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Reduced SIRT1 and SIRT2 Expression Promotes Adipogenesis of Visceral Adipose Stem Cells in Human Obesity.

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Running title: SIRT and human adipose stem cells adipogenesis

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Abstract

Background/Objectives: The histone deacetylases SIRT1 and SIRT2 have been shown to be involved in the differentiation of rodent adipocyte precursors. In light of the differences in gene expression and metabolic function of visceral (V) and subcutaneous (S) adipose tissue (AT) and their resident cells, the aim of this study was to investigate the role of SIRT1 and SIRT2 in the differentiation of adipose stem cells (ASCs) isolated from SAT and VAT biopsies of non-diabetic subjects with varying levels of BMI.

Methods: Human ASCs were isolated from paired SAT and VAT biopsies obtained from 83 of non-diabetic subjects and 92 obese individuals.

Results: Visceral but not subcutaneous ASCs from obese subjects showed an intrinsic increase in both adipogenesis and lipid accumulation when compared to ASCs from non-obese subjects, and this was associated with reduced SIRT1 and SIRT2 mRNA and protein levels. Moreover, adipose tissue mRNA levels of *SIRT1* and *SIRT2* showed an inverse correlation with BMI in the visceral but not subcutaneous depot. Overexpression of *SIRT1* or *SIRT2* in visceral ASCs from obese subjects resulted in inhibition of adipocyte differentiation, whereas knockdown of *SIRT1* or *SIRT2* in visceral ASCs from non-obese subjects enhanced this process. Changes in *SIRT1* or *SIRT2* expression and adipocyte differentiation were paralleled by corresponding changes in *PPARG*, *CEBPA* and other genes marking terminal adipocyte differentiation.

Conclusions: These observations indicate that reduced SIRT1 and SIRT2 expression in visceral ASCs may promote visceral adipose tissue expansion in human obesity by enhancing the differentiation capacity of these adipocyte precursors.

Introduction

Obesity is a major risk factor for developing cardiometabolic disorders, including type 2 diabetes, dyslipidemia, hypertension and cardiovascular disease. It is widely accepted that the obesity-related cardiometabolic alterations are strongly associated with the expansion of visceral adipose tissue (VAT), whereas the subcutaneous adipose tissue (SAT) is viewed as neutral and may even be protective [1, 2]. Differences in anatomical location with different patterns of venous drainage and innervation only in part explain the adverse metabolic impact of VAT compared to SAT, that is likely due to the intrinsic characteristics of the VAT cells (3-6). We and others have shown that adipose-derived stem cells (ASCs) possess intrinsic depot-specific characteristics in terms of gene expression patterns, hormone signaling and metabolic features [3–7], but how this intrinsic diversity may contribute to the propensity to expansion of adipose tissue remains unclear. At a cellular level, both VAT and SAT expand during childhood and adulthood, as a consequence of hypertrophy of preexisting adipocytes due to triglyceride accumulation (lipogenesis) and adipocyte hyperplasia induced by recruitment and differentiation of ASCs (adipogenesis) [8, 9]. Spalding et al. estimated that the fat cell turnover varies greatly throughout life in humans, with a median rate of 8.4 ± 6.2% per year [8]. In addition, the number of new adipocytes added each year was reported to be 2-fold higher in obese compared to lean subjects [8], underlying the key role of adipose tissue turnover in younger as well as older obese individuals.

The family of enzymes known as Sir2-related proteins or sirtuins represents one of the leading targets controlling lipogenesis and adipogenesis [10]. Sirtuins are highly conserved NAD-dependent deacetylases and/or ADP ribosyl transferases that target histones, transcription factors, and co-regulators, to adapt gene expression and metabolic activity in response to changes in cellular energy state [10]. The founding members of the family, SIRT1 and SIRT2, have been implicated in the molecular control of lifespan, cell cycle, fatty acid oxidation in

the liver, nutrient availability in the hypothalamus and more recently in the protection from the metabolic syndrome [11]. SIRT1 and SIRT2 are expressed in the white adipose tissue and modulate adipogenesis by affecting the transcriptional activity of PPARG, the ligand-activated transcription factor that functions as the "master" regulator of this process [10]. In 3T3-L1 cells with overexpression or knockdown of *Sirt1,* adipogenesis was attenuated or enhanced, respectively [12]. Similarly, repression of PPARG by overexpression of *Sirt2* inhibited adipocyte differentiation, whereas reducing *Sirt2* levels had opposite effects [13]. Furthermore, in mice with adipocyte-specific *Sirt1* knockout exposed to high fat diet, an augmentation of adipose tissue mass occurred as a consequence of both increased adipocyte hypertrophy and hyperplasia [14]. Consistent with the role of SIRT2 as a negative modulator of adipogenesis and lipogenesis in 3T3-L1 cells, Bordone et al. showed that mice moderately overexpressing *Sirt2* were leaner than controls and more metabolically active, and displayed lower serum levels of cholesterol, adipokines, insulin and glucose [15]. However, as of today, the role of SIRT1 and SIRT2 in adipocyte differentiation has been studied only in rodent cells and mouse models, whereas information in human cells and in human obesity is very limited.

Considering the reported differences in gene expression and metabolic function of VAT and SAT and their resident cells, and the differential expansion of these two adipose tissue depots often observed in human obesity with individuals displaying prevalent accumulation of visceral vs. subcutaneous fat, it is reasonable to hypothesize that regulation of adipogenesis and lipogenesis may be different in VAT and SAT. Here, we investigated the role of SIRT1 and SIRT2 in human adipocyte differentiation by analyzing ASCs isolated from paired SAT and VAT biopsies of non-diabetic subjects with varying levels of BMI. We show that expression of SIRT1 and SIRT2 is reduced specifically in VAT, VAT ASCs and ASCs-derived adipocytes of obese subjects.

Materials (or Subjects) and Methods

Subjects. VAT and SAT biopsies were obtained from 83 non-obese subjects [50 males and 33 females; age, 53 ± 12.2 years; BMI, 25.1 ± 2.7 kg/m^2] who underwent elective laparoscopic cholecystectomy, and 92 obese subjects [27 males and 65 females; age, 50 ± 10.2 years; BMI, 40.5 ± 7.1 kg/m^2] who underwent laparoscopic sleeve gastrectomy. Subjects affected by diabetes or severe systemic illness and who were on medications known to affect adipose tissue mass or glucose metabolism were considered ineligible. The protocol was approved by the Independent Ethics Committee at the Azienda Ospedaliero Universitaria Policlinico Consorziale, University of Bari School of Medicine (approval no. 152/2012) and was conducted according to the principles expressed in the Declaration of Helsinki. The nature and potential risks of the study were explained to all subjects before obtaining their written informed consent.

Isolation, cell culture and adipogenesis of ASCs. Paired human VAT and SAT biopsies were processed, as previously reported [4, 5], in order to obtain ASCs. The ASCs were grown and differentiated into adipocytes as previously published [4, 16, 17].

Analysis of lipid droplets number, Oil Red O and Nile Red staining. Ten digital images (20x magnification) from non-overlapping fields were acquired from each slide, and the number of lipid droplets was calculated using Image J software [18]. Oil Red O and Nile Red staining was performed as previously described (4,16,17).

Gene expression analysis by quantitative RT-PCR. RNA isolation from cells and whole adipose tissue, RNA quantitation and cDNA synthesis were carried out as previously described [4, 5, 19]. Specific primers, shown in electronic supplementary material [ESM] Table 1, were designed using the Primer Express 3.0 program (from Applied Biosystems, Foster City, CA, USA) and purchased from PRIMM (Milan, Italy) and Eurofins Genomics

(Ebersberg, Germany).

Immunoblotting. Cells lysates were obtained and analyzed by immunoblotting as previously described [4, 16]. The list of the antibodies used is shown in ESM Table 2.

Adenoviral infection. Recombinant adenoviruses encoding *SIRT1* or *SIRT2* cDNA for overexpression (Ad*SIRT1*, Ad*SIRT2*) or short hairpin RNA to *SIRT1* or *SIRT2* for knockdown (Ad*SIRT1*sh, Ad*SIRT2*sh) were obtained from Vector Biolabs (Malvern, PA, USA). An empty adenovirus (Ad-GFP), used as control for the infection, was obtained as previously described (16,20). Transduction of ASCs with adenoviruses was carried out according to previously reported procedures [16, 20]. Gene and protein overexpression and knockdown were confirmed by qRT-PCR and immunoblotting, respectively.

Statistical analysis. Data were analyzed by the Student's t test if comparing a single variable, ANOVA test if comparing multiple variables or Pearson correlation analysis if measuring linear correlation between two variables, and are presented as mean ± SD. All statistical analyses were carried out using the Minitab 16.0 Statistical Software (from Minitab Inc., State College, PA, USA) considering a p value <0.05 as statistically significant.

Results

Baseline characteristics of the study participants.

Between May 2012 and March 2017 more than 250 subjects were screened. Eligible subjects (83 non-obese and 92 obese) were enrolled in the study and underwent VAT and SAT biopsies. The clinical characteristics of the experimental subjects are shown in Table 1. As expected, BMI, waist circumference, fasting insulin, insulin resistance index and fasting triglycerides were significantly higher in obese subjects compared with non-obese subjects ($p<0.05$ vs. non-obese; Table 1). By contrast, age, plasma fasting glucose, cholesterol and inflammatory parameters were not different between the two groups (ESM Table 3).

Lipogenesis and adipogenesis of human ASCs.

To examine lipogenesis and adipogenesis, paired SAT- and VAT-derived ASCs were obtained from non-obese and obese subjects and differentiated into mature adipocytes. Lipid content, total number of cells accumulating lipids, and characteristics of lipid droplets were assessed in the cell cultures. No differences in lipid accumulation ($p = 0.53$) and proportion of new adipocytes ($p = 0.83$) assessed by Oil Red O and Nile Red staining, respectively, were observed between SAT ASCs from non-obese compared to obese subjects (Fig. 1a and c). Similarly, lipid droplet count was similar in SAT ASCs from non-obese and obese subjects ($p = 0.45$; Fig. 1a and c). In contrast, lipid accumulation was 2-fold higher in VAT ASCs from obese compared to non-obese subjects when differentiated into mature adipocytes (*$p<0.05$ vs. non-obese; Fig. 1b). The greater lipid staining intensity observed in these VAT cells from obese vs. non-obese subjects was accounted for by an increased lipid droplet number (*$p<0.05$ vs. non-obese; Fig. 1b) rather than lipid droplet size (data not shown). Consistently, the percentage of new adipocytes differentiated *in vitro* was 2-fold higher in VAT ASCs isolated from obese compared to non-obese subjects (*$p<0.05$ vs. non-obese subjects; Fig.

1d). Furthermore, as showed in multi-group comparison analysis, both the rate of total lipid accumulation and estimated number of mature adipocytes were significantly higher in SAT than VAT ASCs (§$p<0.05$; ESM Fig. 1), however only in VAT ASCs the lipogenic and adipogenic potential was found to be augmented in obesity (Fig. 1 and ESM Fig. 1). Since insulin is an important component of the differentiation medium to which ASCs are exposed, insulin signaling was also assessed by measuring AKT phosphorylation. However, both at baseline and after insulin stimulation, the levels of AKT phosphorylation did not show any significant difference in SAT ($p = 0.90$) or VAT ASCs ($p = 0.95$) from non-obese compared to obese subjects (ESM Fig. 2).

Expression of SIRT1 and SIRT2 in SAT and VAT.

To identify genes that could potentially contribute to the observed differences in the lipogenic/adipogenic potential, the expression levels of SIRT1 and SIRT2, which are reportedly involved in the modulation of murine adipogenesis [12, 13], were assessed next. mRNA and protein levels of SIRT1 and SIRT2 were not different in SAT ASCs and ASCs-derived mature adipocytes from non-obese and obese subjects (*SIRT1*: $p = 0.8$ in ASCs; $p = 0.1$ in adipocytes. *SIRT2*: $p = 1.0$ in ASCs; $p = 0.2$ in adipocytes. SIRT1: $p = 0.82$ in ASCs; $p = 0.48$ in adipocytes. SIRT2: $p = 0.79$ in ASCs; $p = 0.06$ in adipocytes. Fig. 2a and c). In contrast, a significant reduction of SIRT1 and SIRT2 mRNA and protein levels was observed both in VAT ASCs and ASCs-derived mature adipocytes from obese subjects compared to non-obese controls (*$p<0.05$ vs. non-obese; **$p<0.01$ vs. non-obese; Fig. 2b and d).

Because reduced expression of SIRT1 and SIRT2 was apparently associated with enhanced lipogenic and adipogenic behavior of VAT ASCs from the obese individuals, and this change persisted in adipocytes differentiated *in vitro*, we investigated the potential inverse correlation between SIRT1 and SIRT2 expression in SAT and VAT and the extent of overweight and

abdominal fat accumulation *in vivo* in subjects with a wide BMI range, from 19 to 52 kg/m^2 (Fig. 3). No correlation was observed between *SIRT1* or *SIRT2* mRNA levels in SAT and BMI or waist circumference, either in male or female subjects (Fig. 3a). By contrast, in both male and female subjects there was a negative correlation between *SIRT1* and *SIRT2* mRNA levels in VAT and the BMI (*SIRT1*: r = -0.352, p = 0.001 for all; r = -0.463, p = 0.004 for men; r = -0.305, p = 0.031 for women) (*SIRT2*: r = -0.365, p = 0.0001 for all; = r = -0.456, p = 0.004 for men; r = -0.341, p = 0.013 for women), as well as between *SIRT1* and *SIRT2* mRNA levels in VAT and the waist circumference (*SIRT1*: r = -0.389, p = 0.0001 for all; r = -0.443, p = 0.008 for men; r = -0.374, p = 0.010 for women) (*SIRT2*: r = -0.458, $p = 4.70*10^{-6}$ for all; r = -0.520, p = 0.001 for men; r = -0.438, p = 0.002 for women) (Fig. 3b). Thus, reduced expression of SIRT1 and SIRT2 can be observed in ASCs, differentiated adipocytes and whole adipose tissue specifically of the visceral depot, and this is associated with increased adipocyte differentiation assessed *in vitro* and abdominal fat accumulation *in vivo*.

Modulation of *SIRT1* and *SIRT2* expression in VAT ASCs and adipocyte differentiation.

Next, SIRT1 and SIRT2 protein levels were increased in VAT ASCs from obese subjects using recombinant adenoviruses (Ad*SIRT1* and Ad*SIRT2* to overexpress SIRT1 and SIRT2 proteins, respectively) to assess whether this would restrict the enhanced lipogenic and adipogenic potential of these cells (Fig. 1b and d). Following transduction of VAT ASCs with Ad*SIRT1* or Ad*SIRT2*, SIRT1 or SIRT2 protein levels were increased approximately 2.5-fold (*p<0.05 vs. non-infected ASCs; ESM Fig. 3a and b). The VAT ASCs with forced expression of SIRT1 or SIRT2 were then exposed to the adipogenic induction medium and followed-up until terminal differentiation. Notably, in Ad*SIRT1* ASCs and Ad*SIRT2* ASCs, the mRNA and protein levels of SIRT1 and SIRT2 were increased approximately 2-fold, respectively, during most of the differentiation process (*p<0.05 vs. Ad*GFP* ASCs; **p<0.01 vs. Ad*GFP* ASCs;

Fig. 5b and c, Fig. 6b and c), and this change was specific since it did not involve the alternate sirtuin molecule (Fig. 5b, Fig. 6b).

The number of lipid droplets, extent of lipid accumulation and rate of adipocyte conversion, respectively, were significantly reduced by approximately 50% in the VAT ASCs from obese subjects overexpressing *SIRT1* as compared to control cells transduced with Ad*GFP* (*p<0.05 vs. Ad*GFP* ASCs; **p<0.01 vs. Ad*GFP* ASCs; Fig. 4a and Fig. 5a). In addition, SIRT1 overexpression prevented the induction of *PPARG2* and *CEBPA* expression, two key transcription factors for adipocyte differentiation. Specifically, *PPARG2* mRNA levels were significantly reduced by 50% at day 16 and 80% at day 30, respectively, during adipogenesis induction, and *CEBPA* mRNA levels showed a slight decrease at day 16 and a more evident 70% reduction at day 30 in Ad*SIRT1* compared to Ad*GFP* ASCs (*p<0.05 vs. Ad*GFP* ASCs; Fig. 5b). Moreover, protein expression of SIRT1 was also decreased by approximately 2-fold at days 16 and 30 following the induction of adipogenesis (*p<0.05 vs. Ad*GFP* ASCs; Fig. 5c). Similarly, overexpression of *SIRT1* also hindered the induction of genes marking terminal adipocyte differentiation, which are controlled by *PPARG2* and *CEBPA*, since mRNA levels of *SREBF1C*, *FASN*, *ADIPOQ* and *SLC2A4* were markedly lower at days 16 and 30, respectively, in Ad*SIRT1* compared to Ad*GFP* ASCs (*p<0.05 vs. Ad*GFP* ASCs; Fig. 5b).

Similar effects were observed in VAT ASCs from obese subjects following *SIRT2* overexpression (Fig. 4b and Fig. 6a-c). Overexpression of *SIRT2* reduced lipid droplet number and attenuated lipid accumulation and adipocytes conversion by approximately 50% (*p<0.05 vs. Ad*GFP* ASCs; **p<0.01 vs. Ad*GFP* ASCs; Fig. 4b and Fig. 6a), and also prevented the proper induction of early and late markers of adipogenesis, including *PPARG2*, *CEBPA*, *SREBF1C*, *FASN*, *ADIPOQ* and *SLC2A4*, with reduced mRNA levels of these genes at day 16 and especially at day 30 of differentiation, when compared with control ASCs (*p<0.05 vs. Ad*GFP* ASCs; Fig. 6b). Altogether, these results show that reduced expression

of SIRT1 and SIRT2 plays a causal role in promoting lipogenesis and adipogenesis in VAT ASCs from obese subjects.

Finally, an attempt was made to recapitulate the adipogenic phenotype of obese VAT ASCs in in control VAT ASCs from non-obese donors. To this purpose, *SIRT1* or *SIRT2* mRNA levels were selectively diminished in VAT ASCs from non-obese subjects using recombinant adenoviruses encoding human *SIRT1* or *SIRT2* shRNA (Ad*SIRT1*sh or Ad*SIRT2*sh). Infection of VAT ASCs with Ad*SIRT1*sh or Ad*SIRT2*sh produced an average 50% reduction in SIRT1 or SIRT2 protein expression, respectively, compared to control (**p*<0.05 vs. non-infected VAT ASCs¸ ESM Fig. 3c). With this approach, it was possible to achieve persistent knockdown of *SIRT1* or *SIRT2* expression in these VAT ASCs during differentiation (**p*<0.05 vs. Ad*GFP* ASCs; ***p*<0.01 vs. Ad*GFP* ASCs; Fig. 5e and f, Fig. 6e and f), with specificity over the alternate sirtuin molecule (Fig. 5e, Fig. 6e).

As hypothesized, VAT ASCs isolated from non-obese subjects, when exhibiting *SIRT1* knockdown, showed a significant 30-50% increase in lipid droplet number, lipid content and adipocyte conversion (**p*<0.05 vs. Ad*GFP* ASCs; ***p*<0.01 vs. Ad*GFP* ASCs; Fig. 4c and Fig. 5d), as well as higher mRNA levels of *PPARG2*, *SREBF1C*, *FASN*, *ADIPOQ* and *SLC2A4* during adipocyte differentiation, when compared with control cells (**p*<0.05 vs. Ad*GFP* ASCs; Fig. 5e). Similarly, 35-50% greater lipid droplet count, lipid accumulation and adipocyte conversion rate were observed in VAT ASCs from non-obese subjects with knockdown of *SIRT2* compared to control cells (**p*<0.05 vs. Ad*GFP* ASCs; ***p*<0.01 vs. Ad*GFP* ASCs; Fig. 4d and Fig. 6d). The VAT ASCs, upon downregulation of *SIRT2*, also showed increased *PPARG2*, *SREBF1C*, *FASN*, *ADIPOQ* and *SLC2A4* expression during adipogenesis, particularly at day 30, compared to control cells (**p*<0.05 vs. Ad*GFP* ASCs; Fig. 6e and f). Thus, downregulation of *SIRT1* and *SIRT2* drives fat storage and white adipocyte differentiation in control VAT ASCs.

Discussion

The key role of SIRT1 and SIRT2 in metabolic disorders has been shown by multiple studies demonstrating an association between reduced SIRT1 [21–24] and SIRT2 [25, 26] expression and obesity and/or type 2 diabetes. Here we extend these results, showing that SIRT1 and SIRT2 expression is specifically downregulated in the VAT in humans and inversely correlated with BMI and waist circumference. The observed obesity-related reduction of SIRT1/2 in VAT was found to be associated with reduced mRNA and protein levels of SIRT1 and SIRT2 also in VAT ASCs, and these cells displayed increased adipogenic potential with augmented rates of triglyceride accumulation, number of lipid droplets and capacity to generate new adipocytes *in vitro*. Moreover, stably forced expression of *SIRT1* or *SIRT2* in VAT ASCs from obese individuals restricted their adipogenic potential, highlighting a causal role of the reduction of SIRT1 and SIRT2 levels in the observed obese adipocyte phenotype. By contrast, in SAT, *SIRT1* and *SIRT2* mRNA levels were not correlated with either BMI or waist circumference, and the resident ASCs did not show any changes in expression levels of SIRT1 and SIRT2 or lipogenesis/adipogenesis rates in obesity. These findings support previous observations in mice that identified SIRT1 and SIRT2 as important regulators of adipogenesis [12, 13].

In this study, ASCs from SAT were more prone to differentiate into mature adipocytes than ASCs from VAT, even though the proportion of differentiated SAT ASCs was not enhanced in obesity (Fig. 1 and ESM Fig. 1). These results are in line with previous studies, showing that SAT ASCs differentiate into mature adipocytes more readily than their VAT counterparts [6, 27, 28]. On the other hand, the lack of obesity-related changes in the SAT ASCs is apparently in contrast with the results of other investigators, who reported a negative correlations between the BMI and differentiation capacity of SAT preadipocytes [29, 30]. However, Landgraf et al. showed that the percentage of differentiated preadipocytes from the

SAT was not different in obese compared with lean children [31], in line with the results of our study in adult subjects. Indeed, few studies have explored the relationship between the degree of obesity and *in vitro* differentiation capacity of ASCs by comparatively assessing ASCs from paired SAT and VAT biopsies [29, 30, 32]. Other studies have also suggested that the limited expandability of SAT in obese subjects could be genetically determined and related to abdominal fat distribution. For instance, in obese adolescents with high VAT/SAT ratio, the lack of SAT expandability, assessed by measuring the *in vitro* preadipocyte adipogenic rate, was found to be associated with the extent of VAT accumulation [33]. In line with these data, Wang et al., showed that mice undergoing a prolonged exposure to a high fat diet (HFD) developed a high capacity for adipogenesis in VAT, whereas SAT mass maintained an extremely low rate of adipogenesis [34]. Similarly, in *Sirt1* adipocyte-specific knockout mice at more prolonged stages of HFD, only the epididymal fat retained adipogenic capacity related specifically to its ability to undergo hyperplasia, which was not seen in SAT [14]. Altogether, these results suggest the possibility that nutritional overload may enhance the expandability of VAT specifically over SAT, and that this may occur via repression of SIRT1/2 expression in the resident ASCs.

It is of note that reduced levels of both SIRT1 and SIRT2 were found not only in ASCs but also in *in vitro* differentiated adipocytes (Fig. 2), indicating persistent, possibly epigenetic regulatory effects on *SIRT1* and *SIRT2* genes in human obesity. Indeed, inflammatory changes associated with obesity may affect the activity and expression of SIRT1/2 [35]. For example, inflammatory factors, such as lipopolysaccharide, collagen, interferon-γ and tumour necrosis factor-α, have been shown to reduce SIRT1/2 expression, and thereby leading to decreased anti-inflammatory effects mediated by these histone deacetylases (36,37).

The ability of *Sirt1* or *Sirt2* overexpression to limit adipocyte differentiation is well-established in mouse cells and animal models [12, 13]. In line with these studies, in VAT

ASCs from the obese subjects, stable overexpression of *SIRT1* or *SIRT2* inhibited adipocyte differentiation and was also associated with reduced expression of early and late transcriptional factors or markers of adipogenic differentiation, such as *CEBPA*, *PPARG2*, *SCL2A4*, *ADIPOQ*, *FASN* and *SREBF1C* (Figs. 5 and 6). This is consistent with earlier findings in rodent preadipocytes showing that both SIRT1 and SIRT2 act upstream of CEBPA and PPARG at a very early step controlling adipogenesis. Upregulation of *Sirt1* was shown to trigger lipolysis and loss of fat by inhibiting the nuclear receptor PPARG and attenuating the PPAR response to an exogenous ligand [12, 38]. Similarly to *Sirt1*, overexpression of *Sirt2* has been shown to suppress adipogenesis by increased level of *Foxo1* deacetylation, which in turn increases the ability of FOXO1 to bind *Pparg* promoter and repress *Pparg* transcriptional activity [13, 39]. During 3T3-L1 adipocyte differentiation, CEBPB is induced early to transactivate the expression of CEBPA and PPARG [40]. In addition, disruption of the *Cebpb* gene in mice caused decreased fat mass because of impaired adipose tissue development [40, 41]. Thus, based on this model, it is not surprising that overexpression of SIRT1 and SIRT2 in the VAT ASCs led to decreased expression of PPARG and CEBPA and restricted the capability of these ASCs to differentiate into mature adipocytes, and it is of interest that such an effect is being observed for the first time in cells from obese donors. This suggests that obesity-related changes in adipogenic potential largely rely on reduced SIRT1/2 expression and can thus be corrected by augmenting the cellular levels of these histone deacetylases. On the other hand, an increase in adipogenic potential of the VAT ASCs, typical of the obesity-associated phenotype, can be achieved by knocking down SIRT1 or SIRT2, as shown in cells from non-obese donors, in which *PPARG2*, *CEBPA* and the typical marker genes of mature adipocytes were also more prominently induced (Fig. 5).

Wnt signaling has reportedly been shown to regulate adipogenesis and adipocyte function [42]. Secreted frizzled-related proteins (SFRPs) are a family of secreted proteins (SFRP1-5)

that bind and inhibit Wnt and have been also implicated in adipogenic differentiation [43]. When comparing non-obese and obese subjects, 4 of 5 SFRP family members were found to be differentially expressed in human white adipose tissue [44]. Interestingly, in obese individuals, SFRP4 expression was significantly increased in VAT but not in SAT, and this was positively correlated with BMI and insulin resistance [44]. More recently, *Sfrp4* knockdown in preadipocytes isolated from epididymal white adipose tissue of C57BL/6J mice was shown to reduce lipid accumulation and adipocyte differentiation in association with diminished mRNA levels of adipogenic markers, including PPARG and SLC2A4 [45]. Since SIRT1 deacetylates SFRPs and represses their expression thus activating Wnt signaling and suppressing adipogenesis [46], it can be postulated that reduced levels of SIRT1/2 in visceral ASC from obese subjects may increase SFRP4 levels, allowing early induction of adipogenic transcription factors and fostering lipogenesis and adipogenesis in VAT.

Apparently, the effects of SIRT1 and SIRT2 were similar in adipogenesis. To date, potential biological differences in these two sirtuin molecules have not been reported. Moreover, the potential additive or synergic effects of overexpression (or knockdown) of both SIRT1 and SIRT2 on VAT ASCs adipogenic capability could not be investigated, since infection of ASCs with two adenoviral constructs significantly impaired their viability and survival (data not shown).

In conclusion, we show that reduced SIRT1 and SIRT2 expression is a key event in adipose tissue, which occurs in a depot-specific and obesity-related manner and is tightly linked to increase adipogenic differentiation of ASCs. Thus, SIRT1 and SIRT2 are involved in VAT expandability in humans and could be considered as useful therapeutic targets.

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Data availability The authors declare that all data supporting the findings of this study are available within the article and its supplementary information files.

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Author contributions S.P. and S. Porro were responsible for recruiting and characterization of subjects, designed experiments, analyzed data, and wrote the paper. P.N, A.C., C.C, and V.A.G performed experiments and analyzed data; G.M., M.DF. and P.C. performed the adipose tissue biopsies; A.N. and L.L. analyzed data and discussed the manuscript. F.G. was responsible for recruiting and characterization of subjects, designed experiments, analyzed data, and wrote the paper. All authors discussed the results and implications and commented on the manuscript at all stages. F.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figures and tables legends

Figure 1. Markers of lipogenesis and adipogenesis of adipose stem cells (ASCs) isolated from the subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) of non-obese and obese subjects and differentiated *in vitro*, as described in the Research Design and Methods. a, b: *Top Left and Right.* Lipid accumulation in cells assessed by Oil Red O staining. Following staining with Oil Red O, cell extracts were quantified by spectrophotometric analysis measuring optical density at 510 nM; data from non-obese (*white bars*) and obese (*black bars*) subjects are shown (n = 10/group). Representative microscopy images of Oil Red O staining of cell cultures are presented. a, b: *Bottom Left and Right.* Representative microscopy images of lipid droplets are shown. Magnification was 10x. Quantitation of lipid droplet number by morphometric analysis was carried out in cells from non-obese (*white bars*) and obese (*black bars*) subjects. For each cell culture, 10 random microscopic fields were analyzed. The number of samples (n) was as follows: SAT ASCs, non-Obese = 10 and Obese = 9; VAT ASCs, non-Obese = 9 and Obese = 12. c, d: Quantitation of adipocyte conversion of fixed cells evaluated by Nile Red staining. The number of samples (n) was as follows: SAT ASCs, non-Obese (n) = 9; Obese (n) = 10. VAT ASCs, non-Obese (n) = 9; Obese (n) = 10. Representative images of fluorescence microscopy of cell cultures using DAPI and Nile Red staining are shown in the bottom. Magnification 10x. *$p<0.05$ vs. non-obese subjects (Student's *t* test).

Figure 2. SIRT1 and SIRT2 mRNA and protein levels in ASCs isolated from the SAT and VAT of non-obese and obese subjects and in ASCs-derived adipocytes. a and b: quantitation of mRNA levels of *SIRT1* and *SIRT2* in SAT ASCs (*left*) and VAT ASCs (*right*) and in ASCs-derived adipocytes differentiated *in vitro*, as described in the Research Design and Methods. ASCs were isolated from non-obese (*white bars*) and obese (*black bars*) subjects. *RPS18* was used as an endogenous reference gene. The number of samples (n) in a was as

follows: *SIRT1* in SAT ASCs, non-Obese = 14 and Obese = 20; *SIRT2* in SAT ASCs, non-Obese = 14 and Obese = 20; *SIRT1* in SAT adipocytes, non-Obese = 10 and Obese = 11; *SIRT2* in SAT adipocytes, non-Obese = 9 and Obese = 11. The number of samples (n) in b was as follows: *SIRT1* in VAT ASCs, non-Obese = 11 and Obese = 13; *SIRT2* in VAT ASCs, non-Obese = 10 and Obese = 13; *SIRT1* in VAT adipocytes, non-Obese = 8 and Obese = 7; *SIRT2* in VAT adipocytes, non-Obese = 7 and Obese = 6. c and d: Representative immunoblots and the quantitation of SIRT1 and SIRT2 protein levels in SAT ASCs (*left*) and VAT ASCs (*right*) and in ASCs-derived adipocytes differentiated *in vitro*. ASCs were isolated from non-obese (*white bars*) and obese (*black bars*) subjects. Data are presented as relative expression of proteins normalized to ACTB. The number of samples (n) in c was as follows: SIRT1 in SAT ASCs, non-Obese = 9 and Obese = 10; SIRT2 in SAT ASCs, non-Obese = 7 and Obese = 7; SIRT1 in SAT adipocytes, non-Obese = 8 and Obese = 8; SIRT2 in SAT adipocytes, non-Obese = 8 and Obese = 8. The number of samples (n) in d was as follows: SIRT1 in VAT ASCs, non-Obese = 8 and Obese = 11; SIRT2 in VAT ASCs, non-Obese = 6 and Obese = 8; SIRT1 in VAT adipocytes, non-Obese = 8 and Obese = 8; SIRT2 in VAT adipocytes, non-Obese = 6 and Obese = 6. *$p<0.05$, **$p<0.01$ vs. non-obese subjects; §$p<0.05$ vs. undifferentiated ASCs (Student's *t* test).

Figure 3. mRNA levels of *SIRT1* and *SIRT2* in SAT and VAT and correlation with BMI and waist circumference. a: mRNA levels of *SIRT1* (*left*) and *SIRT2* (*right*) in SAT, in women (*white circles*) (n = 49) and men (*black circles*) (n = 44). b: mRNA levels of *SIRT1* (*left*) and *SIRT2* (*right*) in VAT, in women (*white circles*) (n = 49) and men (*black circles*) (n = 44). *RPS18* was used as an endogenous reference gene. Associations were determined using Pearson correlation analysis; *r* and *p* values for the cumulative cohort (all), and separately for men and women are shown in the individual panels.

Figure 4. Effects of *SIRT1* and *SIRT2* overexpression and knockdown on adipogenesis. a and b: VAT ASCs from obese subjects were transduced with Ad*SIRT1* ($9.3x10^7$ PFU/µL for 24 h) or Ad*SIRT2* ($7.5x10^7$ PFU/µL for 24 h), and then induced to differentiate into adipocytes, as described in the Research Design and Methods (n = 8 independent experiments). c and d: VAT ASCs from non-obese subjects were transduced with Ad*SIRT1*sh ($4.8x10^7$ PFU/µL for 24 h) or Ad*SIRT2*sh ($3.3x10^7$ PFU/µL for 24 h), and then induced to differentiate into adipocytes, as described in the Research Design and Methods (n = 8 independent experiments). ASCs transduced with Ad*GFP* were used as control. Cells were analyzed by optical and fluorescence microscopy, and lipid droplet number, lipid amount and adipocytes conversion rates were measured. Representative microscopy images of lipid droplets and Oil Red O staining of cell cultures, and images of fluorescence microscopy using DAPI and Nile Red staining are also shown. Magnification 10x *p<0.05 vs. Ad*GFP* ASCs; **p<0.01 vs. Ad*GFP* ASCs (Student's *t* test).

Figure 5. Effects of *SIRT1* overexpression and knockdown on genes involved in adipocyte differentiation. VAT ASCs from obese subjects or non-obese subjects were transduced with $9.3x10^7$ PFU/µL of Ad*SIRT1* or $4.8x10^7$ PFU/µL of Ad*SIRT1*sh for 24 h to overexpress or knockdown *SIRT1*, respectively, and then induced to differentiate into adipocytes, as described in the Research Design and Methods. ASCs transduced with Ad*GFP* were used as control. a: Effects of *SIRT1* overexpression in VAT ASCs from obese subjects on adipocyte differentiation (n = 8 independent experiments). Cells were stained with Oil Red O. b: Effects of *SIRT1* overexpression in VAT ASCs from obese subjects on mRNA levels of *SIRT1*, *SIRT2*, *PPARG2*, *CEBPA*, *FASN*, *SLC2A4*, *ADIPOQ* and *SREBF1C* at different times of adipocyte differentiation. c: Effects of *SIRT1* overexpression in VAT ASCs from obese subjects on SIRT1 and PPARG protein levels at different times of adipocyte differentiation. Representative immunoblots and quantitation of n = 8 independent experiments are shown. d:

Effects of *SIRT1* knockdown in VAT ASCs from non-obese subjects on adipocyte differentiation (n = 8 independent experiments). Cells were stained with Oil Red O. e: Effects of *SIRT1* knockdown in VAT ASCs from non-obese subjects on mRNA levels of *SIRT1*, *SIRT2*, *PPARG2*, *CEBPA, FASN, SLC2A4, ADIPOQ* and *SREBF1C* at different times of adipocyte differentiation. f: Effects of *SIRT1* knockdown in VAT ASCs from non-obese subjects on SIRT1 and PPARG protein levels at different times of adipocyte differentiation. Representative immunoblots and quantitation of n = 8 independent experiments are shown. Ad*SIRT1* and Ad*SIRT1*sh cells are indicated as *solid squares*, Ad*GFP* cells as *empty squares*. **p*<0.05 vs. Ad*GFP* ASCs; ***p*<0.01 vs. Ad*GFP* ASCs (Student's *t* test).

Figure 6. Effects of *SIRT2* overexpression and knockdown on genes involved in adipocyte differentiation. VAT ASCs from obese subjects or non-obese subjects were transduced with $7.5x10^7$ PFU/µL of Ad*SIRT2* or $3.3x10^7$ PFU/µL of Ad*SIRT2*sh for 24 h to overexpress or knockdown *SIRT2*, respectively, and then induced to differentiate into adipocytes, as described in the Research Design and Methods. ASCs transduced with Ad*GFP* were used as control. a: Effects of *SIRT2* overexpression in VAT ASCs from obese subjects on adipocyte differentiation (n = 8 independent experiments). Cells were stained with Oil Red O. b: Effects of *SIRT2* overexpression in VAT ASCs from obese subjects on mRNA levels of *SIRT1*, *SIRT2*, *PPARG2*, *CEBPA, FASN, SLC2A4, ADIPOQ* and *SREBF1C* at different times of adipocyte differentiation. c: Effects of *SIRT2* overexpression in VAT ASCs from obese subjects on SIRT2 and PPARG protein levels at different times of adipocyte differentiation. Representative immunoblots and quantitation of n = 8 independent experiments are shown. d: Effects of *SIRT2* knockdown in VAT ASCs from non-obese subjects on adipocyte differentiation (n = 8 independent experiments). Cells were stained with Oil Red O. e: Effects of *SIRT2* knockdown in VAT ASCs from non-obese subjects on mRNA levels of *SIRT1*, *SIRT2, PPARG2, CEBPA, FASN, SLC2A4, ADIPOQ* and *SREBF1C* at different times of

adipocyte differentiation. f: Effects of *SIRT2* knockdown in VAT ASCs from non-obese subjects on SIRT2 and PPARG protein levels at different times of adipocyte differentiation. Representative immunoblots and quantitation of n = 8 independent experiments are shown. Ad*SIRT2* and Ad*SIRT2*sh cells are indicated as *solid squares*, Ad*GFP* cells as *empty squares*. **p*<0.05 vs. Ad*GFP* ASCs; ***p*<0.01 vs. Ad*GFP* ASCs (Student's *t* test).