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Stem cells isolated from adipose tissue of obese patients show changes in their transcriptomic profile that indicate loss in stemcellness and increased commitment to an adipocyte-like phenotype

Blanca Oñate¹, Gemma Vilahur¹, Sandra Camino-López¹, Alberto Díez-Caballero², Carlos Ballesta-López³, Juan Ybarra⁴, Fabrizio Moscatiello⁵, Javier Herrero⁵ and Lina Badimon^{1*}

Abstract

Background: The adipose tissue is an endocrine regulator and a risk factor for atherosclerosis and cardiovascular disease when by excessive accumulation induces obesity. Although the adipose tissue is also a reservoir for stem cells (ASC) their function and “stemcellness” has been questioned. Our aim was to investigate the mechanisms by which obesity affects subcutaneous white adipose tissue (WAT) stem cells.

Results: Transcriptomics, in silico analysis, real-time polymerase chain reaction (PCR) and western blots were performed on isolated stem cells from subcutaneous abdominal WAT of morbidly obese patients (ASCmo) and of non-obese individuals (ASCn). ASCmo and ASCn gene expression clustered separately from each other. ASCmo showed downregulation of “stemness” genes and upregulation of adipogenic and inflammatory genes with respect to ASCn. Moreover, the application of bioinformatics and Ingenuity Pathway Analysis (IPA) showed that the transcription factor Smad3 was tentatively affected in obese ASCmo. Validation of this target confirmed a significantly reduced Smad3 nuclear translocation in the isolated ASCmo.

Conclusions: The transcriptomic profile of the stem cells reservoir in obese subcutaneous WAT is highly modified with significant changes in genes regulating stemcellness, lineage commitment and inflammation. In addition to body mass index, cardiovascular risk factor clustering further affect the ASC transcriptomic profile inducing loss of multipotency and, hence, capacity for tissue repair. In summary, the stem cells in the subcutaneous WAT niche of obese patients are already committed to adipocyte differentiation and show an upregulated inflammatory gene expression associated to their loss of stemcellness.

Keywords: Human adipose-derived stem cells, Subcutaneous adipose tissue, Cardiovascular risk factors, Transcriptome, Inflammatory genes

* Correspondence: lbadimon@csic-iccc.org

¹Cardiovascular Research Center, CSIC-ICCC, Hospital de la Santa Creu i Sant Pau, IIB-Sant Pau, Barcelona, Spain

Full list of author information is available at the end of the article

Background

White adipose tissue (WAT), traditionally viewed as an energy storage tissue, is now recognized as an endocrine organ that harvests and serves as repository of mesenchymal stem cells, the adipose-derived stem cells (ASC), for physiological cell renewal and spontaneous repair. Henceforth, these ASC have the potential to differentiate towards multiple tissue lineages, produce a large variety of growth factors and present immunomodulatory properties [1]. Mesenchymal stem cells (MSC) homing from bone marrow to peripheral tissue is probably the most specialized organ repairing process. During the last few years it has been possible to isolate MSC from different tissues [2].

Obesity, a chronic pathological condition, is a risk factor for cardiovascular disease, but paradoxically, it seems to protect against morbi/mortality from heart failure [3]. The hypothesis of a repository of stem cells in WAT of heart failure-obese patients that may serve as a source for spontaneous repair has never been tested. It has been recently suggested that any alteration of the stem cell homeostasis by constant and repetitive trauma, and chronic disease could provoke a persistent disequilibrium inside the stem cell reservoir leading to an irreversible and premature decrease of the stem cells regenerative potential reducing their "stemcellness" [2]. Because a growing body of evidence suggests that depot-specific variations in resident stem cells are retained despite in vitro culture processes [3], we investigated the isolated stem cells from the adipose tissue niches to identify their changes due to obesity. Indeed, we have previously reported that adipose-derived stem cells (ASC) from obese patients show a differentiation potential and are less proangiogenic than ASC from non-obese individuals [4]. However, how obesity affects ASC and which are the affected genes remain unclear.

Differences in gene expression between subcutaneous and visceral WAT have been reported. As such, genes involved in energy homeostasis and adipogenesis are profoundly altered in obese WAT [5-8]. Additionally, in obese individuals' mature adipocytes, the major WAT-cell types have shown to present an altered inflammation-related gene expression profile [9]. In addition, studies in subcutaneous preadipocytes from type 2 diabetes patients and obese Pima Indian subjects have reported diminished expression of genes involved in differentiation and an upregulation of inflammation-related genes [10,11]. Moreover, obesity has shown to dysregulate the stemness gene network of omental-ASC [12]. However, there are few studies investigating how obesity affects the transcriptome of resident stem cells reservoir.

In this study, we investigated the gene expression profile and the involved biological functions in ASC of

obese patients using a systems biology approach. ASC were isolated from WAT to avoid the contribution of differentiated cells in subcutaneous WAT to the transcriptomic signature. We studied gene expression profile of isolated subcutaneous ASC from patients with obesity and clustering of cardiovascular risk factors and ASC from non-obese healthy individuals. We used a bioinformatic approach with "in silico" analysis to identify biological functions and target genes potentially altered in ASC. Further validation of candidate genes has identified potential targets associated to a reduced regenerative potential.

Results

Patient demographics and ASC phenotypic characterization

ASC were harvested from morbidly obese patients [Body mass index (BMI) = 44.44 ± 1.29 kg/m²], and non-obese individuals (BMI = 22.26 ± 0.88 kg/m²). Although both groups were comparable in age (morbidly obese patients = 41.56 ± 3.07 years; non-obese individuals = 38.28 ± 2.48 years), morbidly obese patients showed significantly ($p < 0.05$) elevated glucose (142.13 ± 11.95 mg/dl vs 61.54 ± 7.35 mg/dl), creatinine levels (0.83 ± 0.07 mg/dl vs 0.55 ± 0.1 mg/dl) and total cholesterol/high density lipoprotein cholesterol (HDL) ratio (5.92 ± 0.48 vs 3.98 ± 0.38), and lower HDL cholesterol levels (33.2 ± 3.05 mg/dl vs 48.32 ± 2.82 mg/dl) as compared to non-obese individuals. No differences were detected in triglyceride, total cholesterol, low density lipoprotein cholesterol (LDL), urea, total proteins and hepatic enzymes levels.

Flow cytometry analysis of isolated ASC from morbidly obese patients (ASC_{mo}) and non-obese individuals (ASC_n) revealed similar ASC phenotype in both groups of cells at passage 3, when the transcriptomic analysis was performed. Both cell types strongly expressed typical ASC surface antigens such as CD90 (Thy-1 cell surface antigen), CD29 (Integrin beta 1), CD44 (Hyaluronic acid receptor), and CD105 (Endoglin) and were negative for CD45, a hematopoietic cell marker, and for CD34, a marker of bone marrow cells, hematopoietic stem cells, endothelial progenitor cells and muscle stem cells (Additional file 1: Table S1). Interestingly the flow cytometry analysis at passage 0 demonstrated that passage 3 cells had similar ASC surface markers levels (CD105, CD29, CD90 and CD44) with respect to passage 0 cells. However, a significant reduction in CD34 and CD45 was found indicating that the passage 3 cells used for transcriptomic analysis were a homogeneous ASC population (Additional file 1: Table S1).

Transcriptomic analysis

In order to compare gene expression patterns between ASC_{mo} and ASC_n, a comprehensive transcriptomic

analysis was performed using Affymetrix whole-transcript expression array. To visualize gene expression data we performed Principal Component Analysis (PCA) that demonstrated the independence of transcriptomic signature in ASCmo and ASCn (Additional file 2: Figure S1). To further explore genes differentially expressed between ASCmo and ASCn, we used modified analysis of variance (ANOVA) analysis. With a p-value < 0.2, a 60% False Discovery Rate (FDR) and a fold change > 1.5, 637 differentially expressed transcripts were detected while with a p-value < 0.1, a 60% FDR and a fold change > 1.5, 457 gene transcripts were detected (Additional file 3: Figure S2). Among the 637 detected genes, 319 exhibited an increased expression in ASCn (fold change range: 1.5 to 7.2) while 318 were overexpressed in ASCmo (fold change range: 1.5 to 18).

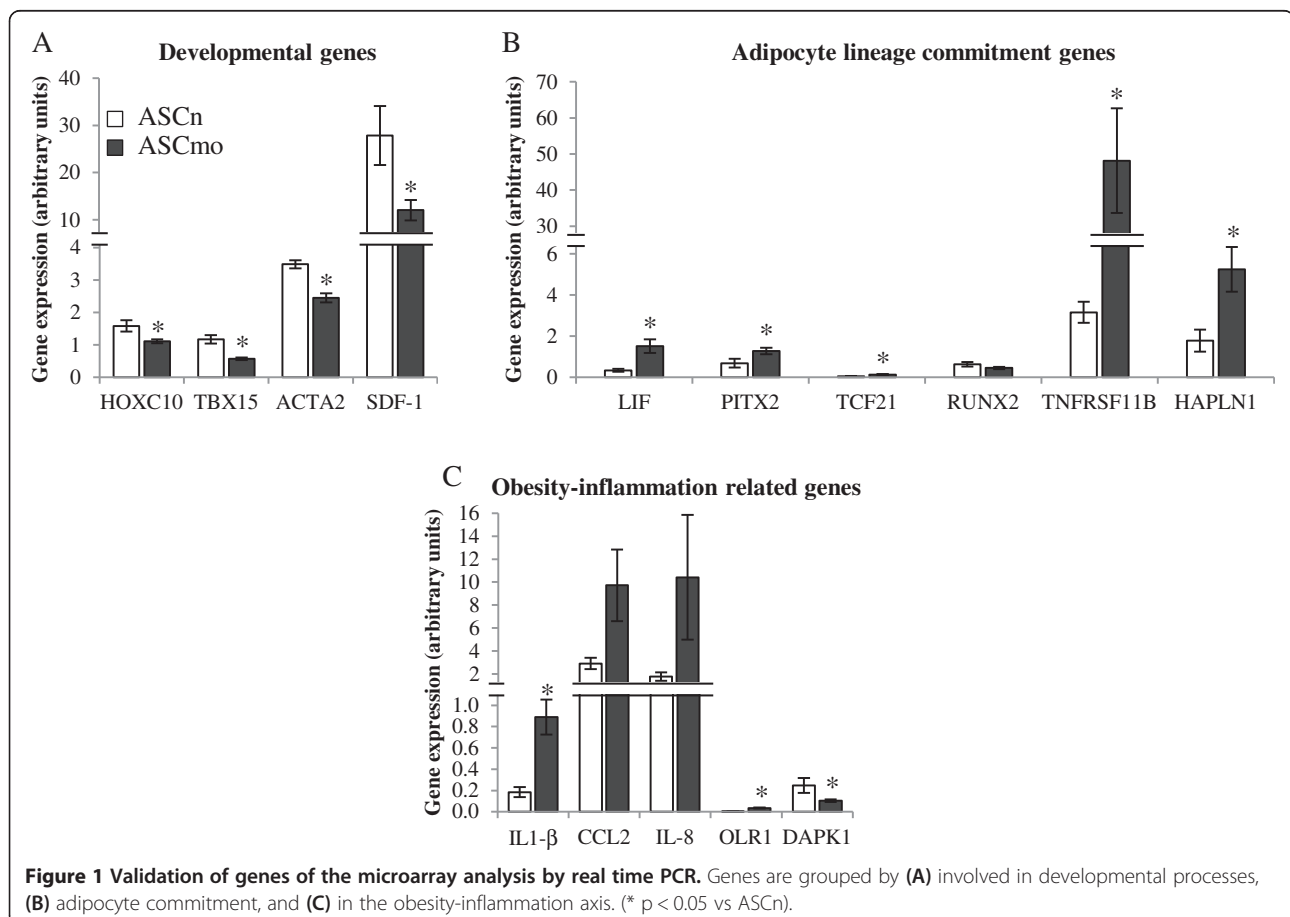
ASCmo and ASCn gene expression patterns

Ingenuity Pathway Analysis (IPA) data identified the most relevant modifications in gene expression in ASCmo. These genes were further validated by real-time PCR (Figure 1). Developmental genes were down-regulated in the ASCmo

compare to ASCn since a significantly reduced expression was observed in HOXC10 (Homeobox C10; -1.42 fold), TBX15 (T-box 15; -2.04 fold), ACTA2 (α -smooth muscle actin; -1.42 fold) and SDF-1 (Stromal cell-derived factor 1/CXCL12; -2.32 fold) (Figure 1A).

Moreover, ASCmo presented a significant up-regulation in genes that induce adipocyte lineage commitment or regulates adipogenesis, such as TCF21 (Transcription factor 21; 2.62 fold), PITX2 (Paired-like homeodomain transcription factor 2; 1.88 fold), LIF (Leukemia inhibitory factor; 4.4 fold), TNFRSF11B (Osteoprotegerin; 15.23 fold) and HAPLN1 (Hyaluronan and proteoglycan link protein 1; 2.94 fold). Contrary RUNX2 (Runt-related transcription factor 2; -1.4 fold), that induces osteoblastogenesis, showed a trend towards a decrease expression in ASCmo compared to ASCn (Figure 1B).

There was an up-regulation in the expression of pro-inflammatory molecules such as IL-1 β (Interleukin-1 β ; 4.81 fold), IL-8 (Interleukin-8; 5.9 fold) and CCL2 (monocyte chemotactic protein-1; 3.35 fold), and OLR1 (oxidized low density lipoprotein receptor 1; 7.62 fold) in ASCmo. The dual pro-apoptotic/pro-survival molecule



DAPK1 (Death-associated protein kinase 1, -2.40) is down-regulated in ASCmo compared to ASCn (Figure 1C).

Effects of cardiovascular risk factor clustering

Correlation analysis between gene expression and BMI or clustering of cardiovascular risk factors (morbid obesity, high blood pressure, smoking, previous cardiovascular event, dyslipidemia and/or type 2 diabetes) was

performed. Expression levels of ACTA2 (R: -0.69), TBX15 (R: -0.68), SDF-1 (R: -0.46) and DAPK1 (R: -0.43) negatively correlated with BMI increase ($p < 0.05$); whereas LIF (R: 0.46), PITX2 (R: 0.44), TNFRSF11B (R: 0.58), IL-1 β (R: 0.45) and OLR1 (R: 0.51) correlated positively ($p < 0.05$) (Figure 2). Furthermore, ACTA2 (Rho: -5.97), HOXC10 (Rho: -5.29) and TBX15 (Rho: -0.671) negatively correlated with cardiovascular risk factor clustering ($p < 0.05$); while LIF (Rho: 0-477), PITX2 (Rho:

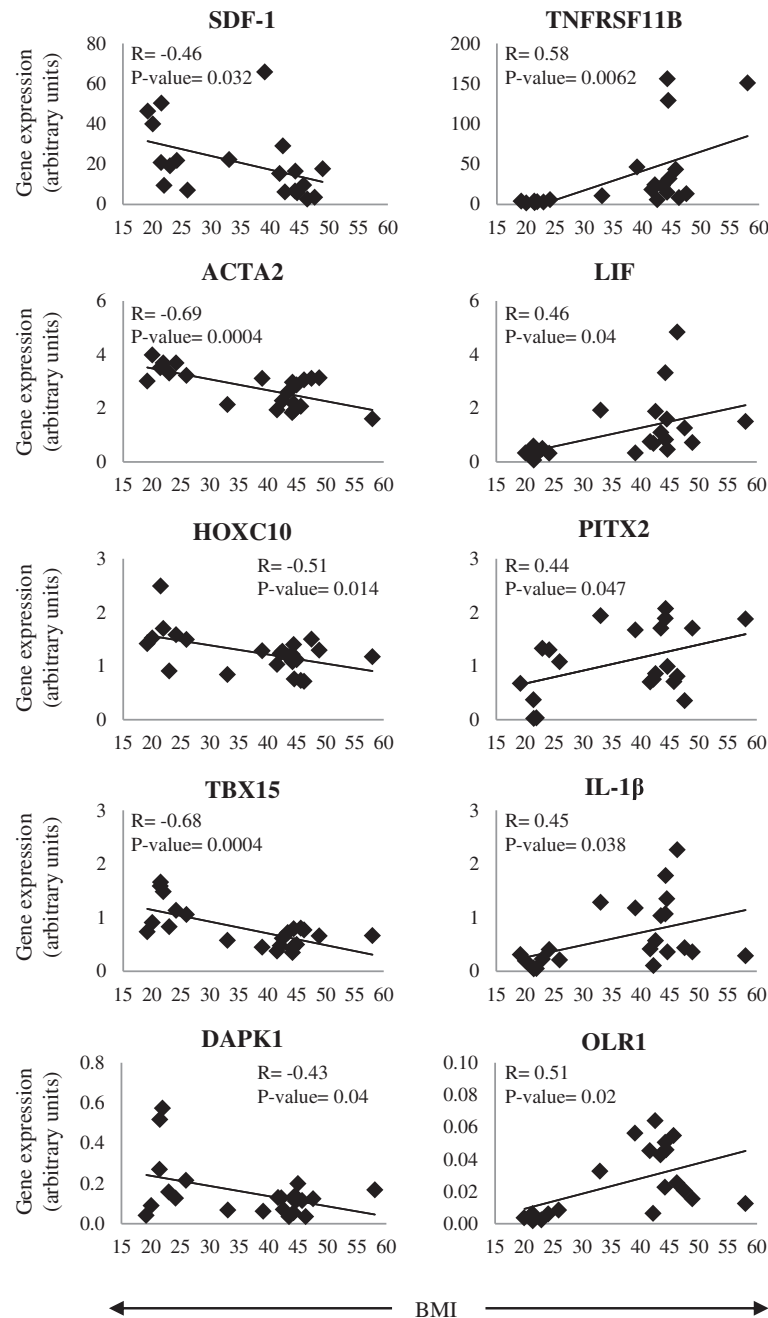


Figure 2 Correlation between expression of validated genes and body mass index (BMI). Correlations were determined by linear correlations.

0.459), OLR1 (Rho: 0.485), TNFRSF11B (Rho: 0.805), IL-1 β (Rho: 0.52) and IL-8 (Rho: 0.644) correlated positively ($p < 0.05$) (Figures 3 and 4).

SMAD activation differs between ASCn and ASCmo

Bioinformatic systems biology analysis predicted the SMAD pathway as a potential candidate in the activation/repression of the modified genes in obesity. In order to analyze SMAD pathway activation, we assessed nuclear translocation of phospho-Smad3. We show that ASCmo present a lower activation of SMAD pathway, in fact, phospho-Smad3 nuclear translocation were significantly reduced in the ASCmo (Figure 5).

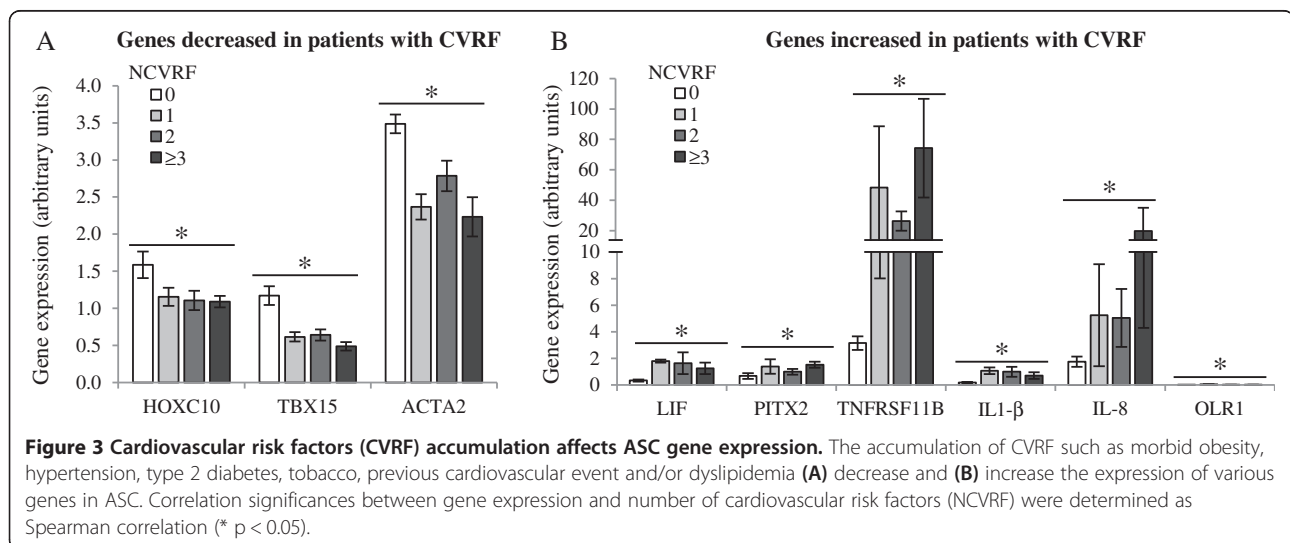
Discussion

In this study we demonstrate that obesity affects WAT-resident ASC by differentially regulating their gene expression. The transcription of both genes involved in development as well those involved in adipocyte differentiation is significantly modified. Moreover, ASC from obese patients already show upregulation of inflammatory genes. Interestingly, ASC from obese patients with metabolic syndrome (and clustering of cardiovascular risk factors) show further impairment in their transcriptomic profile than ASC from obese metabolically healthy patients.

ASC in morbidly obese patients show similar ASC surface markers (CD44, CD90, CD29 and CD105) to ASC from non-obese individuals. However, we had previously shown that ASCmo have significantly different cell proliferation, differentiation and angiogenic potential [4]. Here we show that ASC from the subcutaneous WAT of morbidly obese patients adopt a significantly modified expression profile with major changes on genes that regulate cell survival, cell differentiation, tissue integrity and metabolic pathways. Interestingly, the ASC

(passage 3; both from non-obese and obese patient abdominal fat) were negative for the surface marker CD34, a marker described for bone marrow cells, hematopoietic stem cells, endothelial progenitor cells and muscle stem cells [13]. In fact, the expression of CD34 by ASC has been a matter of discussion because of contradictory reports and findings [14]. While some authors have described an ASC population that express CD34 [15-17], some others have not been able to detect this marker [1,18,19].

Two highly conserved families of genes involved in embryonic development show down-regulation in ASC from morbidly obese patients: the homeobox (HOXC10) and T-box (TBX15). The HOX code is mainly a “biological fingerprint” of different cell types reflecting a continuation of embryonic patterning [20]. Despite the essential role played by HOX genes in embryonic development, hematopoiesis, cancer, angiogenesis and wound repair, there is limited information on HOX genes in a variety of MSC. Similarly, Tbx15 is one of the fundamental developmental mesodermal transcription factors that is highly expressed in both human and rodent adipose tissue [21]. Here we described for the first time that obesity down-regulates expression of TBX15 and HOXC10 in subcutaneous WAT resident stem cells. The expression of both genes negatively correlates with BMI and cardiovascular risk factor clustering therefore potentially marking a reduced developmental potential of the more differentiated cells of the morbid obesity fat depot. The same result is shown with ACTA2, an ASC gene marker. It has been shown that mice ASC expressing ACTA2 present multilineage differentiation ability compared to ASC that do not express ACTA2 which only display adipogenic commitment [22]. The fact that expression of these three genes are found significantly decreased in ASC from morbidly obese patients



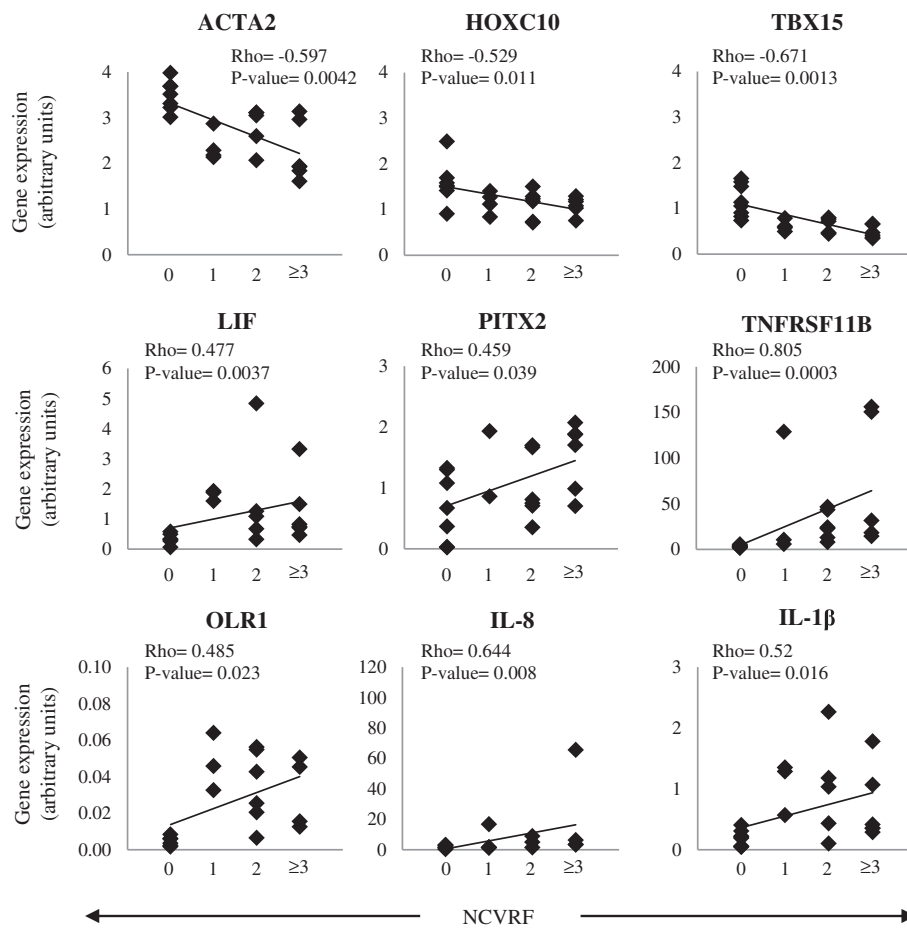


Figure 4 Correlation between gene expression and the presence of various cardiovascular risk factors (CVRF). CVRF such as morbid obesity, hypertension, type 2 diabetes, tobacco, previous cardiovascular event and/or dyslipidemia were correlated with gene expression. Correlation significances were determined by Spearman correlation (Rho coefficient). NCVRF, number of cardiovascular risk factors.

indicates that obesity impairs the multipotency of the stem cells homed into their subcutaneous WAT.

Stem cell migration is a key process for development, spontaneous regeneration and homing. It has been described that the CXCR4-SDF-1 axis regulates cellular

trafficking [23] and that ASC produce and secrete SDF-1 [24]. Circulating SDF-1 levels are decreased in diet-induced obese mice and in adolescents with elevated waist circumference and BMI measures [25]. In this study we show that ASC in morbidly obese patients

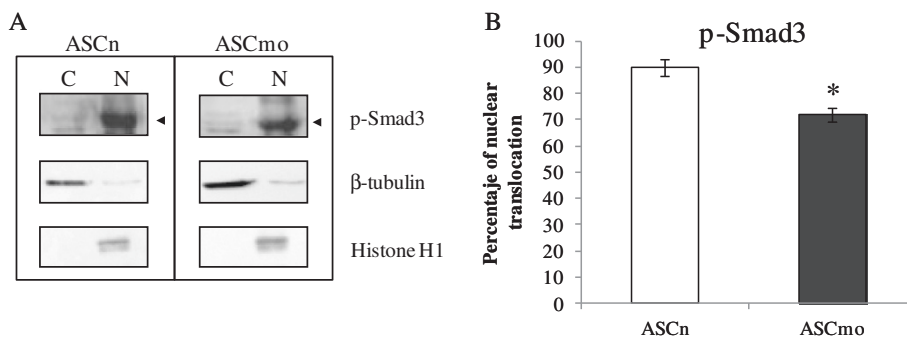


Figure 5 Nuclear translocation of phospho-Smad3 differs between ASCn and ASCmo. (A) Western blot analysis of cytoplasmic-membrane (C) and nuclear (N) fraction of ASCn and ASCmo. β -tubulin and Histone H1 were used as a membrane-cytoplasmic and nuclear fraction marker, respectively. (B) ASCmo presented a reduced nuclear translocation of phospho-Smad3 compared to ASCn (* $p < 0.05$ vs ASCn).

show a significant down-regulation in SDF-1 expression, and that the effect is BMI-dependent and correlates with cardiovascular risk factors clustering. These results indicate that obesity seems to impair ASC trafficking and homing.

Given that adipocytes share a common mesenchymal precursor cell with osteoblasts, entrance into the adipocytic or osteoblastic lineage is thought to be mutually exclusive [26]. In fact, our results revealed that the adipogenic lineage is stimulated in ASCmo while osteoblastogenesis is inhibited. We have observed that TCF21 expression, a white WAT-selective marker, is increased in ASCmo. Opposite, RUNX2, an osteoblast marker, presented a non-significant trend to decrease in ASCmo. TCF21 is the first useful positive selection marker for white preadipocytes and plays an important role in adipogenesis. It has been shown that TCF21 suppresses myogenesis [27]. Of interest, PITX2 that is known to upregulate the expression of TCF21 [28], is up-regulated in ASCmo explaining the increase in TCF21 expression.

On the other hand, we detected that ASCmo presented an increased expression in LIF and TNFRSF11B genes. Various studies of LIF in adipose tissue have suggested that adipogenesis could be modulated by this gp130 cytokine at certain stages of development while regulating the expression of genes associated with lipid metabolism [29]. Furthermore, TNFRSF11B (Osteoprotegerin) is a glycoprotein of the tumor necrosis factor (TNF) receptor superfamily that was originally discovered as bone resorption inhibitor [30]. Its expression is high in human WAT, and is increased during 3T3-L1 adipocyte differentiation and under TNF- α and IL-1 β stimulation [31]. In humans, plasma TNFRSF11B levels are elevated in patients with cardiovascular risk factors such as type 2 diabetes and abdominal obesity [32]. We have also detected that both LIF and TNFRSF11B levels positively correlate with BMI and the clustering of cardiovascular risk factors. Moreover, RUNX2 is involved in inhibiting adipocyte differentiation and appears to be down-regulated during 3T3-L1 adipocyte differentiation [33] and obesity reduces bone RUNX2 expression leading to bone marrow adiposity [34]. Interestingly, it has been published that ASC from WAT of obese patients show abnormal upregulation of peroxisome proliferator-activated receptor- γ (PPARG), a gene that reveals a high adipogenic potential [12,35]. All together these observations indicate that morbid obesity not only affects ASC multilineage potential but commit ASC to adipogenic lineage.

Recent studies demonstrate that during adipogenesis and development of obesity, the extracellular matrix (ECM) undergoes structural remodeling affecting adipose tissue expandability and functional integrity [36]. Analysis of the secretome of human ASC identified

various components of the ECM synthesis and remodeling [37]. The glycosaminoglycan hyaluronan is an integral component of ECM with an important role in organizing and maintaining the integrity of the ECM. Although hyaluronan regulates differentiation of mesenchymal lineage cells to chondrocytes and osteoblasts [38], it has been also shown that hyaluronan increases adipogenesis efficiency likely because it provides an appropriate ECM [39]. In fact, it has been demonstrated that hyaluronan synthesis, in 3T3-L1, appears at the stages where early adipocyte markers develop and correlates with triglyceride accumulation [40]. Interestingly we have detected in ASCmo higher levels of HAPLN1, a member of the hyaluronan and proteoglycan binding link protein gene family that stabilizes the interaction between hyaluronan and versican, two ECM components that contribute to the viscoelastic properties observed during 3T3-L1 adipogenic differentiation [40]. More experiments are needed to address how obesity and cardiovascular risk factors modify the adipose tissue ECM and its expandability.

Obesity is associated with a low-grade chronic inflammation [41]. Paracrine and autocrine signaling in WAT-cells may further enhance inflammation, ultimately increasing proinflammatory cytokines production [41]. Yet, it seems that adipocytes and mainly stromal vascular cells appear to be involved in the local production of such cytokines [11,42]. Here we show that obesity induces inflammatory gene up-regulation already in the stem cells. IL-1 β , IL-8 and CCL2 genes are up-regulated in ASCmo. In fact, while we detected that IL-1 β increase is affected by BMI, IL-8 expression is correlated with cardiovascular risk factors clustering. IL-1 β and IL-8 are considered the prototypical proinflammatory cytokines involved in stimulating the synthesis and secretion of a variety of other interleukins, and also participates in a variety of cell functions [42]. Furthermore, high circulating levels of IL-8 and CCL2 have been found in obese subjects increasing the risk of coronary artery disease and type 1 or 2 diabetes [43]. In fact, IL-8 released from subcutaneous WAT and omental-ASC displayed a positive correlation with BMI [12,44], whereas CCL2 expression was higher in subcutaneous adipocytes of obese than non-obese Pima Indians [9].

Furthermore, we report for the first time that ASCmo show increased expression of OLR1 (LOX-1), a lectin-like receptor for oxidized-LDL. OLR1 is expressed in highly vascularized tissues, plays critical roles in the development of atherosclerosis and related disorders, and is upregulated in hypertensive, dyslipidemic, and diabetic animals and humans [45,46]. A recent study reported that oxidized-LDL-OLR1 interaction induces the generation of reactive oxygen species and stimulates pro-inflammatory gene expression [47]. Thus, the effects

of OLR1 in obesity could be related to a complex state of inflammation and adipocyte death. In this study we identified that although OLR1 expression levels in ASC is low, morbid obesity and clustering of cardiovascular risk factors significantly increases ASC OLR1 expression.

Finally, we have observed a significantly reduced expression of DAPK1 in ASCmo. The death-associated protein kinase-1 (DAPK1) is the prototypic member of a family of death-related kinases. However, DAPKs can also act as a survival factors and block apoptosis in response to certain cytokine signals modulating the balance between pro-apoptotic and pro-survival pathways [48].

To address the signaling pathways differentially activated in ASCmo, the validated gene expression data was analyzed in silico with the IPA software to obtain the most probable networks and biological pathways. Candidate proteins identified by IPA were validated by western blot. As such, a lower activation of Smad3 was found in the stem cells of the subcutaneous WAT of obese patients. Interestingly, the transcription factor Smad3 plays a key role in the inhibition of adipocyte differentiation and in the development of insulin resistance [49,50]. This integrated bioinformatic systems biology approach led us to propose a network that regulates the differential gene expression pattern in stem cells found in subcutaneous fat of morbidly obese patients (Figure 6).

Our data does not help to explain the obesity paradox. Indeed, studies of patients with chronic heart disease suggest that overweight and obese patients may paradoxically have better outcomes than lean patients. This

results may associate adiposity with enhanced cardiovascular autoreparative potential [51]. However our previous results [4] and these here reported indicate that obesity renders ASC less multipotent leaving the paradox unsolved. An alternative not yet fully explored possibility is that obesity could promote MSC recruitment in order to perpetuate adipose tissue formation [2], and consequently lead to increase number of circulating MSC which in turn, upon organ damage, could migrate to the site of injury for repair [52].

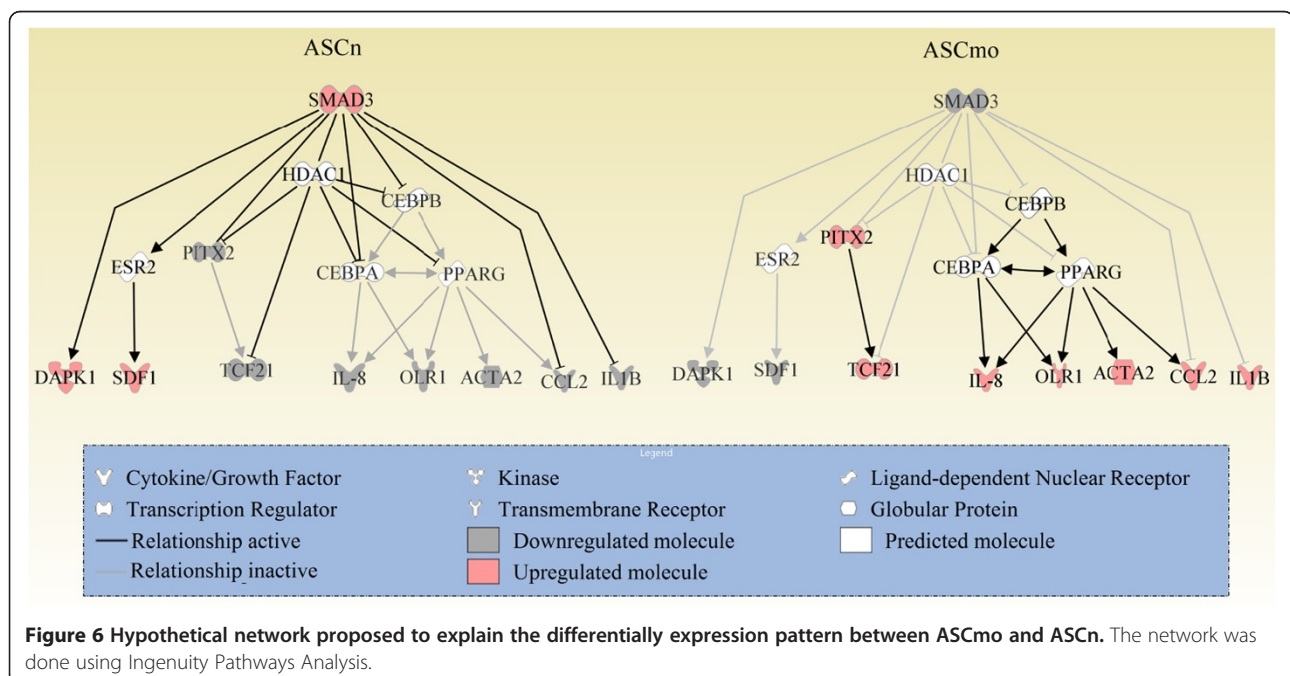
Conclusions

In summary, obesity produces a detrimental effect on its resident stem cells. In fact, the ASC undifferentiated multipotent state is impaired in obese patients with respect to non-obese individuals. This study provides a global systems biology analysis of highly modified genes expressed in ASC from patients with obesity and cardiovascular risk factor clustering that shows how the WAT niche affect resident stem cells. A remaining question that deserves further investigation is whether obesity affects the mesenchymal stem cell transcription potential in the adipose tissue reservoir or stem cells are already conditioned by obesity in the patient's bone marrow.

Methods

Subjects

Subcutaneous abdominal WAT was obtained via surgical resection from morbidly obese patients (BMI > 40 kg/m²; n = 16) that underwent bypass gastric surgery, and from non-obese individuals (BMI < 25 kg/m²; n = 8)



who underwent abdominal lipectomy. Tissues were obtained with informed consent and the study protocol was approved by the Centro Medico Teknon's Ethical Committee consistent with the principles of the Declaration of Helsinki. Blood samples from all subjects were obtained at the time of intervention in order to evaluate the following biochemical parameters: glucose, triglyceride, total cholesterol, HDL, LDL, urea, total proteins, glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, and creatinine levels. Patients used regular medication as recommended in the guidelines if it was necessary. In order to analyze the effect of cardiovascular risk factors (morbid obesity, high blood pressure, smoking, dyslipidemia, type 2 diabetes and/or previous cardiovascular event), individuals were clustered according to the accumulated cardiovascular risk factors: 0) non-obese individuals, 1) morbid obesity, 2) morbid obesity and one additional risk factor (high blood pressure or dyslipidemia), ≥ 3) morbid obesity and two or more risk factors (patients with metabolic syndrome).

ASC isolation and culture

Isolation of ASC_{mo} and ASC_n was performed as we have previously described [4]. Briefly, AT was washed, minced and digested with 1 mg/ml Collagenase I-A (Sigma-Aldrich; St. Louis, MO, USA) in Dulbecco's modified Eagle's Medium high glucose (Gibco; Life Technologies, Inc., Grand Island, NY, USA) for 1 h at 37°C with gentle agitation. The digested tissue was sequentially filtered through a 100 μ m mesh and centrifuged for 10 min at 1200 rpm at room temperature. The supernatant, containing mature adipocytes, was aspirated and the pellet was identified as the stromal vascular fraction (SVF). SVF was resuspended in culture medium (Dulbecco's modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin; Life Technologies, Paisley, UK) and seeded at 2×10^5 cells/cm². Finally, these cells were incubated overnight at 37°C, 5% CO₂ under hypoxic conditions (1% O₂); 24 h after incubation, the medium was changed to remove non-adherent cells. These primary adherent cells are referred as ASC passage 0 (cultured for 7 days until they reached 75-90% confluence). For further passages cells were detached by trypsin digestion.

Flow cytometry characterization

For further characterization, cell surface antigen phenotype was performed on ASC_{mo} and ASC_n at passage 0 and passage 3. The following cell-surface epitopes were marked with anti-human antibodies: CD105-fluorescein isothiocyanate (GeneTex), CD90-fluorescein isothiocyanate (FITC), CD29-phycoerythrin (PE), CD44-FITC, CD34-PE-Cy[™]5 and CD45-PE (BD Biosciences; San

Diego, CA, USA). Mouse isotype antibodies served as control (BD Biosciences). Cellular events (30000) were acquired and analyzed by Fluorescence-activated cell sorting using Coulter EPICS XL flow cytometer (Beckman Coulter) running Expo32 software (Beckman Coulter).

Transcriptomic analysis

The whole genome expression was assessed by independently run microarrays in isolated human ASC_{mo} (n = 3) and ASC_n (n = 3) cultured under hypoxic conditions (passage 3) using GeneChip Human Gene 1.0 ST arrays (Affymetrix; Santa Clara, CA, USA). Total cell RNA was extracted using silica-membrane columns (RNesasy Mini Kit, Qiagen; Valencia, CA, USA). RNA quantification was determined spectrophotometrically using the Nanodrop ND-1000 (Thermo Fisher Scientific; Waltham, MA, USA). RNA quality was measured using Agilent 2100 Bioanalyzer technology (Agilent Technologies; Santa Clara, CA, USA) with the Agilent RNA 6000 Nano Kit (Agilent Technologies). Only RNA samples with RIN > 7 (RNA integrity number) values were chosen for microarray experiments. Using the Ambion WT Expression Kit (Ambion, Life Technologies, Paisley, UK) 200 ng of total RNA (mixed with poly-A controls; Affymetrix) were converted to double strand DNA, in two steps, in order to obtain cRNA by an in vitro transcription. Single strand DNA was obtained from 10 μ g of cRNA. Then 5.5 μ g of single strand DNA were fragmented and labelled with biotin using the WT Terminal Labeling Kit (Affymetrix). Hybridization controls from the Hybridization, Wash and Stain Kit (Affymetrix) were added to the sample. Every sample was hybridized to GeneChip Human Gene 1.0 ST array during 16 h at 45°C and 60 rpm, according to manufacturer's instructions. Hybridization, washing, staining and scanning of microarrays were performed according to Affymetrix instructions using the Affymetrix GeneChip 3000 7G System (645 Hybridization Oven, 450 Fluidic Station and GeneChip 3000 7G Scanner).

Raw data were pre-processed with Robust Multiarray Average (RMA) method [53]: background correction, quantile normalization and median polish summarization of the probes. Bioinformatics was used to analyze the extracted log₂ expression data. The raw data from the array experiments has been deposited in NCBI's Gene Expression Omnibus [54] and is accessible through GEO Series accession number (GSE48964; <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE48964>).

Microarray quality control and statistical analyses were performed using the Partek Genomics Suite software (Partek Inc., St Louis, Missouri, USA). PCA procedure was used to represent samples variability, which defines a group of Principal Components in order to explain the

variability existing between the samples. ANOVA, modified by Partek Inc, was used for statistical comparisons between groups. Derived p-values were adjusted for multiple testing with the method of Benjamini and Hochberg [55]. Adjusted p-value, FDR <0.6 and fold change $\geq \pm 1.5$ were used to obtain differential expressed genes. Some of the modified genes were validated using real-time PCR in ASC from additional non-obese individuals (n = 8) and morbidly obese patients (n = 16). Differential expressed genes were associated with biological functions and/or diseases based on literature and the Ingenuity Knowledge Base. Differential expressed genes were analyzed through the use of IPA (Ingenuity® Systems, www.ingenuity.com). Right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone. The data set from our study was used to construct hypothetical protein interaction based on a regularly updated Ingenuity Pathways Knowledge Base. The networks are displayed graphically as nodes (individual proteins) and edges (the biological relationships between the nodes). The relationships include direct and indirect protein interactions.

Real time PCR validation

In order to validate the generated data, genes of interest that showed a statistically significant difference in expression were validated using custom Taqman Low Density Arrays and real-time PCR assays according to manufacturer's instructions (Applied Biosystems, Life Technologies). Relative gene expression values were calculated by the $\Delta\Delta C_t$ method [56]. The raw gene expression values were normalized according to the expression of TATA-binding protein (TBP) gene.

Western blotting

ASC subcellular fractionation and western blot analysis were performed to analyze proteins levels and nuclear translocation in non-obese individuals (n = 7) and morbidly obese patients (n = 9).

Subcellular fractionation analysis was performed to evaluate phospho-Smad3 nuclear translocation. Nuclear and membrane-cytoplasmic proteins were extracted from ASCn and ASCmo cultured under hypoxic conditions as previously described [57]. The extraction was performed on ice to avoid denaturation of the proteins. Equivalent amounts of proteins were subjected to electrophoresis on 10-12% gels and then transferred to nitrocellulose membrane. After blocking in 5% nonfat milk 1 h at room temperature, blots were incubated with anti-phospho-Smad2/3 goat polyclonal (Santa Cruz Biotechnology; Santa Cruz, CA, USA), anti- β -tubulin rabbit polyclonal (Abcam; Cambridge, UK) and anti-Histone H1 mouse monoclonal (Millipore; Billerica, MA,

USA) at 4°C overnight. Secondary antibodies consisted of horseradish peroxidase-conjugated antibodies (Dako; Barcelona, Spain) and were detected using SuperSignal chemiluminescence system (Pierce; Rockford, IL, USA). β -tubulin and Histone H1 were used as a membrane-cytoplasmic and nuclear fraction marker, respectively. Protein expression was determined using Image Lab™ Software (BIO RAD). Nuclear translocation was calculated as a relation between nuclear protein vs total protein (nuclear + membrane/cytoplasmic protein) quantification.

Additional statistical analyses

Results are expressed as means \pm standard error mean. Statistical Analyses were performed using StatView® software (SAS Institute Inc.). The statistical significance was assessed by unpaired Student's *t*-test. Correlation analyses between gene expression and BMI or number of cardiovascular risk factors were determined by linear regression and Spearman correlation, respectively. P-value < 0.05 was considered statistically significant.

Additional files

- Additional file 1: Table S1.** Phenotypical characterization of ASC. Flow cytometry analysis of ASCn and ASCmo at passage 0 and 3 were performed in order to characterize ASC populations. Data are presented as means \pm standard error mean.
- Additional file 2: Figure S1.** Principal component analysis (PCA) of genomic expression data from ASC from different donors.
- Additional file 3: Figure S2.** Heat map of genes displaying 1.5-fold difference in expression between ASC individuals groups.

Abbreviations

ASC: Adipose-derived stem cells; ASCn: ASC derived from non-obese individuals; ASCmo: ASC derived from morbid obese patients; BMI: Body mass index; CVRF: Cardiovascular risk factors; FDR: False discovery rate; HDL: High density lipoprotein cholesterol; LDL: Low density lipoprotein cholesterol; MSC: Mesenchymal stem cells; PCA: Principal component analysis; PCR: Polymerase chain reaction; SVF: Stromal vascular fraction; WAT: White adipose tissue.

Competing interests

The authors declare that they have no competing interest.

Authors' contributions

BO performed experiments, interpreted results and wrote the manuscript; GV designed strategy for experimental planning and reviewed the manuscript; SC-L performed experiments and revised the manuscript; JY was in charge of medical patients follow-up; AD-C, CB-L, FM and JH were surgeon in charge of bariatric surgery sample collection; LB planned, evaluated and interpreted experiments and wrote the article. All authors read and approved the final manuscript.

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Author details

¹Cardiovascular Research Center, CSIC-ICCC, Hospital de la Santa Creu i Sant Pau, IIB-Sant Pau, Barcelona, Spain. ²Quirúrgica cirujanos Asociados, Centro Médico Teknon, Barcelona, Spain. ³CLB Centro laparoscópico Doctor Ballesta, Barcelona, Spain. ⁴Resistencia a la Insulina SL, Barcelona, Spain. ⁵Department of Plastic Surgery, Centro Médico Teknon, Barcelona, Spain.

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