Adipocyte size as a determinant of metabolic disease and adipose tissue dysfunction

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Running Title*: Adipocyte size and metabolic disease*

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ABSTRACT

Obesity is a heterogeneous disease and is associated with comorbidities such as type 2 diabetes mellitus, cardiovascular diseases and cancer. Several studies have examined the role of dysfunctional adipose tissue in the pathogenesis of obesity, highlighting the contrasting properties and impact of distinct adipose tissue compartments, sometimes with contradictory results. Dysfunctional adipose tissue involves enlargement, or hypertrophy, of pre-existing fat cells, which is thought to confer increases in cardiometabolic risk, independent of the level of obesity *per se*. In this article we critically analyze available literature which examined the ability of adipocyte cell size to predict metabolic disease and adipose tissue dysfunction in humans. Many studies demonstrate that increased fat cell size is a significant predictor of altered blood lipid profiles and glucose-insulin homeostasis independent of adiposity indices. The contribution of visceral adiposity to these associations appears to be of particular importance. However, available studies are not unanimous and many fat depot-specific aspects of the relationship between increased fat cell size and cardiometabolic risk or parameters of adipose tissue dysfunction are still unresolved. Methodological factors such as the approach used to express the data may represent significant confounders in these studies. Additional studies should consider the fact that the relationship between fat cell size and common adiposity indices is non-linear, particularly when reaching the obese range. In conclusion, our analysis demonstrates that FCS is a significant predictor of the cardiometabolic alterations related to obesity. We propose that adipocyte hypertrophy, especially in the visceral fat compartment, may represent a strong marker of limited hyperplasic capacity in subcutaneous adipose tissues, which in turn is associated with the presence of numerous cardiometabolic alterations.

ABBREVIATIONS AND GLOSSARY

3T3-L1: Mouse embryonic preadipocyte cell line; ApoB: apolipoprotein B; ATGL: adipose triglyceride lipase; BFM: body fat mass; BMI: body mass index; CD11b: macrophage and neutrophil cell marker; CD11c: dendritic cell and macrophage cell marker; CD31: endothelial cell marker; CD68: macrophage cell marker; DEXA: dual-energy X-ray absorptiometry; FCS: fat cell size; GLUT4: Glucose transporter type 4; HOMA-IR: homeostatic model assessment of insulin resistance; HIF-α: hypoxia-inducible factor 1; HSL: hormone-sensitive lipase; IGF-1: insulin-like growth factor 1; IL-6:interleukin 6; IRS-1: insulin receptor substrate 1; MeSH: Medical Subject Heading; NAFLD: non-alcoholic fatty liver disease; NF-κB: nuclear factor kappa B; NGT: normal glucose tolerant; OM: omental; PCOS: polycystic ovary syndrome; PLIN: perilipin (lipid droplet-associated protein); SC : subcutaneous; SVF: stroma-vascular fraction; T2DM: type 2 diabetes mellitus; TNF-α: tumor necrosis factor alpha; VEGFA: vascular endothelial growth factor A; VEGF-R2: vascular endothelial growth factor receptor 2; vWF: von Willebrand Factor; WHR: waist-to-hip ratio

INTRODUCTION

Over one third of the world population is now struggling with overweight or obesity and the disease burden frequently associated with these conditions (1). Obesity is a well-known risk factor of metabolic diseases such as type 2 diabetes mellitus (T2DM), dyslipidemia, coronary heart disease, hypertension, non-alcoholic fatty liver disease (NAFLD) and stroke (2). It has also been linked with dementia, obstructive sleep apnea and numerous types of cancer (2). However, a significant proportion of obese individuals, 15 to 30% depending on the populations examined and the definition of metabolic health, do not develop alterations associated to obesity, at least in the short term (3, 4). These subsets of apparently healthy obese subjects are named metabolically healthy obese.

Differences in body fat distribution likely account for many discrepancies between metabolically healthy and unhealthy individuals. Jean Vague proposed as early as 1947 that patients with upper-body obesity had an increased risk for metabolic diseases, while individuals with lower-body (femoral-gluteal) obesity had a lower risk of alterations (5). Since then, body fat distribution indices have been recognized as stronger predictors of metabolic alterations compared to overall obesity, emphasizing their impact on global metabolic health (2). Specifically, studies have shown that excess fat accumulation on visceral anatomical structures such as the greater omentum or mesentery is a strong predictor of a detrimental metabolic status; whereas accumulation of gluteal and femoral fat is viewed as protective when total adiposity is accounted for (2). In fact, despite a high body fat mass, low visceral fat accumulation is a major feature of the metabolically healthy profile observed in some obese individuals (6, 7).

Adipose tissue expansion in a given fat compartment occurs through adipocyte hyperplasia, hypertrophy or a combination of both. Recruitment of new cells through differentiation of preadipocytes (hyperplasia) is now generally considered as a mechanism that protects against metabolic alterations. Indeed, adipocyte hyperplasia is associated with preferential accumulation of subcutaneous (SC) adipose tissue and the

gynoid phenotype (8-12). On the other hand, visceral adipocytes and the android fat distribution phenotype are more commonly associated with adipocyte hypertrophy, that is, enlargement of existing fat cells (8, 9, 11, 12).

Increased fat cell size (FCS) has been demonstrated to be associated with metabolic impairment in patients as early as the 70s (13, 14). In 1979 (15), adipocyte hypertrophy was proposed as a valuable parameter to characterize metabolic disturbances. Since then, FCS has been linked to numerous variables assessing adipose tissue biology and also with metabolic alterations independently of total adiposity (9, 11, 16-25). To our knowledge, studies on the potential role of adipocyte hypertrophy as a biological marker of metabolic disease and adipose tissue dysfunction have never been reviewed in detail. The present article proposes an extensive analysis of the studies on this topic and demonstrates that adipocyte size may, indeed, represent an important determinant of adipose tissue dysfunction and a potential marker of pathologies that may or may not be linked with total adiposity.

LITERATURE SEARCH

To perform this critical analysis, the first step was to determine all possible Medical Subject Heading (MeSH) terms to be used in searches on the PubMed database. The term «adipocyte» was combined with the following keywords: size, hypertrophy, white, area, diameter, morphology, cellularity, dysregulation and volume. The same approach was then repeated but this time using the term «fat cell» instead of «adipocyte». The articles were selected depending on the following specific criteria: 1) emphasis was placed on human studies directly relevant to the research topic; and 2) only articles on white adipose tissue were selected. Relevant articles from the reference list of identified papers were added. In total, 172studies were retained. They were published between 1970 and March 2015. A record of the articles was kept and they were listed according to the research criteria used to find them. An exhaustive analysis of the population, the methods and major findings of each study was performed. We further selected the studies that had analyzed the ability of FCS to predict obesity-related metabolic complications or

parameters of adipose tissue function. The latter studies represent the main body of literature used for this article. Analyses of cell size variations among sexes, obesity degrees as assessed by body mass index (BMI) and cell sizing techniques were performed using 76 publications in which the variables of interest were available. FCS measurements in these studies were converted to the same unit. Units of volume (µL, pL and μm^3) and units of surface (μm^2) were converted to a diameter in micrometer (µm) assuming a spherical or circular cell shape. Units of mass (µg) were first converted to volume using the density of triolein (density=0.915 g/mL) and then to cell diameter in µm.

METHODS TO MEASURE FAT CELL SIZE

This section provides a brief overview of the main techniques used to assess FCS. We focus mainly on the most commonly used: collagenase digestion, osmium tetroxide fixation and histological analysis. We also provide analyses of FCS variation according to sex, obesity level and measurement technique.

Collagenase digestion

Collagenase digestion has been developed by Rodbell as a mean to separate mature adipocytes from the stroma-vascular fraction (SVF) (26). It is now used as the first step of many experiments such as cell cultures and cell sizing. Briefly, adipose tissue is digested by collagenase and mature adipocytes are separated from the SVF by floatation in an aqueous solution. Pictures of mature adipocytes can be taken with a phase contrast microscope to assess adipocyte size. This method has been the most frequently used. However, some drawbacks have prevented it from becoming a gold standard in determining FCS. Small adipocytes do not float as easily as the average-size cells, due to their low lipid content (27). Additionally, adipocytes tend to break in unfixed tissue because of their fragility (27). However the introduction of adenosine in the solution minimized this bias (28). Centrifugation during the floatation step can bias the recovery of small adipocytes, therefore this step can be omitted (29). Finally, blue methylene staining

may be required to assess viability of the cells and facilitate identification of lipid droplets vs undamaged fat cells (26).

Osmium tetroxide fixation and Multisizer Counter analysis

This technique has been developed in 1968 by Hirsh and Gallian (30) on the basis that osmium tetroxide fixes intracellular lipids and allows staining of very fragile cell types. In brief, adipose tissue can be digested by collagenase and subsequently fixed by osmium tetroxide, or these steps can be simultaneous if a collidine-HCl solution is used to separate the SVF from mature adipocytes instead of collagenase. Adipocytes are then analyzed with a counter allowing cell size measurement by fluctuation of electrical resistance. This technique allows studying large distributions of adipocyte sizes as well as analyzing cell subpopulations like very small fat cells (31, 32). Even if this technique allows fixation of very small adipocytes (15-25μm), a threshold value (most often 25 μm) is used to discriminate between mature adipocytes and artefacts. However, multilobular fat cells tend to rupture during the fixation process which can lead to an underestimation of mean FCS, especially in obese individuals (31). This technique is also time-consuming, it comes at a relatively high price and requires proper handling of osmium tetroxide, a hazardous chemical (27, 30, 31).

Histological analysis

Adipocyte cell size analysis can also be performed on histological slides. Type and concentration of fixatives, paraffin-embedding, time of fixation, slice thickness and type of coloration may vary among laboratories. Of importance, this is the only technique that allows examination of global tissue architecture. However, many potential biases are inherent to this approach and many assumptions have to be made in order to assess cell size. Cell distribution has to be considered uniform, which may not be entirely true in obese and young individuals (27). Furthermore, fixation agents are known to induce significant shrinkage of the cells (33, 34). Likewise, it must be assumed that cells are perfect spheres

showing their largest diameter (27). This technique has been extensively used recently due to the possibility of simultaneously performing immunostaining of various cellular markers.

Other techniques

Other methods have been used in various contexts to appreciate variation in FCS. Flow cytometry as well as scanning electron microscopy have been used by a few teams (35-39). These specialized methods are not in common use to measure FCS in clinical research.

Cell size analysis software

Promising semi-automatic methods have arisen with the purpose of limiting user-dependent biases and time of use (40-42). Information on a few completely automated programs has also been published (43, 44). Obstacles such as the time needed to obtain high-quality images may slightly complicate their use, along with the presence of the SVF and other tissue artefacts in some samples. Moreover, user-dependent variation still needs to be addressed.

Overall, among these approaches to assess FCS, none are without drawbacks. Characterization of very large and very small adipocytes remains a challenge for all methods available. Moreover, the number of adipocytes examined in each study varied from 50 to 20 000, which at times limits study comparisons. As a result, no single method has arisen in the literature as the gold standard for adipocyte cell sizing.

Population and methodological variation

Our survey of the literature demonstrates that adipocyte size varies between men and women as well as between fat depots (visceral vs. SC). Moreover, as a function of increasing obesity level, mean adipocyte size increases, reaching a plateau at a certain level of adiposity. Arner and colleagues have already shown that the relationship between SC FCS and body fat mass (BFM) is curvilinear in men and women (45). Moreover, adipocyte hypertrophy is negatively correlated with adipocyte hyperplasia, when adjusted for

the predicted adipocyte volume at a given BFM, and subjects with higher fat cell size than predicted by the curve have lower rates of adipogenesis (45). This suggests an important impact of adipose morphology, independent of BFM. However, a complete review of FCS variation and obesity indices such as BMI has not been published yet.

Using all the studies in which mean FCS and mean BMI were available, we summarize sex-, depot- and obesity-related variation in adipocyte size in humans and address potential differences related to the method used for analysis. As shown in Figure 1A, omental (OM) and abdominal SC FCS increase in both sexes with increasing obesity level as assessed by BMI and reach a plateau around BMI values of approximately 30 kg/m^2. When plotting data from men (Figure 1B) and women (Figure 1C) separately, we find that the plateau is reached at lower BMI values in men (approximately 25 kg/m^2) compared to women (approximately 35 kg/m^2). Moreover, OM FCS appears to be approximately 20% lower than SC FCS in women, whereas this depot difference is not apparent in men. These findings suggest a clear difference among sexes regarding the expansion of adipose tissue. Men are more prone to hypertrophic obesity, as the plateau is seen at lower BMI values. On the other hand, women show signs of both hypertrophic and hyperplasic expansion as obesity level increases. Similar observations can be made with both the SC and OM adipocyte size curves.

When examining FCS as a function of the measurement technique, we found that the use of histological slides generally leads to lower mean FCS by approximately 15% across all BMI values (Figure 1D). Collagenase digestion and osmium fixation appear to generate similar mean FCS (Figure 1D). These patterns can be observed in both depots as well. All techniques showed a general pattern of BMI-related increase, with a plateau reached at higher obesity levels.

In this analysis, population differences noted in FCS variation closely reflect those observed in many individual studies as reviewed here. Adipocyte size is clearly influenced by sex, anatomical localization

and obesity levels. As discussed in the sections below, these variations need to be considered critically when examining the relationship between fat cell hypertrophy in a given fat compartment and metabolic diseases or parameters of adipose tissue function.

ADIPOCYTE SIZE AS A POTENTIAL BIOMARKER OF CARDIOMETABOLIC ALTERATIONS OR DISEASE ENDPOINTS INDEPENDENT OF COMMON ADIPOSITY INDICES

Common adiposity indices have been widely used to assess the presence of cardiometabolic risk factors in overweight or obese individuals. BMI is certainly the most widely used. However, it has its limitations. It does not take into account body fat distribution and only provides a crude assessment of body composition. Other anthropometric indices have been examined along with more invasive measurements of body fat distribution and/or body composition (2). An important question which has been addressed in many original publications is whether adipocyte size also predicts the metabolic complications frequently associated with obesity or abdominal obesity. Considering that FCS is strongly related to body composition and fat distribution, whether it predicts metabolic alterations independent of overall or regional adiposity has also been tested in a number of studies. The next sections provide a review of the studies that have addressed the link between FCS and cardiometabolic risk factors or disease endpoints.

Blood lipid profile

Imbeault et al. (46) reported that when matched for visceral adipose tissue area, abdominal but not femoral SC FCS predicted an altered lipid profile in men but not in women. Specifically, men with large adipocytes had hypertriglyceridemia and higher LDL-apolipoprotein B (ApoB) levels. Another study reported in patients with first-degree relatives of T2DM individuals that elevated SC FCS correlated with lower HDL-cholesterol concentration, but not with high total cholesterol or LDL-cholesterol concentrations when adjusted for BMI (20). In homozygous twins discordant for obesity level, after adjustment for total body fat mass (BFM), SC FCS correlated positively with LDL-cholesterol

concentrations (47). Interestingly, in the obese twin characterized by adipocyte hypertrophy, higher LDL-cholesterol and lower HDL-cholesterol levels were found compared to the lean co-twin, but this difference was not found in twins characterized by hyperplasia. However, in both cases, the obese co-twin had 61% larger adipocytes than the lean twin (47). In obese women, Ledoux et al. (21) did not find a correlation between FCS in either the SC or visceral depot and blood lipid concentrations when adjusting for BMI or waist-to-hip ratio (WHR).

An elegant study by Hoffsted et al. (16) showed in obese women that OM, but not SC FCS, was associated with plasma apolipoprotein B, total cholesterol, LDL-cholesterol and triglyceride concentrations independent of BMI, BFM and body fat distribution indices obtained by dual-energy X-ray absorptiometry (DEXA). In another study, visceral and SC fat cell volumes were positively correlated with triglyceride levels and negatively with HDL-cholesterol concentrations in bariatric patients after control for total BFM (9). In a sample of women ranging in adiposity from lean to moderately obese, after control for BMI, total BFM and visceral adipose tissue area measured by computed tomography, we found that OM FCS predicted triglyceride concentration whereas SC FCS did not (17). Yet, in female and male Indians, another group reported no significant correlation between total cholesterol and triglyceride concentrations and SC or OM FCS after adjustment for BMI, BFM, waist circumference, total abdominal adipose tissue area and SC adipose tissue area (48). However, in that particular study, associations between visceral adipose tissue accumulation and metabolic parameters were scarce as visceral adipose tissue only correlated with HDL-cholesterol and triglyceride concentrations in men. In sum, a number of studies have shown associations between high FCS either in the SC or visceral fat compartment and an altered lipid profile. Some of these associations appeared to be independent of total or abdominal adiposity in some cases. Further studies in both genders taking into account ethnicity as well as the OM and SC fat compartment are needed to generalize these findings.

Glucose homeostasis, insulin resistance and T2DM

The relationship between SC FCS and insulin resistance is well documented. A number of studies have shown a clear association between abdominal SC FCS and markers of insulin resistance, in both women and men (11, 15, 24, 45, 49-54). In overweight South African women with normal glucose tolerance (NGT), no link was found between abdominal or gluteofemoral SC FCS and insulin resistance markers such as basal plasma insulin, insulin area and glucose area after a 100 g oral glucose load (55). Nevertheless, another study demonstrated that SC FCS was increased in obese with T2DM when compared with obese control subjects matched for BMI, age, sex and ethnicity (56). Also, when controlling for BMI, SC FCS was positively correlated with homeostatic model assessment of insulin resistance (HOMA-IR) and negatively with post-glucose insulin sensitivity (57).

This was challenged by McLaughlin et al. (58) who reported that mean diameter of the larger SC cells assessed with osmium tetroxide was not different between insulin resistant vs insulin sensitive patients (119 vs. 115 μm respectively). However, insulin resistant patients were characterized by a larger population of small cells (a high ratio of small-to-large cells), a feature that can only be assessed with the osmium technique and which could possibly reflect arrested development of the very small adipocytes toward fully mature cells (58). Another study found no link between SC FCS and HOMA-IR (21). On the other hand, in a sample of T2DM patients matched to NGT patients for BMI, Pasarica et al. found larger SC fat cells measured with osmium tetroxide in T2DM patients (124 μm diameter compared to 115 μm respectively) (59). In that study, there was no depletion of the small cell population in the diabetic subgroup and mean FCS was positively correlated with HOMA-IR. Similar results were obtained by Yang et al. (60). Mean fat cell volume was inversely correlated with insulin sensitivity measured by euglycemic, hyperinsulinemic clamp after adjustment for BMI in a sample of first-degree relatives of T2DM patients (60). In non-obese first-degree relatives of T2DM patients, SC FCS was negatively correlated with GLUT4 protein expression and positively with circulating insulin levels whereas BMI was not (61). We found that SC GLUT4 mRNA abundance was lower in women with hypertrophic obesity (62). Moreover, another group reported that after adjustment for BMI and percent body fat, SC FCS was

associated with insulin resistance, but not with fasting glucose concentration in first-degree relatives of T2DM patients (20). Arner and colleagues found that in women characterized by SC adipocyte hypertrophy, adipocyte volume, adjusted for BFM, was an important and independent correlate of HOMA-index and fasting insulin levels (45).

Depending on how anthropometric adjustments are performed, SC FCS is not always an independent predictor of alterations in indices of glucose-insulin homeostasis. Our group reported that SC FCS was not a significant predictor of fasting glucose or fasting insulin in non-obese women when adjusted for adiposity and visceral adipose tissue area measured by computed tomography (17). The adjustment for variation in visceral adipose tissue area was critical in the latter analysis, raising the possibility that lack of control for this variable in other studies may have led to the finding of significant associations. Similarly, Azuma et al. (18) found in T2DM patients that increased FCS was associated with insulin resistance independent of total BFM, but when they adjusted for body fat distribution measurements (leg fat mass or trunk fat mass), the relationship became non-significant. These results are not unanimous. Glycemia, insulinemia and glucose disposal rate were associated with SC FCS independently of age, BMI, BFM or body fat distribution indices obtained by DEXA in a sample of obese women (16). Similar results were found in obese men and women, after adjustment for total body fat content (9). SC adipocyte hypertrophy also explained the difference in glucose disposal rate between South Asians and Caucasians, after control for total body fat content, intraperitoneal and SC fat (19). In another study, SC FCS was associated with markers of insulin resistance in NGT patients but not in T2DM patients, indicating that this relation may become non-linear when a certain degree of disease severity is reached (24). Such phenomena could contribute to the discrepancies noted among some studies.

The relationship between visceral FCS (OM adipocytes in most studies) and insulin resistance has also been of interest. Our group was the first to report a relationship between fasting insulin, HOMA-IR and OM FCS in non-obese women (17). However, once adjusted for adiposity and body fat distribution, there

was no significant association. The close correlation between OM FCS and visceral adipose tissue area measured by computed tomography explained the latter finding. Similar results were obtained in female and male Indians when adjusting for BMI, BFM, waist circumference, total and SC adipose tissue area (48). Another study found an association between fasting glycemia, HOMA-IR (63) and OM FCS; however statistical adjustment for anthropometric values was not performed in that study (52).

In obese patients, the association between visceral FCS and markers of insulin resistance remains uncertain. OM FCS seems to predict fasting glycemia, fasting insulin and HOMA-IR independent of BMI or WHR in a few studies (21-23). Hoffstedt et al. found that visceral adipocyte hypertrophy was not associated with plasma insulin, fasting glycemia and glucose disposal after control for age, BMI, BFM and DEXA-measured body fat distribution indices (16). The same group found that visceral fat cell volume (to a lesser extent than SC FCS) was correlated with insulin levels, insulin-induced glucose disposal and insulin sensitivity after control for total BFM, in both sexes (9). To add to these results, no significant relationship was found between OM FCS and HOMA-IR in non-diabetic obese adults, yet OM FCS was associated with these variables independently of BMI and WHR *in vitro* when glucose uptake in freshly isolated adipocytes stimulated by insulin was measured (24). In morbidly obese patients with insulin resistance, a greater proportion of adipocytes exceeding 100 μm diameter was also observed along with a lower proportion of small adipocytes compared to insulin sensitive patients (7, 52, 64). This relation was challenged recently by van Beek and colleagues (65), who reported no difference in adipocyte size between subjects with T2DM compared to those with NGT.

Overall, many studies have reported significant associations between SC FCS and various markers of insulin resistance. Some reported that this association was independent of concomitant elevations in total BFM, pointing toward a specific role of adipocyte hypertrophy, and perhaps adipose tissue dysfunction, as a marker of insulin resistance. On the other hand, most of the studies that adjusted their analysis for well-measured body fat distribution markers such as visceral adipose tissue accumulation have shown that

the association between SC FCS and indices of insulin resistance was no longer significant. We propose that excess visceral adipose tissue accumulation and SC fat cell hypertrophy may represent markers of a common phenomenon: limited hyperplasic capacity of adipose tissues. Yet, the relationship with visceral adipocyte hypertrophy remains equivocal in most studies. The non-linear nature of the relationship between FCS and obesity level or insulin resistance may contribute to explain discrepancies among the various studies that examined this relationship.

Metabolic syndrome features

Three studies examined the association between FCS and the number of metabolic syndrome features. In bariatric surgery patients, no difference was noted in FCS of patients with or without the metabolic syndrome (25, 66). The first study assessed the metabolic syndrome on the basis of the Harmonized criteria (67). Three features or more were required to qualify as metabolically unhealthy. The second study considered metabolic health status on the following criteria: 1) fasting glucose >100 mg/dL; 2) total insulin> 19 μU/mL; 3) triglycerides >150 mg/dL; 4) HDL-cholesterol <39 mg/dL; 5) systolic blood pressure >140 mmHg; and 6) diastolic blood pressure >90 mmHg. Patients were considered as metabolically unhealthy when at least one feature was present. Visceral adipocyte hypertrophy was observed in patients with low HDL-cholesterol levels and high fasting glucose concentrations in one of these studies (25). Also, the number of metabolic syndrome features increased with visceral FCS (25), consistent with another study (22) (adapted version of (68)) that associated adipocyte hypertrophy with a detrimental metabolic state in bariatric patients. The fact that only severely obese patients were studied may have led to low level associations between FCS and the number of metabolic syndrome features, considering that FCS values reach a plateau in the obese range. Another study reported that high mesenteric FCS was associated with a 1.79 increased in the risk of having metabolic syndrome (ATP III criteria) in overweight men (63). More studies are needed to assess whether FCS is associated with the onset of metabolic syndrome independent of adiposity.

NAFLD and liver fat accumulation

Six studies found an association between liver fat accumulation and SC adipocyte size (22, 47, 69-72). Two studies reported the opposite (73, 74). One of these studies included a homogenous population of T2DM patients (74) and the other examined males before and after weight gain (73). When patients were matched for BMI, age, sex and/or BFM, SC adipocyte hypertrophy was an independent marker of liver fat (47, 69-72). Moreover, OM adipocyte hypertrophy could independently predict stages of NAFLD (22, 75). In one study, the extent of fatty liver disease was not predicted by FCS, but this finding was consistent with the lack of association between FCS and chronic intermittent hypoxia, the best and only predictor of liver disease stages in that sample (76).

Polycystic ovary syndrome (PCOS)

There are a few studies on adipocyte hypertrophy and PCOS in the literature. Two teams noted a 25% increase of SC FCS in PCOS women compared to controls when matched for age and BMI (77, 78). Another group found similar results for visceral adipocyte size, even if women with PCOS were younger than controls in this particular study (79). Moreover, in PCOS women, lipolysis responsiveness was reduced in SC and increased in visceral adipocytes when compared to controls (79). Additional studies are required to assess whether adipocyte hypertrophy in PCOS could represent the missing link between adipose tissue dysfunction and the metabolic alterations observed in this condition.

Cardiovascular endpoints

To our knowledge, there is no data available on FCS and cardiovascular endpoints. One study related visceral adipocyte volume positively to arterial stiffness, a known cardiovascular disease risk factor (80). Mean SC gluteal FCS has also been positively correlated with mean arterial blood pressure (81). Ledoux et al. found in patients with hypertension that mean OM and SC abdominal cell size were increased (21). Other studies did not report such an association.

FCS as a predictor of metabolic alterations in weight loss studies

A large number of studies has been performed on weight loss through various modalities (bariatric surgery, diet and/or exercise) and have documented the impact of such interventions on adipocyte size reductions. However only a few studies have attempted to link the favorable metabolic effect of weight loss to reduced FCS. Some studies have shown that weight loss-induced decreases in FCS were associated with changes in plasma levels of leptin, adiponectin, glucose, insulin, triglycerides, cholesterol, LDL-cholesterol as well as with changes in HOMA-IR, glucose disposal rate and systolic/diastolic blood pressure independently of total BFM (70, 82-85).

Cancer

The link between obesity and the risk of mortality from cancer is well known (86, 87). However, the biological processes underlying this association are still being investigated. Breast adipose tissue has been of interest recently due to its relative proximity with cancer cells. Hypertrophic adipocytes can induce a permanent pro-tumorous microenvironment as a source of: 1) growth factors such as estrogen, IGF-1 and leptin; 2) constant energy supply; and 3) pro-inflammatory cytokines such as IL-6 and TNF-α. In fact, *in vitro* studies have shown that mature adipocytes are able to sustain and promote tumor growth (88). Therefore, hypertrophic mammary adipocytes could reflect dysfunctional adipose tissue contributing to a microenvironment that favors cancer growth. Only one team has examined FCS in mammary fat tissue in women with breast cancer and reported a positive correlation with BMI and other markers of inflammation such as increased NF-κB binding and number of macrophages (89). More studies are necessary to understand the link among obesity, FCS and breast cancer.

ADIPOSE TISSUE DYSFUNCTION AND HYPERTROPHIC ADIPOCYTES

An increasingly large body of evidence points toward dysfunctional adipose tissue as a major determinant of metabolic impairments in individuals with abdominal obesity. Hypertrophy of the adipose cells may represent a marker and perhaps to some extent a driver of adipose tissue dysfunction. After providing an

overview of how FCS relates to metabolic abnormalities, we now review the basis of adipose tissue dysfunction and its relationship with adipocyte hypertrophy.

Lipid metabolism

The traditional belief has been that lipid metabolism is stimulated in large adipocytes, both from the standpoint of fatty acid uptake and fatty acid release through lipolytic pathways. For example, elegant studies comparing populations of small (35 μm) vs. large (50 μm) diameter adipocytes from the same anatomical location have shown that fatty acid synthase and lipoprotein lipase activities were increased in large compared to small adipocytes when expressed per number of cells (90). In that study, the lipolytic response to β-adrenergic agonist isoproterenol was also increased in large vs. small adipocytes. Interestingly, the beta(1)-integrin/ERKs signalling pathway was also activated in large adipocytes and had been proposed as a putative mechanism of the adaptation of adipose tissue functions to cell size (90, 91). Consistent with these results we found that adipocyte isoproterenol-stimulated lipolysis was higher in women with hypertrophic adipocytes, independent of overall adiposity and body fat distribution (62). Furthermore, Laurencikiene et al. demonstrated that hormone-induced lipolysis rates were increased in large (100 μm) compared to small (82 μm) diameter SC cells. They also observed that protein level of HSL, PLIN and ATGL was increased in large adipocytes (92). In contrast, opposite results were obtained in lean and obese children, in whom a significant negative correlation was found between basal lipolysis of isolated adipocytes (expressed per number of cells) and abdominal SC FCS (54). The latter association was lost upon statistical control for BMI and lipolytic responsiveness to isoproterenol was not associated with FCS (54). A study using an original fluorescence-based technique to assess lipid uptake in individual cells has reached opposite conclusions with SC adipose tissue explants from monkeys under insulin-stimulated conditions (93). Specifically, small cells of the explants responded to insulin by increasing lipid uptake, whereas adipocytes with cell diameters >80-100 μm were insulin resistant. Data were expressed per cell area in that study. It was proposed that such a mechanism could protect adipocytes from lipid overload and restrict further expansion of adipose tissue (93). Additional studies in 3T3-L1

cells have shown highly dynamic lipid trafficking among cellular compartments and between lipid droplets (94). Interestingly, rates of exchange were lower in cells with larger lipid droplets compared to those with smaller lipid droplets, suggesting that cells with large lipid droplets are less efficient in transporting and possibly metabolizing fatty acids than those with small lipid droplets (94). An *in vivo* study was performed by the group of Jensen and collaborators to assess rates of lipid uptake in small, medium or large adipocytes with radioactive and stable-isotope-labelled fatty acids (95). Interestingly, when expressed per lipid weight, no difference in lipid uptake was found among small (83 μm), medium (103 μm) or large (117 μm) diameter adipocytes. On the other hand, when expressed per cell number, larger adipocytes had higher rates of lipid uptake (95). These experiments did not include insulin-stimulated conditions.

Considering the above-cited studies, it is difficult to reach firm conclusions regarding the impact of cell size on lipid metabolism in adipose tissue. Much confusion may arise from the method chosen to express the data. For example, in a study of diacylglycerol acyltransferase activity in isolated microsomal fractions from SC and OM adipose tissues, we have shown no relationship between maximal activity of this enzyme and adipocyte size when data were expressed per cell number (96). Yet, when expressed per mg of tissue, activity of the enzyme in OM tissue was lower in patients with excess visceral adipose tissue accumulation and large OM adipocytes (96). Consistent results were obtained in other *in vivo* studies by Votruba and collaborators showing that net lipid uptake from a meal expressed per adipose tissue weight but not per cell was decreased as a function of adipocyte size in both upper and lower body SC fat (97). These results suggest that a given amount of tissue seems to take up fewer fatty acids and synthesize triglyceride less efficiently when adipocytes are larger, despite lack of a difference or increased metabolism on a per cell basis. The study by Serra and collaborators (98) also reported a positive relationship between abdominal SC FCS and adipose tissue heparin releasable lipoprotein lipase activity (expressed per number of cells) in the same depot, but only in obese women with a low relative accumulation of visceral fat. The authors concluded that high visceral fat accumulation relative to total

abdominal fat reflected lower triglyceride storage capacity at least in the SC fat compartment (94). Inconsistencies among studies may also be explained by a loss of heterogeneity in insulin-stimulated lipid uptake of individual cells with increasing FCS and levels of obesity. This has been demonstrated by Varlamov and collaborators in white adipose tissue explants of rhesus macaques (99). Additional factors such as an obesity-related decrease in the ability to increase adipose tissue blood flow in response to a meal may also interfere with lipid storage in a given fat compartment (100), which could contribute to explain some of the discrepancies among *in vitro* and *in vivo* studies.

Glucose metabolism

Some studies have shown that there is no difference in small vs. large fat cells from the same patient for protein levels of IRS-1 and GLUT4 (101, 102), whereas another study reported increased GLUT4 protein in large vs small adipocytes from the same compartment (90). Insulin stimulation significantly increased GLUT4 translocation in small (81 μm) but not in large (114 μm) diameter cells from the same individual indicating an insulin resistant state consistent with the reduction of insulin-stimulated lipid uptake described in the previous section on lipid metabolism (101). Consistent with these results, we reported a decrease in mRNA transcript of GLUT4 in SC adipose tissue of women with hypertrophic OM adipocytes compared to those with adipocyte hyperplasia (62).

Adiponectin and leptin

Some studies have shown that expression and secretion of adipose-derived cytokines or adipokines may vary as a function of adipocyte size and location (103, 104). A full review of all cytokines is beyond the scope of this review. This section focuses on leptin and adiponectin, two adipokines secreted almost exclusively by adipocytes. Inflammatory cytokines will be discussed in the subsection on inflammation.

Many reports have shown a negative association between SC FCS and adiponectin release (19, 61, 69, 103, 105, 106), even after adjustment for SC adipose tissue area (19). One study reported no association

between OM FCS or SC FCS and adiponectin levels (24). No association was found between SC FCS and serum adiponectin in another study in children (54). Another group reported that this association was non-significant when expressed as a function of cell surface (103). In South Asians, adiponectin decreased at lower obesity levels compared to Caucasians, likely due to ethnicity-related differences in FCS or in body fat distribution (69). Of interest, our group found that adiponectin release by isolated OM mature adipocytes (expressed as a function of lipid weight of the cell suspension) was reduced with increasing BFM and OM FCS (104). On the other hand, adiponectin release by SC fat cells remained unaffected by differences in total BFM or SC FCS (104).

Adipocytes appear to secrete more leptin as their size increases, at least for those derived from the SC fat compartment (19, 24, 103, 107-109). This is consistent with other studies showing that SC FCS is an independent predictor of plasma leptin concentration (24) and that SC FCS is the second most important predictor of leptin concentration after total BFM (107). SC FCS predicted 16.2% of the variance in leptin levels (109). SC FCS seems to be related to leptin levels, even when expressed per cell surface units (103) an association that is fully explained by differences in SC adipose tissue areas (19). However, in OM adipocytes, the association is equivocal. OM fat cells express lower levels of leptin mRNA (110) and it was thought that FCS and/or differences in tissue innervation contributed to this phenomenon. Recent studies showed no correlation (24) or even a negative relationship (111) between serum leptin levels and OM FCS.

Inflammation

Hypertrophic adipocytes overexpress NF-κB (112) and TNF-α (106, 113, 114) independent of BMI and total BFM (115). Other studies demonstrated that levels of inflammation marker C-reactive protein were correlated with the presence of larger adipocytes in obese individuals (51, 106, 114). However, this association was not found in lean individuals when they were analyzed separately from the obese subgroup (114).

The presence and number of adipose tissue macrophages in adipose tissues relate to its dysfunction and systemic inflammation. Macrophages in crown-like structures are often observed in adipose tissues of mice on a high fat diet, but their occurrence in human fat is far less common (116). We observed only a few crown-like structures in obese and more specifically in SC adipose tissue (116). No correlation was found between the number of crown-like structures and adipocyte hypertrophy in humans (65, 74, 76, 117).

We examined 40 women for whom SC and visceral adipose tissue was obtained and assessed the number of macrophages in the tissue using the CD68 marker and found positive correlations between percentage of macrophages in SC and OM adipose tissue as well as SC and OM FCS respectively (116). We also examined the CD11c and CD11b markers to distinguish subtypes of macrophages. We found that visceral and SC adipose tissue area was strongly correlated with CD11b and Cd11c expression in SC tissue, but not in visceral fat. Consistent with this result, OM and SC FCS were associated with expression of both CD11b and CD11c, but in SC tissue only. Increased SC FCS was associated positively with CD68+ macrophages in children. When FCS was categorized into tertiles, there was a threefold increase in the number of macrophages between the first (<116 um) and last (>130 um) FCS tertile (54). Further characterization of adipose tissue immune cells is needed to better understand how cell size relates to the low-grade, chronic inflammation state of obesity.

Hypoxia/Angiogenesis

Hypoxia and mitochondrial dysfunction could explain some of the mechanisms underlying adipose tissue dysfunction. Reactive oxygen species production could trigger an inflammatory response and cellular death. Hallgren et al. found that large diameter cells (88 μm) from both lean and obese individuals consumed more O_2 than small diameter cells (66 μm) regardless of the phenotype (118). This finding was not supported by recent work by Yin et al. who also showed that for lean patients, FCS increases

549 consumption rate of O_2, but this relation was not present in obese patients (119). Moreover, obese patients
550 had a lower O_2 consumption rate than their lean counterpart, regardless of FCS (119). This finding is
551 consistent with a few other studies that found no link between FCS and angiogenic capacity as measured
552 by expression of vWF, HIF-α or VEGFA (21, 61, 120). One study reported a positive association between
553 FCS and VEGF-R2 expression and with the number of vessels per 10 adipocytes (121). We also
554 examined women characterized by OM adipocyte hypertrophy and found higher expression of vWF and
555 CD31 in both fat compartments compared to women with hyperplasia (62). Another study found a
556 negative association between FCS (OM/SC) and capillary density (122). The relationship between
557 dysfunctional adipocytes and angiogenesis necessitates further investigation since it appears in the obese
558 phenotype, but its association with FCS remains unclear.
559
560 ***Adipogenesis***
561 A complete review of adipogenesis is beyond the scope of this article and has already been done by our
562 group (123). Adipogenic capacity is depot- and obesity-specific (123) and is apparently reduced in
563 hypertrophic obesity (124).
564
565 Elegant studies by Arner and colleagues have shown that adipocyte hypertrophy is negatively correlated
566 with adipocyte hyperplasia, when adjusted for the predicted adipocyte volume at a given BFM (45).
567 Subjects with higher FCS than predicted by the regression curve had a lower rate of adipogenesis. These
568 individuals also had a more deleterious metabolic profile. These results support the hypothesis that each
569 individual has a different plateau of maximum FCS and when it is reached, metabolic alterations arise.
570
571 In our recent analysis of published data, we confirmed the notion that adipogenesis is reduced in
572 overweight and obese women with adipocyte hypertrophy (123). Moreover, in another study, (124), we
573 initiated *in vitro* primary preadipocyte cultures of cells obtained from the SC and OM adipose tissues of
574 35 women. We assessed adipogenic capacity by measuring lipid accumulation (oil red staining) and

G3PDH activity as a terminal differentiation marker. We found that lower SC preadipocyte differentiation capacity was related to increased OM FCS, excess visceral adipose tissue accumulation, high VLDL lipid content and slightly elevated fasting glycemia (124). These results are consistent with the notion that limited expandability of SC adipose tissue is related to adipocyte hypertrophy, particularly in the visceral fat compartment, and the concomitant presence of metabolic alterations.

CONCLUSIONS AND PERSPECTIVES

In conclusion, our analysis demonstrates that FCS may represent a significant biomarker of the cardiometabolic alterations related to obesity. In particular, many studies demonstrated that FCS predicts alterations in the blood lipid profile and glucose-insulin homeostasis independent of adiposity indices. The contribution of visceral adiposity to these associations seems to be of particular significance. We propose that excess visceral adipose tissue accumulation and adipocyte hypertrophy, either in the SC or visceral fat compartment depending on the studies, may represent strong markers of a common phenomenon: limited hyperplasic capacity of adipose tissues, which in turn associates with the presence of numerous cardiometabolic alterations (Figure 2).

However, we also emphasize that available studies are far from unanimous, in particular when addressing the relationship between FCS and parameters of adipose tissue function. A number of methodological factors such as the approach used to express the data may have confounded these analyses. Additional factors to be considered for future studies on this topic include analyses of cell subpopulations and the plateauing of cell size with increasing obesity levels. These issues are addressed below.

Most investigators have used average FCS as the primary variable in their analyses, which has become the default standard. However, this measure has been revealed to be incomplete when global tissue physiology is studied. Other approaches to better characterize the dynamics of cell populations have already been discussed, including osmium fixation and Multisizer Counting. This approach could

represent a very useful method to characterize cell populations in more detail. Interestingly, the fraction of very small cells has become of interest in patients with severe obesity when it was found to be increased in the presence of the metabolic syndrome. Adipose tissue expansion could lead to changes in the various cell populations that may be missed when examining mean FCS. Analysis of cell size distribution patterns, of the ratio of small-to-large cells as well as minimal and maximal cell sizes are potential alternatives.

Another important factor to consider in analyses based on adipocyte size is the patient population under study. As shown in Figure 1, mean FCS increases with adiposity level and reaches a plateau at higher obesity levels. Accordingly, studies in obese and severely obese individuals have shown that mean FCS only slightly differs among these subgroups of patients. In these cases, it is expected that the predictive ability of FCS will be reduced. The relationship between FCS and adiposity indices becomes non-linear, which may limit the effectiveness of regression studies to control for the effect of adiposity. More importantly, the association between FCS and metabolic parameters may also become non-linear in the obese or very obese range. This phenomenon could indicate that the 'cardiometabolic pathology' has reached another stage, where adipose tissue storage capacity is very limited and other features such as ectopic fat accumulation will become better predictors of metabolic status than FCS *per se*. Further studies are needed to better understand the impact of adipocyte hypertrophy on metabolic health and the ways to manage it.

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DECLARATION OF INTEREST

The authors have nothing to disclose.

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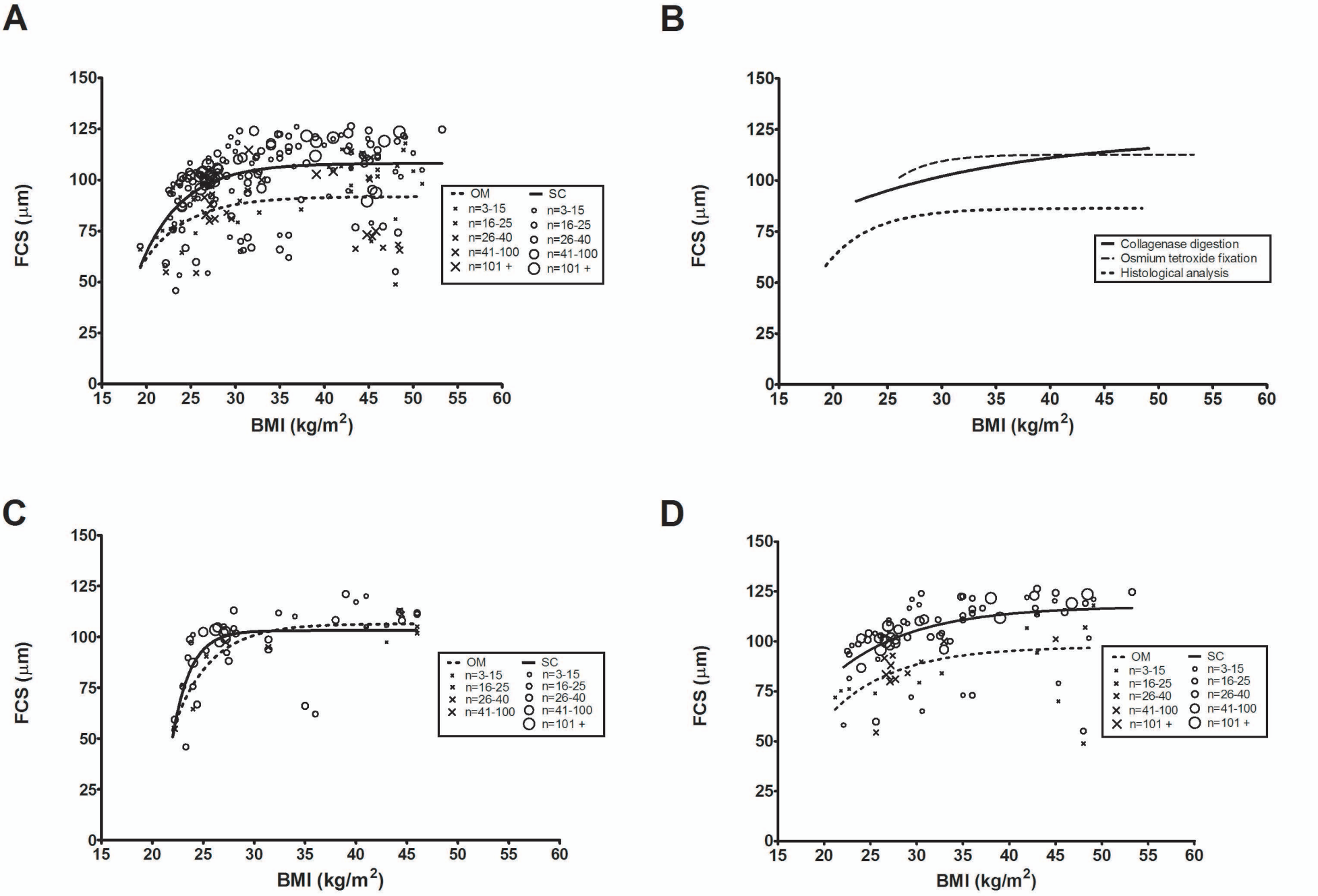
FIGURE HEADINGS

FIGURE 1. Mean FCS of the abdominal subcutaneous (SC) and omental (OM) depot in 176 subgroups of individuals in 78 published studies. Sizes of the symbols reflect study samples size. Published studies on FCS were screened and inclusion criteria were availability of the BMI and FCS for the same subgroup. A total of 6355 individuals are included in the analysis. Non-linear regressions were determined taking study sample size into consideration. **A.** OM and SC FCS in both in males and females. **B.** OM and SC FCS as a function of BMI in males. **C.** OM and SC FCS as a function of BMI in females. **D.** FCS variation as a function of BMI and the technique used to assess FCS. Each curve combines OM and SC FCS. Collagenase digestion, n=4778; Osmium tetroxide fixation, n=1052; Histological analysis, n=2533.

FIGURE 2. Obesity is a multifactorial disease characterized by expansion of adipose tissue occurring through adipocyte hypertrophy (enlargement of pre-existing cells) or adipocyte hyperplasia (generation of new cells through adipogenesis). Limited expandability of adipose tissue through hyperplasia leads to increases in FCS (adipocyte hypertrophy), which represents a critical marker of central adiposity, adipose tissue dysfunction and concomitant metabolic disease risk.


A
B
C
D
FCS (μm)
BMI (kg/m2)
OM
SC
n=3-15
n=16-25
n=26-40
n=41-100
n=101 +
Collagenase digestion
Osmium tetroxide fixation
Histological analysis

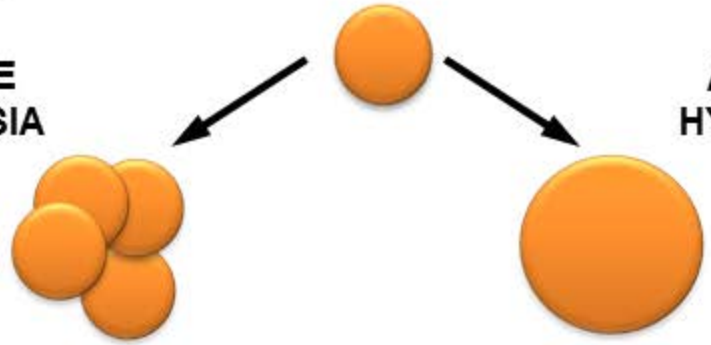
BEHAVIORIAL-GENETIC/EPIGENETIC-ENVIRONNEMENTAL

OBESITY

ADIPOSE TISSUE EXPANSION

ADIPOCYTE HYPERPLASIA

ADIPOCYTE HYPERTROPHY



INSULIN SENSITIVE, EFFICIENT ADIPOGENESIS, PERIPHERAL FAT ACCUMULATION…

INFLAMMATION, ALTERED ADIPOKINES SECRETION, IMPAIRED ADIPOGENESIS, ECTOPIC FAT ACCUMULATION, IMPAIRED GLUCOSE AND LIPID METABOLISM, ER STRESS…

NON-METABOLIC COMPLICATIONS

HYPERTENSION, DYSLIPIDEMIA,CHD, METS, T2DM, PCOS, NAFLD, CANCER (BREAST, COLON, ENDOMETRIAL, ETC.)