metabolic
310 variables than fat cell size assessed by collagenase digestion and histological analysis. This may be due to
311 the larger number of cells examined or the use of curve-fitting techniques both of which could decrease
312 background noise associated with individual data points. Small differences between techniques may
313 explain discrepancies observed among available studies for the association between adipocyte diameter
314 and cardiometabolic variables (1). Furthermore, correlations between glucose homeostasis and fat cell
315 size were stronger in OM compared to SC adipose tissue regardless of the technique used, as previously

316 reviewed (1). Adipocyte hypertrophy in both depots may represent a critical determinant of cellular
317 function and cardiometabolic risk (1).

318

319 Limitations of the study should be acknowledged. The first one is the sample population. Men were not
320 studied and differences between male and female adipocyte characteristics in both depots have already
321 been discussed in the literature (reviewed in (1)). Since sampling of both abdominal depots requires
322 general anesthesia, recruitment of healthy men covering a large range of BMI values and age undergoing
323 elective surgery without major metabolic alterations or chronic diseases such as cancer is a challenge in
324 the scientific community. Women in this study were covering a large range of obesity level and the
325 number of women with obesity or with severe obesity was low (n=13, 21.7% and n=1, 1.7%,
326 respectively). However, participants were generally healthy apart from their gynecological condition.
327 Another limitation of the study was the exclusion of women for whom adipose tissue architecture was too
328 damaged to perform analysis after fixation and paraffin-embedding. A total of 15 (13%) slides were
329 excluded which represents a significant amount and should be taken in consideration when small patient
330 samples are investigated. In comparison, for collagenase digestion and osmium fixation methods, only
331 two samples (1.7%) were excluded due to technical difficulties.

332 Conclusion

333 In conclusion, histological analysis led to smaller adipocyte diameters and osmium tetroxide fixation led
334 to higher adipocyte diameter than collagenase digestion in all BMI categories. However, associations
335 between median adipocyte diameter and adiposity measurements was only slightly affected by the method
336 used, and osmium tetroxide fixation led to stronger associations with cardiometabolic risk factors. Each
337 technique obviously has its advantages and disadvantages, which must be understood and acknowledged
338 by investigators when designing their study (**Table 4**).

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FIGURE LEGENDS

Figure 1: Techniques used to assess adipocyte diameter all presented a Gaussian curve. Adipocyte distribution from formalin-fixed paraffin-embedded H&E colored slides (HIS) (A); from collagenase digestion (CD) (C); or osmium tetroxide fixation (OS) and Multisizer analysis (E). Cells larger than 250 µm were removed in this Figure only, so that the x axes are more extensive in the region of 0 to 250 µm. Depot-specific differences were observed in the three techniques (B, D, F). Subcutaneous (SC) median adipocyte diameter was larger than omental (OM) median adipocyte diameter, n=46, Student paired t-test, ***$p<0.0001$.

Figure 2: Correlations between collagenase digestion median adipocyte diameter, histological analysis median adipocyte diameter or osmium tetroxide fixation median adipocyte diameter in omental (OM) (A, B, C) or subcutaneous (SC) (D, E, F). Collagenase digestion and histology analysis in OM ($r=0.60$, $p<0.0001$) (A) and SC adipose tissue ($r=0.43$, $p<0.0018$) (D). Osmium fixation and histology analysis in OM ($r=0.83$, $p<0.0001$) (B) and SC adipose tissue ($r=0.49$, $p<0.0003$) (E). Osmium fixation and collagenase digestion in OM ($r=0.71$, $p<0.0001$) (C) and SC adipose tissue ($r=0.80$, $p<0.0001$) (F).

444 **Figure 3**: Omental (OM) (A) and subcutaneous (SC) (B) median adipocyte diameter in each BMI
445 category (Lean<25 kg/m^2, Overweight ≤25-<30 kg/m^2, Obese ≥30 kg/m^2); linear mixed-model,
446 ***p<0.0001. Statistically different from the lean subgroup (a) and/or the overweight subgroup (b) from
447 the same technique. P <0.05 were considered significant.

448

449 **Figure 4**: Omental (OM) (A) and subcutaneous (SC) (B) median adipocyte diameter in each BMI
450 category (Lean<25 kg/m2, Overweight ≤25-<30 kg/m2, Obese ≥30 kg/m2); linear mixed-model,
451 ***p<0.0001. HIS median diameters were corrected according to Weibel et al. (2, 3). Statistically
452 different from the lean subgroup (a) and/or the overweight subgroup (b) from the same technique. P<0.05
453 were considered significant.

454

TABLES

Table 1: Characteristics of the 60 women of the study

Variables	Mean	±	SD	Range (min-max)
Anthropometrics				
Age (yrs)	46.8	±	5.9	35.2-59.4
Weight (kg)	69.9	±	11.2	54.7-107.8
BMI (kg/m²)	27.1	±	4.4	20.3-41.1
Waist circumference (cm)	92.4	±	11.4	68.0-123.5
Body composition				
Total body fat percentage (%)	34.9	±	5.0	16.7-45.1
Total body fat mass (kg)	25.2	±	7.0	9.6-47.3
Lean body mass (kg)	36.0	±	5.0	36.0-58.9
Trunk fat mass (kg)	11.6	±	4.0	3.3-24.1
Trunk lean mass (kg)	24.4	±	2.7	18.5-31.1
Limb fat mass (kg)	13.0	±	3.6	5.4-24.0
Limb lean mass (kg)	17.4	±	2.4	12.5-24.6
Trunk fat mass/limb fat mass	0.9	±	0.2	0.5-1.4
Adipose tissue area (cm^2) [a]				
Total	407	±	140	92-725
Subcutaneous	311	±	103	71-568
Visceral	97	±	45	21-278
Plasma lipid profile				
Cholesterol (mmol/L)				
Total	4.91	±	0.82	3.21-6.99
VLDL	0.39	±	0.27	0.05-1.23
LDL	3.07	±	0.76	1.65-4.94
HDL	1.46	±	0.39	0.83-2.57
Triglycerides (mmol/L)				
Total	1.13	±	0.55	0.40-3.32
VLDL	0.65	±	0.48	0.12-2.75
LDL	0.22	±	0.07	0.11-0.40
HDL	0.26	±	0.07	0.14-0.49
Cholesterol/HDL-cholesterol	3.49	±	0.85	2.01-5.52
Glucose homeostasis				
Fasting glucose (mmol/L)	5.3	±	0.4	4.5-6.6
Fasting insulin (mmol/L) [b]	7.3	±	4.0	1.5-21.4
HOMA index [b]	1.7	±	1.0	0.3-5.0

HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; HOMA, homeostasis model assessment index; [a]n=59, [b]n=58,

Table 2: Pearson correlation coefficients between OM or SC adipocyte diameters and anthropometric variables

	Omental adipose tissue			Subcutaneous adipose tissue		
Variables	**CD**	**HIS**	**OS**	**CD**	**HIS**	**OS**
Anthropometrics						
Weight	0.48**	0.66***	0.57***	0.58***	0.47**	0.59***
BMI	0.51**	0.66***	0.57***	0.59***	0.47**	0.60***
WC	0.61***	0.71***	0.66***	0.52**	0.54***	0.63***
Body composition						
Total body fat %	0.34#	0.66***	0.64***	0.62***	0.51**	0.63***
Total body fat mass	0.45*	0.72***	0.66***	0.65***	0.53***	0.66***
Lean body mass	0.45*	0.49**	0.37*	0.37*	0.32#	0.38*
Trunk fat	0.46***	0.69***	0.61***	0.60***	0.57***	0.62***
Limb fat	0.25	0.53***	0.46**	0.52**	0.36*	0.48**
Trunk fat mass/limb	0.36#	0.36*	0.33#	0.27#	0.43*	0.34#
Adipose tissue area						
Total	0.58***	0.82***	0.74***	0.61***	0.59***	0.70***
Visceral	0.62***	0.80***	0.78***	0.55***	0.49**	0.68***
Subcutaneous	0.52**	0.76***	0.68***	0.61***	0.59***	0.66***

#p<0.05, *p<0.01, **p<0.001, ***p<0.0001

BMI, body mass index; WC, waist circumference; CD, collagenase digestion; HIS, histological analysis; OS, osmium tetroxide fixation

Log10 or boxcox-transformed variables: weight, BMI, total body fat mass, fat-free mass, body composition (total body fat, total body fat mass, lean body mass, trunk fat, limb fat, trunk fat mass/limb), adipose tissue area (total, visceral and subcutaneous).

Table 3: Pearson correlation coefficients between OM or SC adipocyte diameters and metabolic variables

	Omental adipose tissue			Subcutaneous adipose tissue		
Variables	**CD**	**HIS**	**OS**	**CD**	**HIS**	**OS**
Glucose homeostasis						
Fasting glucose	0.31#	0.30#	0.30#	0.25	0.12	0.21
Fasting insulin	0.40*	0.37#	0.50**	0.15	0.31#	0.31#
HOMA-IR	0.42*	0.39*	0.52**	0.18	0.30#	0.32#
Plasma lipid profile						
Total cholesterol	-0.08	0.04	-0.02	-0.16	0.05	-0.06
VLDL-C	0.33#	0.35*	0.34#	0.19	0.46**	0.21
LDL-C	-0.06	0.18	0.05	-0.06	-0.01	0.10
HDL-C	-0.21	-0.47**	-0.34#	-0.38*	-0.22	-0.42*
Total TG	0.25	0.30#	0.30#	0.24	0.45*	0.20
VLDL-TG	0.28#	0.31#	0.32#	0.25	0.45**	0.22
LDL-TG	0.17	0.36#	0.23	0.11	0.24	0.09
HDL-TG	0.15	0.04	0.07	0.00	0.17	-0.09
Total cholesterol to HDL-C	0.16	0.51**	0.34#	0.30#	0.30#	0.39*
Adipokines/cytokines						
Leptin	0.37*	0.55***	0.60***	0.53***	0.53***	0.53***
Adiponectin	-0.40*	-0.48**	-0.57***	-0.28#	-0.17	-0.39*
TNF-α	-0.07	-0.08	-0.18	-0.04	-0.04	-0.04
IL-6	0.11	0.17	0.29#	0.20	0.08	0.32#

#p<0.05, *p<0.01, **p<0.001, ***p<0.0001

HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; HOMA, homeostasis model assessment index; CD, collagenase digestion; HIS, histological analysis; OS, osmium tetroxide fixation; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha

Log10 or boxcox-transformed variables: fasting insulin, HOMA-IR, VLDL-C, HDL-C, Total TG, VLDL-TG, LDL-TG, HDL-TG, leptin, adiponectin, TNF-α, IL-6.

Table 4: Summary of the characteristic of each method

	Collagenase digestion (CD)	Histological analysis (HIS)	Osmium tetroxide fixation (OS)
Cost and handling of chemicals	Low cost	Low cost	High cost Hazardous chemical which requires recycling network
Availability of necessary equipment	High	High	Low
Experiment time	Short	Long	Long
Analysis time	Long	Long	Short
Sample conservation (long- term)	Not possible[a]	Possible	Possible
Adipose tissue architecture analysis	Not possible	Possible	Not possible
Number of cells counted	Up to ~250-300	Up to ~50- 250	Between 6,000 and 20,000
Small cell fraction	Detected in some samples	Detected in some samples	Detected
Standardization (between laboratories)	Difficult	Possible	Easy to achieve
Deferred measurement	Not possible[a]	Possible	Possible
Possible biases	Limited floatation of small cells due to low lipid content Cell breaking[b]	Assumption that cells show their largest diameter Cell shrinkage Defining cells, especially small cells	Multilocular cells could be ruptured during fixation process Possibility of residual fragments Possibility of staining dead cells Space occupied by osmium
Advantages	First step to functional experiments Isolation of stromal vascular cell fraction	Possibilities for further analyses such as immunohistochemistry, immunofluorescence, etc	Allows analysis of cell subpopulations

CD, collagenase digestion; HIS, histological analysis; OS, osmium tetroxide fixation.

a: Collagenase-digested cells can be fixed in formaldehyde or osmium tetroxide to postpone analysis and preserve the cells (6).

b: addition of adenosine may limit cell breakage (4).

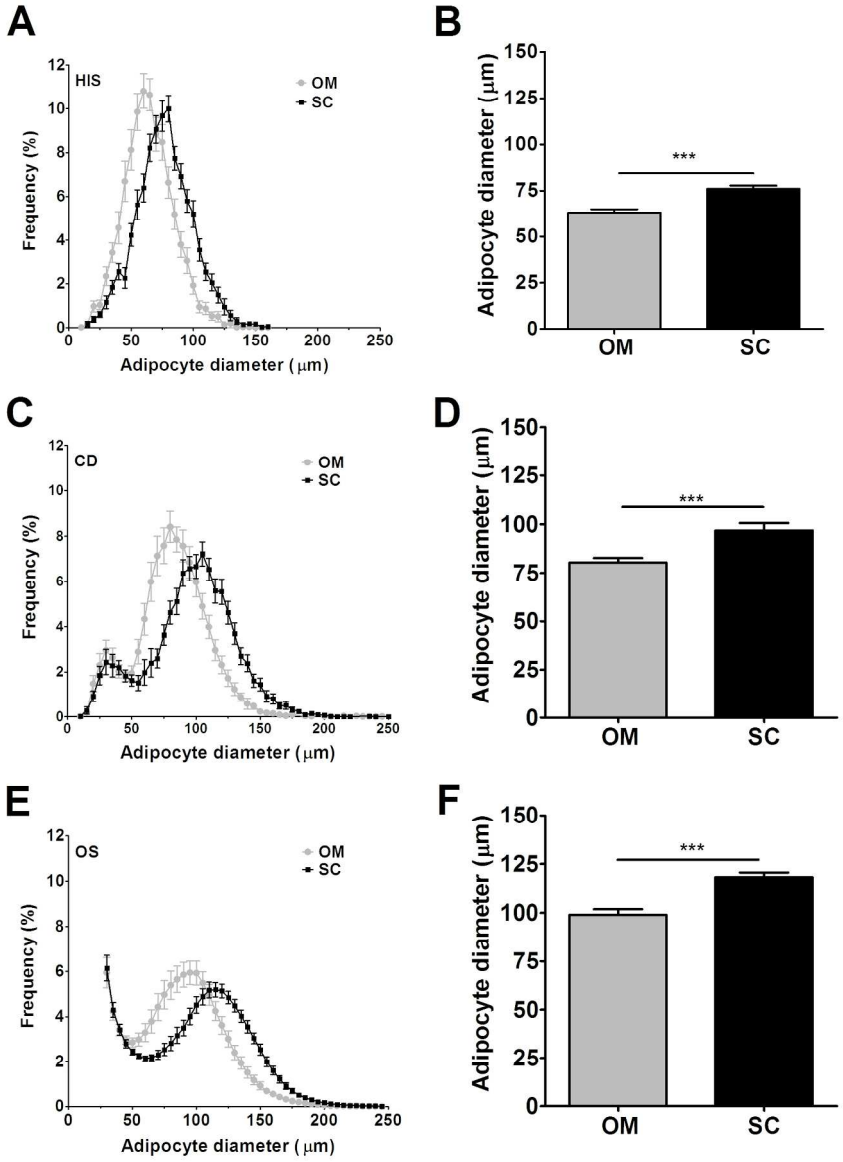

Figure1

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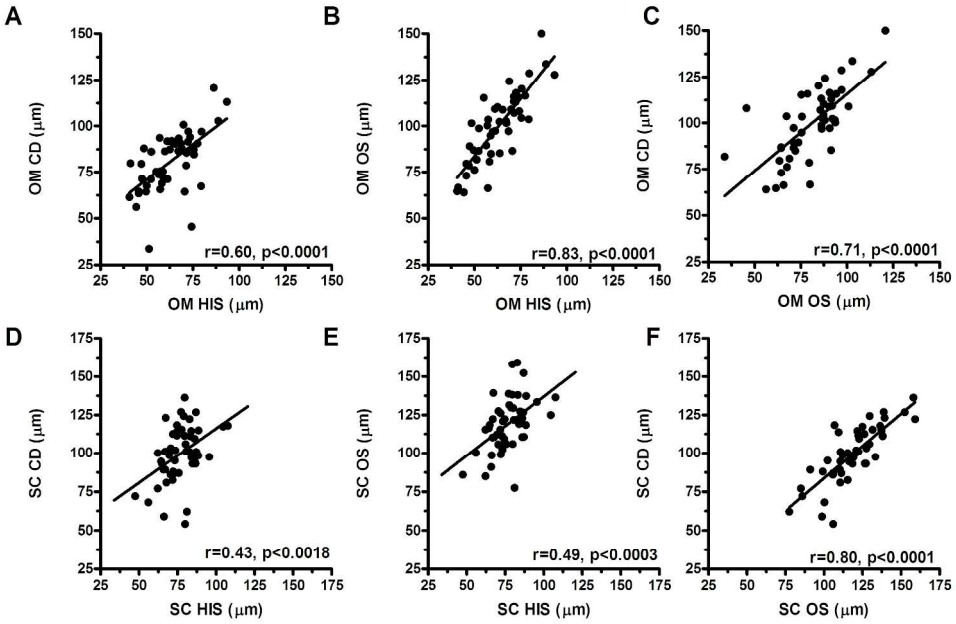

Figure2

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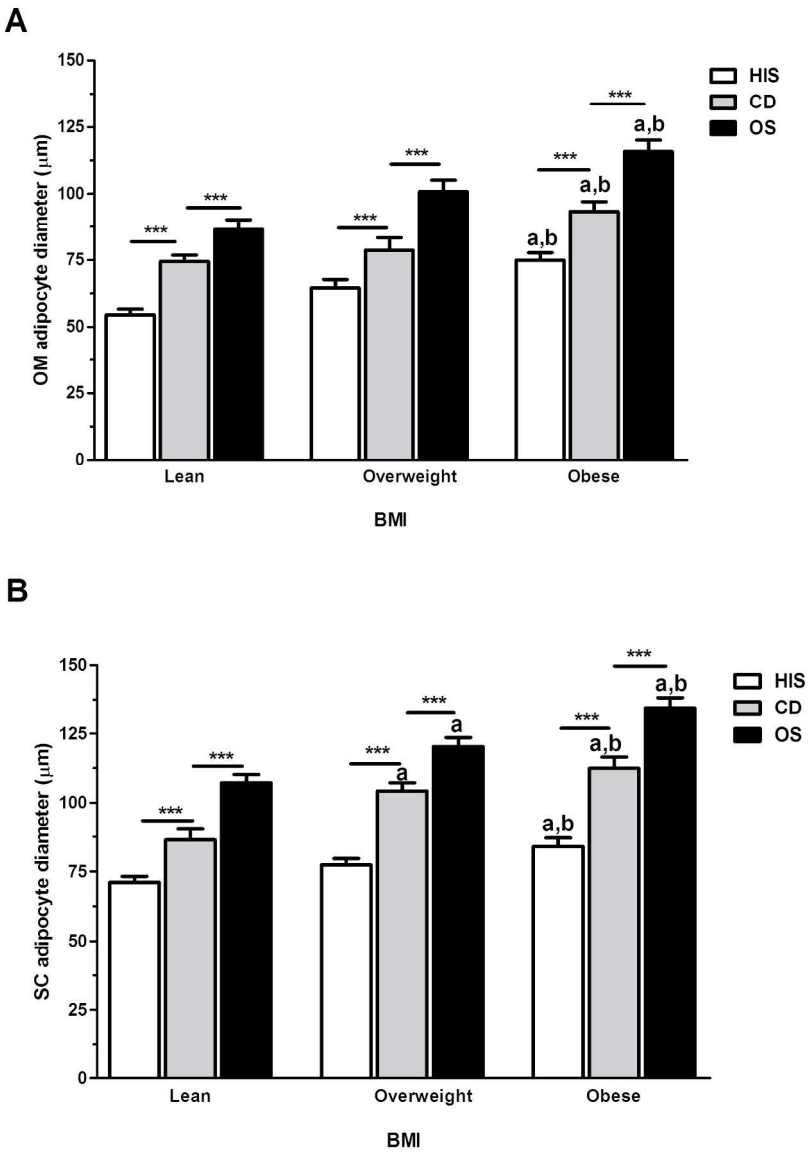

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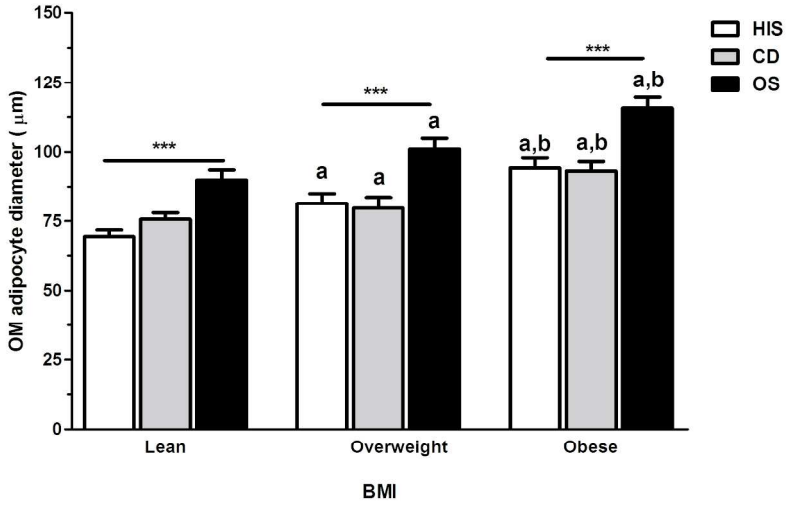

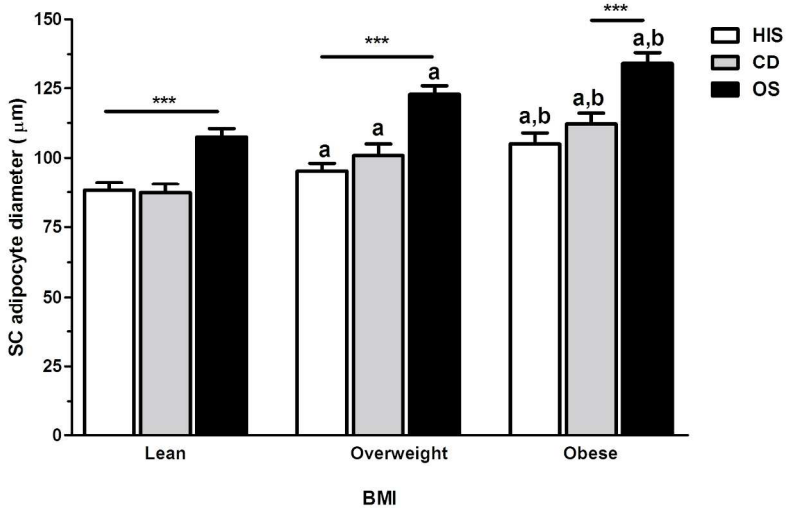

Figure4

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