Adipose cell size: Importance in health and disease

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Abstract

Adipose tissue is necessary to harbour energy. To handle excess energy, adipose tissue expands by increasing adipocyte size (hypertrophy) and number (hyperplasia). Here, we have summarized the different experimental techniques used to study adipocyte cell size and describe adipocyte size in relation to insulin resistance, type 2 diabetes and diet interventions. Hypertrophic adipocytes have an impaired cellular function and inherent mechanisms restrict their expansion to protect against cell breakage and subsequent inflammation. Reduction of large fat cells by diet restriction, physical activity or bariatric surgery therefore is necessary to improve cellular function and health. Small fat cells may also be dysfunctional and unable to expand. The distribution and function of the entire cell size range of fat cells, from small to very large fat cells, is an important but understudied aspect of adipose tissue biology. To prevent dysmetabolism, therapeutic strategies to expand small fat cells, recruit new fat cells and reduce large fat cells are needed.

Keywords insulin; adipocytes; obesity; cell size distribution; diet

Introduction

Humans have the highest percentage of body fat among mammals. In comparison to other hominids body fat percentage is markedly higher, even in physically active persons as in traditional hunter-gatherer populations. At the same time humans have an elevated metabolic rate compared to other hominids, indicating an increased organ metabolic activity (89). This expansion of energy budget in humans occurred with a dietary shift from low-energy food such as leaves and fruits to high-energy food like tubers and meat. Cooking also increased the availability of nutrients and energy after foraging (35, 39). Increased body fat serves as a buffer against calorie deficiency, especially in women with the need for extra energy during reproduction. The capacity to store fat hence has been a major adaptive factor of our species. In modern society with a surplus of energy-dense food and a minimum of effort to obtain this food, the ability to store excess energy intake becomes problematic. Our great appetite for energy-dense food and the efficiency of the gastrointestinal tract to digest and absorb such food is coupled to an ability to store energy in adipose tissue. With a positive energy balance and an expanding adipose tissue metabolic dysfunction may arise leading to diseases like arteriosclerosis, diabetes, osteoporosis and cancer.

Adipose tissue is traditionally considered an organ with low resting energy expenditure (37). Adipose tissue is however a highly dynamic tissue, possessing adipocytes of various size, referred to as small and large adipocytes. The properties

of these cells have been extensively explored, focusing on the relationship between cell size and various disease conditions such as inflammation (97, 109, 118), insulin resistance and diabetes (9, 15, 27, 33, 79, 102, 119). Laforest S *et al.* addressed the correlation between adipose cell size and cellular function as well as metabolic disease and concluded that the size of the adipocyte is an important factor to predict pathophysiological conditions (62).

In the present review, we have described the size variation of small and large fat cells during disease conditions, in particular insulin resistance, diabetes, obesity and during diet intervention. To this end we have included a discussion of the different techniques used for adipose cell size measurements. The data discussed in this review support the notion that the proportion of large and small adipocytes rather than a specific cell size *per se* within the adipose tissue depot is important for maintained cellular function and metabolism.

Adipose cell biology

The white adipose tissue (WAT) is an organ that primarily stores energy as triacylglycerol (lipogenesis) during energy surplus, to be mobilized as fatty acids (lipolysis) when needed. These processes are tightly regulated by insulin and adrenalin, which control the activity of lipogenic and lipolytic enymes. Distinct from WAT, brown adipose tissue (BAT) is specialized for energy expenditure through thermogenesis (16). Browning and beiging of white adipose tissue has gained

attention as a therapeutic strategy to combat obesity (36). However, since to our knowledge there are no studies addressing the size of brown adipocytes or the alteration in cell size during browning of white adipocytes, we will focus on the white adipose tissue in this review.

Most white adipose tissue is distributed in two major depots, the subcutaneous (scWAT) and the visceral white adipose tissue (vWAT) (94). WAT contains both mature adipocytes (roughly 50% total cell number) and a stromavascular fraction, including preadipocytes, endothelial cells, mesenchymal stem cells and macrophages (67). WAT expands both through an increased fat cell volume (hypertrophy) and an increased number of fat cells (hyperplasia) (50). Hyperplasia occurs through differentiation of progenitor cells (adipogenesis) under the influence of various transcriptions factors, among these the peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding protein alpha (CEBPα) (45). There is a constant turnover of adipocytes, with a life-span of around eight years, as established by measuring the ^{14}C-incorporation into DNA (112). Both mature adipocytes and the resident inflammatory cells within adipose tissue secrete various hormones and cytokines, named adipokines (56). The most well-studied adipokines are leptin and adiponectin, which regulate appetite and energy metabolism, respectively (55, 127). Others are either pro-inflammatory, such as interleukin-6 (IL-6) (118) and tumor necrosis factor alpha (TNF-α) (42) or anti-inflammatory, such as

apelin (126). The secretion of free fatty acids and adipokines from adipose tissue cross-talks with other organs to affect whole body metabolism.

Exploring fat cell function

Adipocytes vary dramatically in size, with human white adipocytes ranging from <20 to 300 μm in diameter. This extrapolates to a several thousand fold range in cell volume within the same depot (58). This variation is mainly dependent on cellular triglyceride content. A central question is therefore whether the metabolism of adipocytes is size-dependent. To address this, standard methods for isolation of intact adipocytes have been established (93). However, methods to accurately characterize metabolism of isolated adipocytes with respect to their size are far more complex.

Isolation of viable, intact adipocytes

Early in the 1960's, Rodbell established a method for isolation of intact rat adipocytes from epididymal fat pad, a protocol that is still used in the research field of adipocyte biology (93). The method is based on incubating fat tissue in buffer containing collagenase. The fat cells released from the adipose tissue during digestion are then separated from the denser stromavascular cell fraction by flotation. The resultant single cell population contains adipocytes varying between 50 and 100 μm in diameter as assessed by light microscopy. These early studies concluded that isolated adipocytes may serve as a good model for subsequent

155 investigations of insulin action in adipose tissue. This allows investigation of
156 hormonal responses related to glucose and lipid metabolism.
157
158 Subsequently, a Swedish group headed by Per Björntorp, compared the collagenase
159 method, using the protocol of Rodbell (93), with a microscopy method based on
160 fixation of adipose tissue followed by freeze-cutting the tissue (108), to verify that cell
161 size estimation of adipocytes from human tissue had not been distorted during the
162 isolation procedure (111). The cell size distribution was found similar using either
163 method, with cell diameter varying between 40 and 150 μm. The collagenase method
164 introduced by Rodbell *et al* (93) is a gentle and adequate method for isolation of
165 adipocytes and there is minimal risk to rupture large adipocytes as long as
166 centrifugations are avoided. The collagenase method may therefore appear most
167 useful for metabolic and morphological studies of human adipose tissue. However,
168 the microscopy method (108) is better suited for clinical studies. It is less time-
169 consuming and requires a smaller adipose tissue sample for study. The collagenase
170 method was however later reported to trigger an inflammatory response that could
171 interfere with the signal transduction and gene expression of target molecules
172 involved in lipid metabolism, potentially contributing to the relatively short life-span
173 of the isolated cells (98).
174
175 *Techniques for cell separation according to size*
176 It is difficult to draw conclusions regarding a direct association between function and

size when comparing adipocytes of varying size isolated from different subjects (102, 110, 119). In light of this, a major limitation in characterizing the metabolic function of primary adipocytes in relation to cell size is the technical issue of collecting enough numbers of cells that are well-separated in size from the same subject. Another challenge includes optimization of cell handling, which has to be gentle enough to avoid cell breakage. This is a concern in particular for the very large adipocytes, which contain a huge lipid droplet that makes the cells fragile and prone to breakage. A rapid release of triglycerides will quickly deteriorate the entire cell population. All fat cells irrespective of size have a certain degree of pliability that makes filtration of the cell suspension through filters of different pore size difficult, even though some success has been reported (27, 48, 65, 109) as discussed below.

Flotation and filtration

A strategy to separate isolated cells of different size is to utilize the differences in buoyancy (25, 29). This property was exploited in an early attempt to collect fractions of fat cells of varying sizes from the same fat depot. Meters of dialysis tubing were used (12), resulting in two separated fat cells pools, with a mean diameter of 70 and 95 μm, respectively. Another approach relies on filtration of the cell suspension through nylon mesh of different pore sizes to collect pools of small and large fat cells (27, 48). Through filtration, a successful separation was reported with cells isolated from human subcutaneous fat tissue, producing cell pools with a mean diameter of 58 μm (small cell pool) and 100 μm (large cell pool). However, this technique

199 requires a substantial amount of starting material and yields poor cell recovery in
200 each fraction. For example, in the study by Jernås *et al* (48), although 4-52 g of fat
201 tissue was used for each subject, a limited amount of material was collected at the
202 end of the separation. In another study, filtration was used to separate human fat
203 cells into two pools starting from a similar amount of material, which resulted in
204 slightly less well-distinct fractions, with a mean cell diameter of 81 and 114 μm,
205 respectively (27). This method allowed a sufficient yield of cells for use in cell
206 signalling studies.
207
208 *Flow cytometer*
209 A general belief is that isolated adipocytes are too large and fragile for flow
210 cytometer or similar sorting instrumentation. Nonetheless, in a recent method
211 description by Majka *et al* (75), the authors propose that the modern flow systems
212 have the capabilities needed to sort adipocytes up to 250 μm in diameter with
213 relatively low pressure, thereby reducing mechanical stress. The authors have
214 established a protocol to exclude stromal vascular cells, that can potentially interfere
215 with subsequent analysis (76). Indeed, several papers report successful flow
216 cytometer analysis using primary adipocytes isolated from human (26), mouse (82)
217 and rat adipose tissue (8). Thus, with newer sorting instrumental setups, useful
218 methods have been developed that may become more widely adopted for future
219 characterization of adipocytes of various size.
220

Single Cell Analysis

Methods incorporating use of microscopy have the advantage of functional assessment of single cells of a distinct cell size, though it often includes tedious analysis. Several studies have employed fluorescence microscopy in fixed human adipocytes (27) and live, intact adipocytes isolated from either human (70, 121) or rat adipose tissue (69, 113) for metabolic characterization including hormonal responses. Also, imaging of ex vivo adipose explant from *Rhesus macaques* allowed characterization of fatty acid uptake at a single cell level that included cell size measurements (117). Another technically advanced approach for single cell analysis is patch clamping, which has been established using isolated adipocytes (60, 128). Additionally, the RNA-seq technology has developed rapidly, and is now available at a single cell level (73). A caveat is that even though these methods are, in theory, applicable to adipocytes, these analyses require cell separation, usually carried out by flow sorting, which in itself causes technical problems such as cell breakage. This could explain the lack of published studies using this technique. Considering the vast information that can be gathered by single cell RNA-seq studies, the technical hurdles utilizing this technology for isolated adipocytes will hopefully be resolved. One possibility is the use of a fluid chamber-based system, like Drop seq analysis (74), a method that is applicable for single cell suspensions.

Cell size distribution

In many studies, measurements of cellular and systemic function are correlated with the average fat cell size measured by histological sectioning of intact tissue or isolated cells. This type of size measurement requires only a small amount of tissue/cells and is technically relatively easy to accomplish. However, often this method does not provide causal relationship between cell size and function of individual adipocytes. In addition, it is questionable whether the entire span of cell sizes is detectable using a histological approach, where the very small cell population (<30 μm diameter) easily could be overlooked or underestimated, since the sectioning has to occur at the widest diameter of every cell to obtain a true measurement. Several (semi)-automated image analysis tools have been developed to improve cell size analysis from histologic fat tissue samples (10, 18, 85, 86). This allows faster analysis of an increased cell number compared with manual measurements.

The coulter counter is another commonly used method, where the size of osmium tetroxide-fixed cells is determined by measurement of electric resistance. The method is evaluated to be an accurate method for size measurement in adipose tissue (40), and also for isolated cells from either human or rat adipose tissue (20). A drawback is the use of osmium tetroxide, which is expensive and hazardous. In comparison, the coulter counter measurement has the advantage of measuring a large number of cells (~10000) with a broad size range, 20-300 μm in diameter, whereas histology does not provide the same precision. It also carries a limitation in detecting small fat cells. Caveats raised for the coulter counter method are whether cell debris could

contribute to false-positive measurements of the number of small cells, and the risk of osmium to cause cell swelling (40). Mathematical modelling of data obtained by the coulter counter approach revealed a bimodal cell size distribution (51, 78, 79, 122). It was pointed out that the use of the average cell diameter of the entire cell population could be misleading and that more useful information could be obtained by analysing the mean cell size in each cell population, which was termed a small and a large cell population. Optical analysis on the other hand, usually demonstrates a one-peak Gaussian-like cell size distribution (48, 81), with a reduced frequency of smaller fat (<30 μm) cells. The methods used for analysing adipocytes according to size in both intact tissue and isolated cells are summarized in figure 1. Even though the isolation of fat cells is technically challenging, there is definitely a need for continued research on adipocyte function in relation to cell size.

Fat cells during disease

It is well-known that obesity, which is defined by an excessive white adipose tissue mass, is one of the main risk factors for insulin resistance and type 2 diabetes (92, 104). While the exact mechanisms are not known, it is clear that in obesity the adipocyte itself is unable to regulate excess nutrients, leading to increased circulating levels of fatty acids and glucose, changed adipokine secretion, and dysregulated energy metabolism. In this regard, a limitation in fat tissue expansion, as reviewed by Rutkowski *et al.* (99) and Virtue *et al.* (120), could contribute to systemic insulin resistance and diabetes.

The fact that adipose tissue contains a mixture of adipose cells, that vastly vary in size, makes it complicated to define if there is a set size threshold that is associated with impaired function. When reviewing original research articles, it is also important to consider: 1) what method that was used to determine cell size; 2) if the biologic function is measured in isolated cells or is merely correlated with cell size; and 3) whether cells of different sizes originated from the same subject. In order to compare different cell size measurements across the literature, we have converted data expressed as μg lipid/cell or cell volume (pl), to cell diameter (μm), using the assumption that the cells are spherical with a density of 0.91 (53). In the text, "μm" implies mean adipocyte diameter unless stated specifically to be otherwise. The described differences regarding cell size and their function attained statistical significance in each individual study.

Hypertrophic adipose cells

Increased cell size as a predictor of systemic insulin sensitivity

In general, large, hypertrophic cells are considered less metabolically favorable and are associated with pathophysiologic conditions. Even in the early studies of human adipocytes, increased fat cell size was shown to correlate with impaired whole body metabolic regulation (9, 11) and systemic insulin resistance (33, 61, 101, 102). In a prospective study of Pima Indians, known for their predisposition for developing insulin resistance and type 2 diabetes, increased adipocyte cell size was shown as an

independent marker of type 2 diabetes (119). The average cell size diameter of the entire cell population, as assayed by coulter counter, positively correlated with systemic glucose tolerance (GTT). It was found that an average cell size of 115 μm correlated with normal GTT; 121 μm with impaired GTT; and 125 μm with diabetes (119). In a comparison between Pima Indians and Caucasian children with similar body weight and body fat mass, Pima Indians had increased average adipose cell size, 101 versus 92 μm, as well as increased circulating levels of glucose and insulin (2). In non-diabetic subjects, an increased average adipose cell size, varying between 75 and 130 μm as determined manually by light microscopy, negatively correlated with both cellular and systemic insulin sensitivity independent of BMI (72). In another study, over-feeding of both insulin resistant (IR) and insulin sensitive (IS) moderately obese subjects normalized for BMI was carried out to test whether IS subjects were protected from cell enlargement (77). At the start of the study, the insulin sensitive subjects had smaller adipocytes, but following a modest weight gain (~3 kg), they displayed an increase in average adipocyte size from 108 to 115 μm as assayed by coulter counter. Such individuals also demonstrated impaired insulin-mediated glucose uptake (IMGU) and increased lipolysis (77). In contrast, the insulin resistant subjects had no significant increase in cell size and only slightly further impairment of IMGU. Thus, it was concluded that an increase in adipose cell size could predict the development of insulin resistance and type 2 diabetes (77). In yet another over-feeding study, the subjects starting with smaller adipocytes, assayed between 57 and 115 μm using coulter counter, turned out to have the most impaired

metabolic profile following weight gain. Thus having small adipocytes *per se* was not sufficient to protect against development of insulin resistance (52). It was thought that the small adipocytes expanded more rapidly, whereas the subjects that initially had large adipocytes instead increased the number of cells (hyperplasia). This supports the notion that any increase in adipocyte size, either from small or normal size cells, could result in an impaired metabolic profile (52). Increased subcutaneous adipocyte cell size was also a predictor of type 2 diabetes incidence in a Swedish cohort of ~1300 women followed over 25 years (71). Together, these studies suggest that increase in subcutaneous adipocyte size is an important factor to predicts type 2 diabetes unrelated to obesity (2, 71, 72, 119).

In contrast, Pima/Papago subjects with type 2 diabetes had similar mean adipose cell size, 120 μm, compared to weight matched healthy individuals (53). However, the glucose metabolism and insulin response in adipocytes isolated from type 2 diabetic subjects were markedly reduced (53). Large subcutaneous fat cells were also observed in obese children with hyperinsulinemia (15), with an average cell size of 115 μm compared with 86 μm in lean controls, as assayed by coulter counter. After body weight reduction through dietary restriction over 8 weeks, the average fat cell size in the obese children decreased to an average cell size diameter of 95 μm, but no direct correlation between cell size and insulin level was observed.

Impaired hormonal response in isolated large adipocytes

Direct measurements of cellular hormonal response and metabolism in isolated adipocytes support the concept that enlarged adipocytes are less responsive and less metabolically favorable (1, 33, 61, 102, 122). Insulin responsiveness, measured as % increase of glucose oxidation to CO_2, negatively correlated with cell size, where the mean cell size within each adipose cell tissue sample ranged between 74 and 134 μm, as assessed by coulter counter (102). No difference in the protein level or degree of activation was found for the insulin signaling down-stream intermediates insulin receptor substrate (IRS)-1 and protein kinase B (PKB), when comparing small versus large human adipocytes isolated by filtration from the same non-diabetic subject (27). Even though the total GLUT4 protein content was similar in the small and large cell populations as assessed by microscopy, average diameter 81 and 114 μm respectively, image analysis revealed a 2-fold increase in insulin-induced GLUT4 translocation to the plasma membrane in the small cell population whereas no increase of GLUT4 translocation was found in the large cell population (27). This supported a reduced insulin sensitivity in the large fat cells. In recent studies using live cell imaging with total internal reflection fluorescence microscopy in human fat cells, impaired GLUT4 dynamics correlated with both increased BMI and insulin resistance of the donor subject (121). In a follow-up analysis, there was however no correlation between GLUT4 trafficking and size of fat cells from 25 to 125 μm in diameter, isolated from different subjects (70). In non-diabetic subjects an increase in the average adipocyte size, assayed over a range of 75-130 μm in diameter, correlated

373 with a decrease in insulin-stimulated glucose uptake (72), again supporting a
374 reduced insulin sensitivity with enlargement of fat cells.
375
376 Lipolytic activity, leading to an increased release of circulating FFA is also increased
377 in large human adipose cells (22, 47, 65, 125), an effect regulated by insulin. Using a
378 flotation approach, two cell populations, average diameter of 82 μm of small and 100
379 μm of large fat cells, were collected from human subcutaneous adipose tissue and
380 characterized for their lipolytic activity (65). Both non-stimulated as well as
381 hormone-induced lipolysis was increased in large adipocytes compared to small
382 adipocytes. These findings were supported by increased protein levels of hormone-
383 sensitive lipase (HSL) and adipose tissue triacylglycerol lipase (ATGL), which are
384 responsible for TAG hydrolysis prior to fatty acid release from fat cells (65). Similar
385 findings were observed in a study by Jacobsson B *et al.* (47), where basal and
386 adrenergically stimulated lipolysis correlated positively with cell size, whereas the
387 insulin-induced anti-lipolytic effect was independent of cell size in the size range
388 studied, 70-115 μm, measured by microscopy.
389
390 *Inflammation is associated with increased cell size*
391 Altered adipokine release is tightly associated with an increased inflammatory
392 response in adipose tissues, which in turn can deteriorate insulin signaling in mature
393 adipocytes (63, 97, 118). The positive correlation between fat cell size and expression
394 of the inflammatory genes NF-κB, TNF-γ (6), and expression of TNF receptor (43)

suggest that increased adipocyte size is associated with an increased inflammatory response. Adipokine secretion in relation to cell size was also addressed in a study where human adipocytes isolated from the same subject were fractionated via filtration into four different diameter groups - small (73 μm), medium (98 μm), large (113 μm) and very large (127 μm) (109). Increased pro-inflammatory adipokine release, namely IL-6, IL-8, monocyte chemoattractant protein-1, and granulocyte colony-stimulating factor, was found in the very large fat cell group compared with the small fat cell group, confirming large fat cells to be highly inflammatory. By isolation and separation of human adipocytes through filtration into two pools, large and small cells, 58 and 100 μm mean diameter, Jernås *et al* (48) detected a >4-fold increase of several immune-related genes including serum amyloid A in the large fat cell population. In a recent study, non-obese type 2 diabetic subjects had larger adipocytes, average cell diameter of 104 μm, as assessed by light microscopy of isolated cells, than the healthy controls, average cell diameter of 95 μm, but lipolysis and the secretion of inflammatory proteins measured using isolated adipocytes were similar in the two groups (3). Still, increased adipocyte size positively correlated with both lipolysis and systemic insulin resistance, independent of type 2 diabetes (3). This suggests that the appearance of large fat cells precede the development of diabetes. Release of leptin, but not adiponectin, increased with increasing cell size in mature adipocytes isolated from subcutaneous adipose tissue (109). However, the proportion of small cells in human SAT explants, defined as <69 μm mean diameter, and large cells defined as >131 μm mean diameter by histologic sectioning, positively

417 correlated with adiponectin secretion and circulating serum adiponectin (81). Thus,
418 there seems to be a shift towards an increased pro-inflammatory secretion and
419 decreased anti-inflammatory secretion from the very hypertrophic cells, which could
420 contribute to systemic insulin resistance and type 2 diabetes (119).
421
422 **Dysfunctional small adipocytes**
423 There are also studies that suggest that impaired expansion of small fat cells is
424 associated with insulin resistance (24, 78, 79, 87). The recent approach of studying
425 not only the average cell size but rather the entire cell size distribution including the
426 very small fat cells and the proportion between different cell sizes could be the
427 reason for the emerging concept of an impaired lipid storage capacity in not only the
428 large cells but also the smaller cells. In morbidly obese subjects, type 2 diabetes was
429 associated with an increased proportion of small adipocytes (<50 μm) in omental,
430 mesenteric and subcutaneous fat depots (24). In a study by McLaughlin *et al*. (79),
431 the coulter counter technique was used to illustrate a bimodal cell size distribution
432 in human subcutaneous adipose tissue, with two cell populations, small and large
433 cells. To verify that the population of so-called small cells represented adipocytes,
434 the analysis was repeated in both intact adipose tissue as well as in isolated
435 adipocytes from the same subject (79). In contrast to expectations, the overall mean
436 adipocyte size was similar for cells from insulin-sensitive and insulin-resistant
437 moderately obese subjects normalized for BMI (~30). However, the proportion of
438 small fat cells was higher in the insulin-resistant group, ratio small/large cells 1.66 in

the insulin sensitive group and 0.94 in the insulin resistant group (79). Presumably, the insulin-resistant subjects had small fat cells that failed to expand appropriately, thus being unable to store sufficient fat, resulting in increased ectopic storage of fat in the liver and the skeletal muscle. This idea was supported in a further study where increased insulin resistance as determined by steady-state plasma glucose concentrations correlated positively with an increased proportion of small fat cells and an increased mean cell size within the large cell population. (78). By coulter counter analysis, type 2 diabetic subjects had a greater mean adipocyte size of the very large cells, 144 versus 171 μm, a lower total cell number in the subcutaneous abdominal fat mass, but a greater proportion of small adipocytes compared with weight-matched non-diabetic subjects (87). The idea of dysfunctional small fat cells is not in conflict with the concept of large adipocytes as less metabolically favourable, but emphasizes the importance of examining the distribution of the entire cell size range. Thus, there seems to be an inability of both small and large fat cells to expand, leading to dysmetabolism and insulin resistance, illustrated in figure 2.

Mechanisms behind impaired cell function

Limited cell expansion

The cellular mechanisms behind impaired function of large adipocytes are not yet resolved but are at least in part due to limitation in further cell expansion. During excess energy, the adipocyte has the ability to maximize its volume several thousand

461 times. However, with an increased cell size the adipocyte becomes stiff (106) and the
462 membrane signaling disturbed (88, 107). Various intracellular signaling pathways
463 are activated as demonstrated in 3T3-L1 adipocytes, for example the MEK signaling
464 pathway (107). Also, the regulation of fat cell size could potentially be mediated
465 through volume sensitive adipocyte membrane proteins (17), one of them recently
466 being identified and named SWELL 1 (128). The authors demonstrated that gene
467 silencing of SWELL1 led to a decrease in fat cell size and decreased glucose and lipid
468 uptake in both cultured and primary adipocytes. Adipose-specific SWELL1 knock-
469 out caused systemic hyperglycemia and decreased insulin sensitivity (128). These
470 experiments clearly demonstrate that fat cell enlargement is closely linked to glucose
471 metabolism and when this mechanism fails glucose metabolism is deranged. Mice
472 studies have shown that remodeling of the extracellular matrix by knock-down of
473 Collagen VI reduced the local adipose tissue inflammation and allowed increased
474 adipocytes expansion without impaired cellular insulin signaling and unfavorable
475 metabolic consequences (57).
476
477 The failure of enzymes promoting triglyceride storage within the fat cell may also
478 contribute to impaired fat cell function. Hence, improvement of the triglyceride
479 storage capacity in adipocytes by adipose tissue-specific overexpression of PEPCK,
480 the rate-limiting step in glyceroneogenesis, leads to increased adipocyte cell size
481 allowing fat mass increase without leading to insulin resistance (28). In humans,
482 increased mRNA expression of the lipid droplet-associated proteins Cidea, FSP27

and perilipin in SAT positively correlated with insulin sensitivity index (HOMA-IR) in weight matched obese subjects (91). Since these proteins promote lipid droplet formation and facilitate triglyceride storage (54), their increased expression is expected to favour increased cell size. A perilipin knock-out mouse model had a 30% reduction of fat tissue mass and was protected against high fat-diet induced obesity (116). Still, these mice were prone to develop impaired glucose tolerance and systemic insulin resistance. Together, these data suggest that a limitation of cell expansion is linked with impaired glucose and impaired fat metabolism both at the cellular and at the systemic level.

Impaired differentiation

Several studies have pinpointed that too few mature adipose cells and impaired differentiation of precursor cells is associated with type 2 diabetes (21) in both non-obese (3) and obese subjects (32, 46). Notably, there was no evidence of fewer precursor cells but rather impaired cell lineage commitment and differentiation (3). Others reported a negative correlation between number of precursors and BMI of the subject (46). The canonical Wnt signaling pathway (96) and bone morphogenetic protein 4 (BMP4) (14) regulate the commitment of mesenchymal stem cells and the differentiation of pre-adipocytes into adipocytes. In a study of non-diabetic insulin-resistant first-degree relatives of type 2 diabetic patients, the average adipose cell size negatively correlated with the expression of Wnt1 signaling genes FZD1, GSK3β, and LEF1β-catenin (123). The adipokine Wnt1 inducible signaling pathway

505 protein 2 (WISP2) was demonstrated to inhibit cell lineage commitment by
506 inhibiting BMP4 (34), and increased expression of WISP2 in human subcutaneous fat
507 tissue positively correlated with increased cell size (34). Thus, this could provide a
508 mechanism for impaired differentiation of new adipocytes, and result in increasing
509 the size of already committed adipocytes.
510
511 **Therapies affecting adipose cell size**
512 Metabolic improvements are strongly associated with a reduction of fat cell size.
513 These may occur though calorie-restricted diets/exercise, cholesterol-free diets,
514 bariatric surgery, the use of bioactive compounds and pharmacological compounds
515 stimulating PPARγ. Weight gain seems to be mostly associated with cell expansion
516 (hypertrophy) (112), whereas weight loss leads to a decreased fat cell size
517 independent of the method employed for weight loss; surgical, diet restriction,
518 physical activity or life style intervention (83).
519
520 *Calorie-restricted diets with or without exercise*
521 Life style intervention usually involves restricted diet, such low-calorie diets (LCD),
522 very-low calorie diets (VLCD) either with or without exercise. In one study, 48 obese
523 subjects were divided into four groups and were fed for six months either 1) a
524 healthy control diet, 2) a 25% calorie restricted diet, 3) a 12.5% restricted diet with
525 12.5% increase in total energy expenditure by exercise, or 4) a low-calorie diet until a
526 15% reduction in body weight was achieved (64). Diets were based on American

Heart Association recommendations (≤30% fat). To estimate fat cell size, subcutaneous abdominal needle biopsies were performed and adipocyte size determined by coulter counter technique (38). It was found that in response to 6 months of energy-restricted diet in overweight men and women, body weight, visceral fat mass and fat cell size all decreased, and that addition of exercise further decreased adipose cell size (64). The reduction in fat cell size was most strongly associated with a decrease in body weight and a decrease in percent body fat (64). The intervention also decreased liver fat, but had no effect on muscular fat. Insulin sensitivity was increased to a similar level by diet alone or in conjunction with exercise. The improved insulin sensitivity was linked to body weight reduction, visceral fat reduction and fat cell size reduction, but unrelated to liver fat reduction. It was stated that large fat cell size was associated with impaired adipogenesis and increased TAG content in adipose tissue, liver, muscle and pancreas leading to insulin resistance.

In another 20-week intervention study, overweight women were given a restricted diet alone, together with low-intensity or high-intensity exercise, consisting of 20 min treadmill walking three times per week at 50 respectively 75 % of maximal heart rate (124). The calorie deficiency was adjusted to -2800 kcal/week for all three groups, obtained though diet restriction in the diet group, and through diet- 2400 kcal per week and -400 kcal per week in the exercise groups (124). All three interventions reduced body weight, fat mass and percent fat. To estimate fat cell size,

549 subcutaneous abdominal and gluteal needle biopsies were performed and adipocyte
550 size determined by microscopic evaluation of isolated fat cells (124). Gluteal
551 adipocyte size decreased similarly in all three groups, whereas abdominal adipocyte
552 size did not change in the diet group but was reduced in the exercise groups. It was
553 concluded that in order to affect fat cell size in the abdomen, exercise needs to be
554 added in a life style intervention program. Hence caloric restriction alone protects
555 against abnormal expansion of fat but may be more efficient in combination with
556 physical activity to prevent dysmetabolism and insulin resistance. The authors did
557 not address whether the reduced fat cell size was due to increased hyperplasia or
558 reduced size of existing fat cells.
559
560 *Bariatric surgery*
561 Bariatric surgery has been demonstrated to decrease fat cell size in abdominal
562 subcutaneous adipose tissue. Two years after a gastric bypass in 62 obese women,
563 body weight loss amounted to 33% with a simultaneous shift of abdominal
564 subcutaneous fat cell size to lower diameter (5, 41). In these studies, a subcutaneous
565 fat biopsy was obtained from the abdominal wall. Fat cells were isolated through
566 collagenase treatment, and the diameter of 100 cells measured. The reduced average
567 fat cell size correlated strongly with improved insulin sensitivity ($p=0.0057$), whereas
568 body weight loss and fat mass loss did not (41, 100). The hypothesis that visceral
569 adipose tissue induces insulin resistance was dismissed since removal of the major
570 omentum, constituting a substantial portion of this tissue, during bariatric surgery

571 had no additive effect on insulin sensitivity (5). The mechanism behind normalized
572 fat cell biology after bariatric surgery is unknown, but may be due to reduced
573 inflammatory markers and increased adiponectin levels (41).
574
575 *Cholesterol-free diet*
576 In the obese state, half of the cholesterol pool in the body is stored in the adipose
577 tissue. With adipocyte hypertrophy there is an increased content of body cholesterol,
578 which is stored up to 90 % in the free form at the surface of the triglyceride droplet,
579 the remainder being part of the plasma membrane (90). Dietary cholesterol has been
580 demonstrated to aggravate adipose tissue inflammation in a mouse model of diet-
581 induced obesity, suggesting that high dietary cholesterol can lead to adipocyte
582 dysfunction (115). In monkeys, a better model for human physiology than rodents, a
583 high-cholesterol diet was demonstrated to induce the enlargement of fat cells (19). In
584 this model, the fat cell size was measured in fixed and embedded samples of omental
585 and subcutaneous adipose tissue, using light microscopy. It was found that in
586 omental fat, the mean diameter of the fat cells were 42.3 (±2.4) μm with low
587 cholesterol diet, and increased to 49.0 (±2.7) μm and and 58.9 (±3.1) μm for medium
588 and high cholesterol diets, respectively after 10 weeks (19). At the same time, there
589 was a parallel increase in plasma lipoprotein LDL levels, suggesting that the LDL
590 levels determined the size of the adipocytes. There was no change in adipocyte size
591 of subcutaneous adipose tissue with the medium and high cholesterol diet compared
592 to the low cholesterol diet. Also, a significant increase in adipose tissue expression of

593 the pro-inflammatory genes IL-6 and IL-8 in the high cholesterol diet group relative
594 to the other groups was observed. There was no difference in glucose and insulin
595 sensitivity, suggesting that 10 weeks of feeding the medium and high cholesterol
596 diets was perhaps not long enough to observe such changes (19). Hypertrophic
597 adipocytes have reduced plasma membrane cholesterol, with a ~40% reduction of
598 membrane cholesterol content (expressed as cholesterol/protein) comparing small
599 (~40 μm) and large (~50 μm) adipocytes, when studied by filtration (66). Since serum
600 amyloid A has been shown to reduce cholesterol uptake from HDL particles, an
601 altered cholesterol metabolism could be one of the mechanisms through which
602 serum amyloid A contributes to the insulin resistance reported in enlarged
603 adipocytes. Dietary cholesterol can thus induce visceral adipocyte enlargement,
604 which may explain metabolic dysfunction with high-cholesterol diets.
605
606 *Bioactive compounds*
607 The increasing world-wide prevalence of obesity is partially related to the ready
608 availability of highly palatable food, which increases the incidence of hedonic, non-
609 homeostatic eating. Any treatment that suppresses appetite, in particular appetite for
610 palatable food, will reduce the flow of nutrients to the fat cell. Thylakoids are the
611 green leaf membranes present in all chloroplasts, that transform energy from light to
612 ATP. Upon isolation, highly complex membrane proteins, galactolipids, vitamins
613 and antioxidants are the main components (23). These were found to suppress fat
614 digestion in the intestine exerted by pancreatic lipase/colipase, suppress food intake

615 and reduce body weight as demonstrated both in rodent and in human studies (23).
616 When given to mice fed a high-fat diet, fat cell size was reduced in the thylakoid-fed
617 animals compared to control high-fat fed animals, since some of the fat was retarded
618 in the intestine (114). Thus a reduced flow of nutrients from the intestine to the body
619 reduces fat cell size. Also, an increased energy expenditure leading to loss of energy
620 may reduce fat cell size. A couple of thermogenic compounds are known that lead to
621 body fat loss, without affecting appetite (7). One example of a thermogenic
622 compound is capsaicin, which administrated in combination with exercise led to a
623 decreased fat cell size in mice (84).
624
625 *Pharmaceutical treatment with PPAR-ligands*
626 Thiazolidinediones (TZDs), also called glitazones, are a class of drugs introduced in
627 the late 1990's that increase insulin sensitivity in muscle, fat and liver (103). TZDs
628 exert their effect by acting as synthetic ligands for PPARγ, a member of the nuclear
629 receptor superfamily of transcription factors. TZD promote binding of the PPARγ-
630 retinoid X receptor (RXR) complex to PPARγ response elements (PPRE) in target
631 genes that are involved in adipogenesis, lipid metabolism and glucose metabolism
632 (68). To date, two PPARγ isoforms have been identified, PPARγ1 and PPARγ 2.
633 PPARγ1 is ubiquitously expressed whereas PPARγ2 is mainly expressed in
634 adipocytes and intestine. In adipose tissue, PPARγ2 is the master regulator of
635 adipogenesis (95). PPARγ is not only essential for proper adipocyte differentiation
636 but is also required for function and survival of mature adipocytes in mice (44), even

637 though PPARγ silencing in differentiated 3T3-L1 cells only partially led to impaired
638 adipogenesis (105). Besides adipogenesis, PPARγ regulates gene expression of the
639 GLUT4 transporter that is essential for glucose metabolism, the c-Cbl-associated
640 protein (CAP) involved in insulin signaling, and several adipokines, including
641 adiponectin (31), IL-6 (63) and TNF-α (49). Thus, the insulin sensitizing effect
642 obtained by TZD treatment is multifactorial.
643
644 In humans, decreased insulin resistance after 12 weeks of pioglitazone treatment of
645 overweight/obese nondiabetic, insulin-resistant subjects was associated with an
646 increased proportion of small fat cells, and a 25% increase in the absolute number of
647 these cells, assessed by coulter counter measurements (80). In contrast, a significantly
648 increased subcutaneous adipocyte surface area was associated with improved
649 systemic insulin sensitivity (59). A trend towards an increased proportion of small
650 subcutaneous adipocytes (<50 μm) was shown after 2 months of TZD treatment in
651 type 2 diabetic subjects (13). In the latter study, TZD treatment also improved the
652 anti-lipolytic effect of insulin, and led to a 2.5-fold increase of plasma adiponectin
653 level and a 30% decrease in plasma leptin levels (13). There are, to the best of our
654 knowledge, no studies yet that have addressed the specific effect of TZD's on
655 adipocytes of different sizes. Still, the literature exploring the mechanisms of action
656 of TZD's supports the idea that the insulin sensitizing effect of TZDs, to a large
657 extent, can be explained by increasing the pool of small adipocytes preferentially in
658 the subcutaneous fat depots (4).

659

660 **Perspective and Significance**

661

662

663 The mechanism by which obesity contributes to dysmetabolism is three-fold: 1) an
664 inability of adipose precursor cells to differentiate into mature fat cells 2) an inability
665 of small fat cells to expand and 3) an inability of large fat cells to expand further.
666 Together, this leads to an impaired ability to store excess energy, whereby increased
667 circulating free fatty acids and adipokines cause disturbed systemic metabolism. The
668 picture of fat cell size should be considered dynamic, where the proportion of
669 various fat cell sizes should be measured in order to gain the greatest degree of
670 insight.

671

672 In agreement with earlier studies, hypertrophic adipocytes are associated with
673 impaired cell function and dysmetabolism. This has been documented through
674 dietary intervention studies, demonstrating that a reduced fat cell size following
675 weight reduction is coupled to improved insulin sensitivity. Whether the
676 hypothrophic fat cell regains its normal function per se or whether other factors
677 inherent in newly recruited fat cells are necessary to provide a healthy metabolism
678 needs to be investigated. Of specific interest will be to characterize the very small fat
679 cells, especially in terms of their capacity to expand, in order to understand their
680 relation to metabolic dysfunction.

681

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Figure Legends

Figure 1.

Different techniques used for characterization of adipose cell function according to size. The method to obtain isolated adipocytes in a single cell suspension from adipose tissue through collagenase digestion was established by Rodbell (93). Cell pools of different sizes can be collected by filtration of the cell suspension through nylon mesh of different pore size (27, 48), or by exploiting their differences in buoyancy (12, 25). The separated cell fractions can be further analyzed (RNA or protein expression, glucose uptake and other functional assays). Protocols to sort adipose cells by flow cytometer have been established (26, 75). Microscopy and patch-clamping techniques allow single cell analysis, even though they usually include tedious experimental handling and data processing (60, 128). Correlative studies often include histological cell size measurements of intact adipose tissue. The coulter counter technique is an alternative method to attain data of cell size distribution within a broad size range (40, 79).

Figure 2.

Precursor adipose cells are differentiated into mature adipocytes, which can expand significantly if needed, having a maximal size of 300 μm in diameter. The large and very large adipocytes are less metabolically favourable, with impaired insulin response (27), increased secretion of free fatty acids (FFA) (65) and pro-inflammatory cytokines (109), and decreased secretion of adiponectin (81); together contributing to

1103 a dysregulated systemic energy metabolism. Also, dysmetabolism could be caused
1104 by an impaired differentiation of the precursor cells (21, 30) in combination with an
1105 impaired ability to expand both small and large mature adipocytes (24, 78). The
1106 distribution of fat cell size hence is dynamic, and the proportion of various fat cell
1107 sizes should be considered in relation to health.
1108

Figure 1

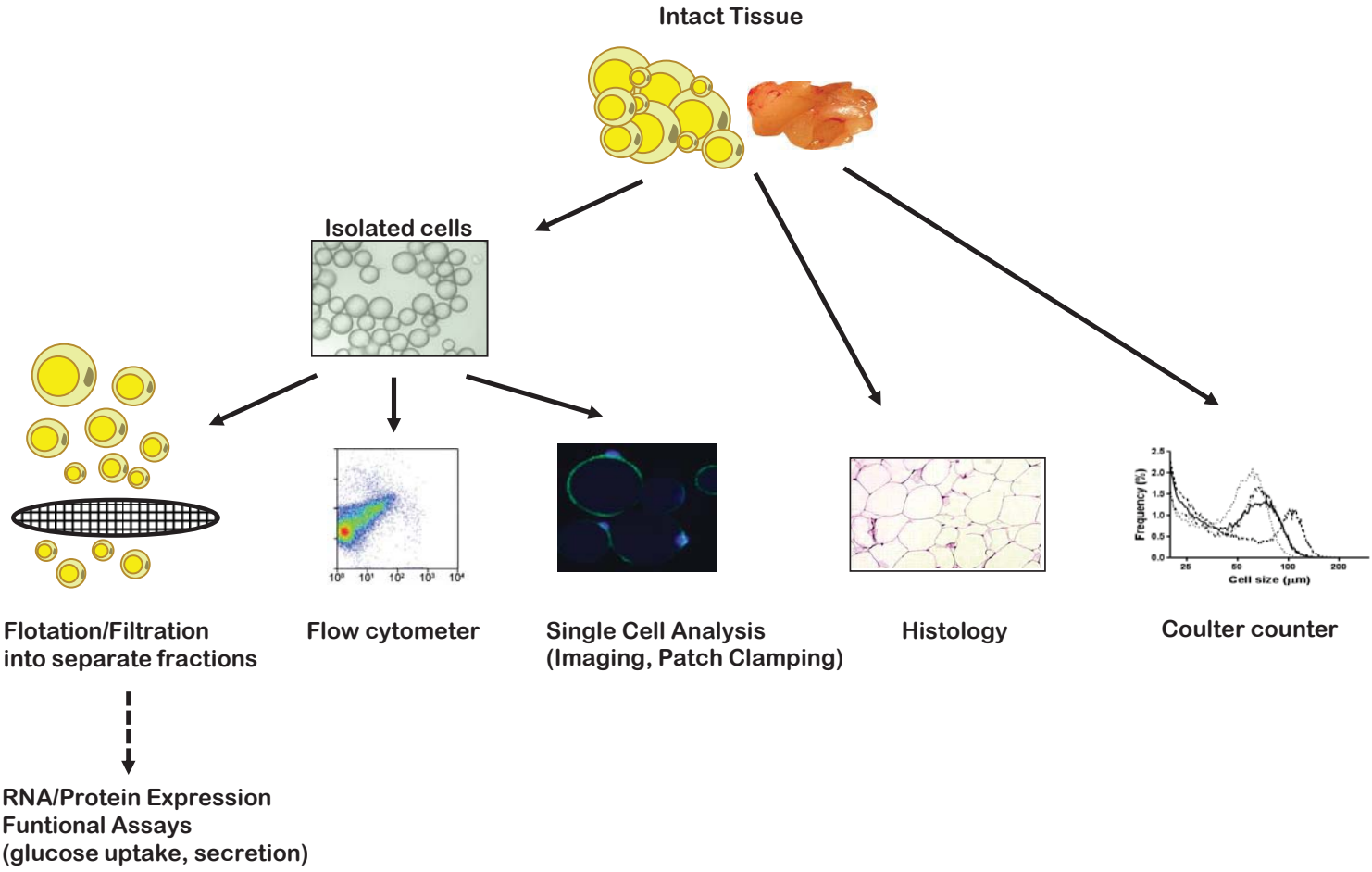

Figure 2