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Sera of Overweight Patients Alter Adipogenesis and Osteogenesis of Bone Marrow Mesenchymal Stromal Cells, a Phenomenon that also Persists in Weight Loss Individuals

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Abstract

Background: Overweight and obesity represents a complex interaction of genetics, diet, metabolism, and physical activity. Abnormal or excessive body fat accumulation increases the risk of pathologies, such as heart disease, diabetes, and high blood pressure.

Methods and results: In a previous paper, we investigated the influence of overweight individuals' sera on in vitro biology of mesenchymal stromal cells, which contain a subpopulation of multipotent stem cells. This report extended the study by evaluating effects of sera collected from weight loss patients on stromal cultures.

Results showed that an increase in fat mass induces physiological changes that negatively impact on stromal stem cell adipogenesis and osteogenesis with a bias toward adipocyte differentiation. This phenomenon occurred with sera from overweight patients and persisted also with sera of individuals that underwent loss of weight. Significant changes in obesity related cytokines were detected in sera of overweight and weight loss compared to healthy weight individuals. These changes may explain some of the biological effects detected on stromal stem cell functions.

Keywords: MSCs; Adipogenesis; Osteogenesis; Overweight; Weight loss; Cytokines; Senescence

Introduction

Overweight and obesity are defined as abnormal or excessive fat accumulation in the adipose tissue, resulting in a high risk to health. On the other hand, visceral obesity is defined by the presence of excess adipose tissue in the abdominal cavity, and is an independent risk factor for several diseases. A common way to measure individuals' obesity is the body mass index (BMI). The metric formula, a person's weight (in kilograms) divided by the square of his or her height (in metres) is used to calculate BMI. Therefore, individuals with a body mass index between 18 and 25 are considered having healthy weight; people with a BMI between 25 and 29 are considered overweight; and people with a BMI of 30 or more are generally defined as obese. Overweight and obesity are major risk factors for a number of chronic diseases, including dyslipidemia, diabetes, cardiovascular diseases, and cancer. These conditions can shorten life span.

Lifestyle modification, consisting of a reduction in energy intake, an increase in energy consumption, and behavioural strategies to support these transformations, is the keystone of weight loss therapy. Many short-term studies have shown that modest weight loss of approximately 5–10% of initial weight can be associated with improvement or, in some cases, regression of obesity-related disorders [1].

Cytokines appear to be major regulators of adipose tissue functions. They can regulate homeostasis, metabolism, and interplay with immune system. In particular, obesity is associated with chronic inflammation [2]. It is well known that, in overweight and obese people, the level of various circulating cytokines, hormones, and other signalling molecules are dysregulated due to presence of a inflammatory status [3]. This may affect the functions of several organs and tissues, including the stem cell niches, which are specific tissue regions that house stem cells and control their self-renewal and lineage production by modulating the concentration of signalling molecules, such as hormones, cytokines, and growth factors [4].

The stroma of mammalian bone marrow is composed of several different elements that support hematopoiesis and bone homeostasis [5]. It includes several types of differentiated cells, progenitors, and stem cells. Among these are mesenchymal stem cells (MSCs), which are primordial cells of mesodermal origin that give rise to adipocytes, chondrocytes, osteocytes, and smooth muscle cells [5,6]. We have previously demonstrated that the dysregulated factors circulating in serum affected the differentiation potential of bone marrow MSCs in overweight people [7]. Indeed, that study showed that in vitro incubation of MSCs with sera of overweight individuals promotes MSC adipogenesis while impairing osteogenesis [7]. There are reports showing that some diseases such as diabetes and obesity may induce a permanent or semi-permanent impairment of stem cell niche functions that is not strictly associated with the acute phase of the disease. In this way, it remains to be determined if negative effects of sera from overweight people still persist after weight loss [8]. In a recent study, Baptista et al. demonstrated that MSCs isolated from adipose

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tissue of individuals who previously had obesity showed more *in vitro* adipogenic potential than those with the same cells type obtained from healthy weight patients [9]. Based on this premise, this study followed overweight people during their programmed diet schedule for losing weight and evaluated the in vitro behaviour of healthy MSCs incubated with sera obtained at the beginning of diet, then three and six months later when their reached healthy BMI.

Materials and Methods

Ethical approval

The experimental procedures followed the rules approved by the Ethics Committee of the Second University of Naples. Specifically, patients were informed of the research and gave permission for the use of serum samples and bone marrow harvests.

Serum samples

Serum samples were collected from three adult men of healthy weight (body mass index (BMI)<25) and three adult men with BMIs>25 (overweight), after informed consent. These latter patients were followed during a weight loss treatment for six months. Serum samples were collected at the first medical visits, then three and six months later. Whole blood samples (10 ml) were collected from patients in Vacutainer test tubes (BD Bioscience, Italy). After collection, the samples were left undisturbed to allow the blood to clot at room temperature. The clots were removed by centrifuging at 1,000 for 10 minutes in a refrigerated centrifuge. The resultant supernatants were designated sera and were collected with a Pasteur pipette. The sexual and age information of patients and healthy people are shown in supplementary file 1.

MSC cultures

Bone marrow samples were obtained from three healthy donors. We separated cells using a Ficoll density gradient (GE Healthcare, Milan, Italy), and the mononuclear cell fraction was collected and washed in PBS. We seeded 1 to 2.5 × 10^5 cells/cm^2 in alpha-minimum essential medium (alpha-MEM) containing 10% fetal bovine serum (FBS) and 1 ng/ml basic fibroblast growth factor (bFGF). After 72 hours, non-adherent cells were discarded and adherent cells were further cultivated to confluency. Cells were then incubated for seven to ten days in growth medium to reach confluence and extensively propagated for the experimental plan, as shown in Figure 1. We verified that, under our experimental conditions, the bone marrow stromal cultures contained MSCs that fulfilled the criteria proposed to define MSCs (CD90+, C73+, CD105, CD45-) [10]. All experiments were carried out on MSC cultures at passage 3.

For evaluation of the patients' serum effects on in vitro MSC functions, cells were incubated for 72 hours in alpha-MEM containing 10% patients' serum and 1 ng/ml β-FGF. At the end of that time, cultures were used for programmed analysis. All cell culture reagents were obtained from Euroclone Life Sciences (Milan, Italy) and Hyclone (UT, Logan, USA) unless otherwise stated.

Cell cycle analysis

For each assay, cells were collected and fixed in 70% ethanol, followed by PBS washes, and finally were dissolved in a hypotonic buffer containing propidium iodide. Samples were acquired on a Guava EasyCyte flow cytometer (Merck Millipore Italy) and analyzed with a standard procedure using EasyCyte software.

Annexin V assay

Apoptotic cells were detected using a fluorescein-conjugated Annexin V kit on a Guava EasyCyte flow cytometer, following the manufacturer's instructions. The kit uses two separate dyes (Annexin V and 7AAD) to identify several types of apoptotic and non-apoptotic cells. Annexin V (red) binds to phosphatidylserine on the external membrane of apoptotic cells, while 7AAD (blue) permeates and stains DNA of late-stage apoptotic and dead cells. Staining allows the identification of three cell populations: non-apoptotic cells (Annexin V- and 7AAD-), early apoptotic cells (Annexin V+ and 7AAD-), and late-apoptotic or dead cells (Annexin V+ and 7AAD+). In our experimental conditions, early and late apoptotic cells were grouped together.

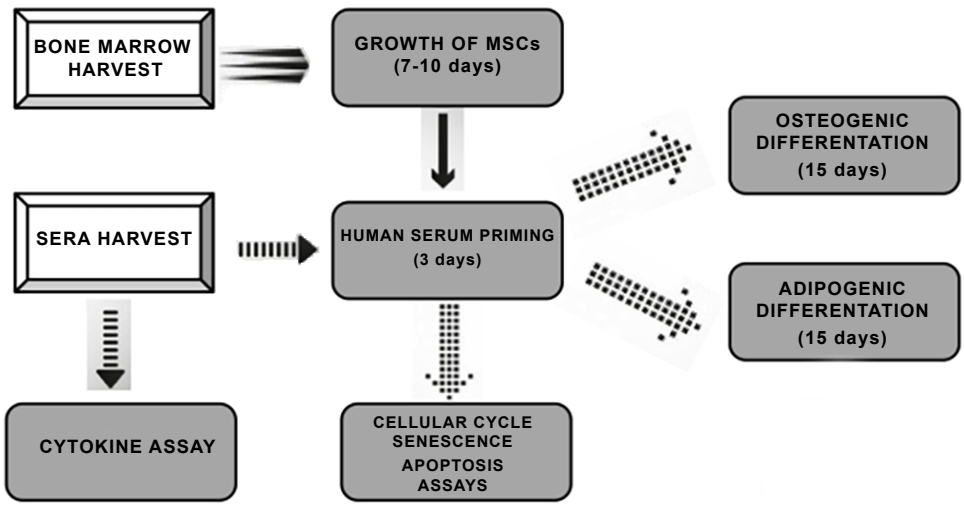

Figure 1: Experimental Plan. Bone marrows were collected from patients and mononuclear cell fractions were used to obtain marrow stromal cultures containing MSCs. Cultures were propagated for seven to ten days and then were treated with OS, HS, LS^3 and LS^6 sera for 72 hours (priming). Following priming, cell cycle, apoptosis and senescence were evaluated. Cultures were then incubated in adipogenic or osteogenic media for 15 days and the differentiation processes were assessed. Cytokines levels in sera patients were evaluated with Human Obesity Antibody Array C1 (RayBiotech, Norcross, GA USA). Mscs: Mesenchymal Stem Cells; HS: Healthy Weight Serum; OS: Overweight Serum; LS^3 and LS^6: Sera from weight loss patients three and six months after first medical examination, respectively.

Senescence-associated beta-galactosidase assay

Cells were fixed for ten minutes using a solution containing 2% (v/v) formaldehyde and 0.2% (w/v) glutaraldehyde. Cells were washed with PBS and then incubated at 37°C for at least two hours with a staining solution (citric acid/phosphate buffer (pH 6), K4Fe(CN)6, K3Fe(CN)6, NaCl, MgCl2, and X-Gal). The percentage of senescent cells was calculated by the number of blue cells (β-galactosidase positive cells) out of at least 500 cells in different microscope fields.

Adipogenic differentiation

MSCs were preliminarily incubated for 72 hours in growth medium containing 10% of patients' sera (healthy individuals, overweight patients at first visit, then after three and six months of diet regimen). Then cultures were stimulated for 15 days in hMSC mesenchymal stem cell adipogenic differentiation medium (catalog n. PT-3004- KT - Lonza, Walkersville, MD, USA). The medium contains insulin (recombinant), dexamethasone, indomethacin, and 3-isobuty-l-methyl-xanthine (IBMX). Lipid droplets were revealed by staining with Oil Red O.

Osteogenic differentiation

After 72 hours of human serum treatment, as reported for adipogenisis, cultures were incubated for 15 days in hMSC mesenchymal stem cell osteogenic differentiation medium (catalog n. PT-3002-KT-Lonza). The medium contains dexamethasone, ascorbate, and glycerophosphate. Calcium deposits in differentiated osteocytes were revealed by staining with alizarin red.

RNA extraction and RT-qPCR

Total RNA was extracted from cell cultures using TRIREAGENT (Molecular Research Center Inc., Cincinnati, OH, USA) according to the manufacturer's protocol. The mRNA levels of osteocyte differentiation markers (Osterix and Osteopontin) were measured by RT-qPCR amplification, as previously reported [11].

Sequences for mRNAs from the nucleotide data bank (National Center for Biotechnology Information, Bethesda, MD, USA) were used to design primer pairs for RT-PCR reactions (Primer Express, Applied Biosystems, Carlsbad, CA, USA) as previously reported [7]. Appropriate regions of GAPDH cDNA were used as controls. Each RT-qPCR reaction was repeated at least three times. A semi-quantitative analysis of mRNA levels was conducted using the GEL DOC UV SYSTEM (Bio-Rad). Primer sequences were designed with Primer Express software (Invitrogen, Milan, Italy).

Cytokine array

The profile of the relative levels of 19 cytokines in the serum samples harvested from the healthy weight, overweight, and weight loss groups was determined using the Human Obesity Antibody Array C1 (RayBiotech, Norcross, GA USA). The nitrocellulose membranes provided by the manufacturer contain 19 capture antibodies spotted in duplicate on the surface. Each membrane also included four pairs of positive control spots and two pairs of negative control spots. A total of 2 ml of the serum samples for each of the three experimental groups was used for hybridization. Hybridizations and signal measurements were performed following the manufacturer's instructions. Array signals were acquired using the Chemidoc system (Bio-Rad Company, Hercules, CA, USA) and the associated software QuantityOne. Array images used for signal quantification (expressed as pixel density) were produced through camera exposure for five minutes. All the membranes were processed simultaneously. All hybridizations were repeated twice.

Statistical analysis

Statistical significance was evaluated using ANOVA analysis followed by Student's t and Bonferroni's tests. A mixed-model variance analysis was used for data with continuous outcomes. All data were analysed with a GraphPad Prism version 5.01 statistical software package (GraphPad, CA, USA).

Results

Table 1 shows the BMI values of overweight patients at their initial medical examinations, and then three and six months later. The main blood serum and biochemical indicators were in the normal range for all the patients at each examination.

In a previous paper, we demonstrated that in vitro biological functions of MSCs were negatively affected by incubation with sera obtained from overweight individuals. We decided to ascertain if loss of weight might reduce the negative impact on MSC biology. We analysed the cell cycle profile, apoptosis, senescence, and differentiation potential of MSCs incubated with sera of overweight people and sera of the same individuals following their loss of weight. As reference, we evaluated the properties of MSCs incubated with sera of individuals with healthy BMI (<25).

To generate a more complete picture of changes occurring in overweight patients following diet regimen, we assessed a panel of circulating cytokines at first medical inspection and later on.

The overall research strategy is depicted in Figure 1. The sera from healthy people, overweight patients at the first visit, and then three and six months later was called HS, OS, LS3, and LS6, respectively.

Cell cycle profile, apoptosis, and senescence unaffected by overweight serum incubation

The cell cycle status of stromal cultures did not change following incubation with sera from overweight patients compared with sera of healthy individuals. Also sera collected after loss of weight did not modify cell cycle profile (Figure 2).

The Annexin assay did not show a significant difference in the percentage of apoptotic cells in cultures treated with LS3 and LS6 as compared to OS and HS treatment (Figure 3). Moreover, the treatment with LS3 and LS6 did not change the senescence process as detected with acid beta-galactosidase assay, as shown in Figure 4.

Adipogenic and osteogenic differentiation

Adult men and women have 3.0 – 4.5 kg of fat mass, and in severely obese people, adipose tissue can constitute 45 kg or more of body weight. In obese status, an increment in fat mass could only result from engagement and differentiation of adipocyte progenitor cells [12]. In

	BMI Index		
	Initial Medical Examination	Three month later	Six month later
Patient A	34.2	29.3	25.7
Patient B	37	32.2	27.8
Patient C	35	30.3	25.2
Average	35.4	30.6	26.2

Table 1: Body Mass Index. The table shows BMI of patients at initial medical examination, three and six months later.

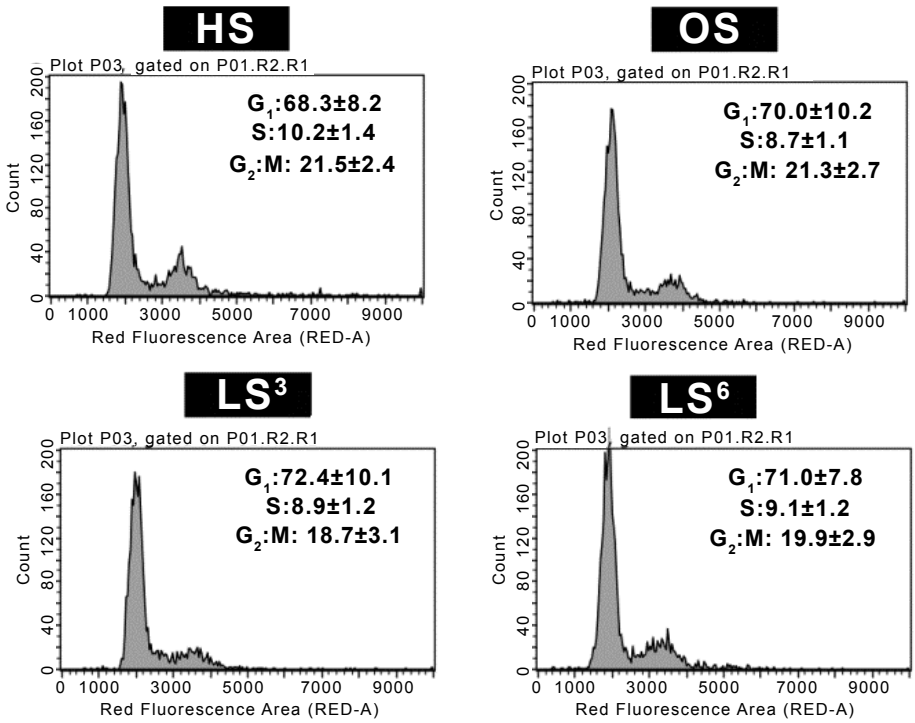

Figure 2: Cell cycle analysis. The picture shows a representative FACS analysis of HS, OS, LS^3 and LS^6 treated MSCs. The percentage of different cell populations (G_1, S and G_2/M) is indicated. Data are expressed with standard deviations. HS: Healthy Weight Serum; OS: Overweight Serum; LS^3 and LS^6: Sera from weight loss patients three and six months after first medical examination, respectively.

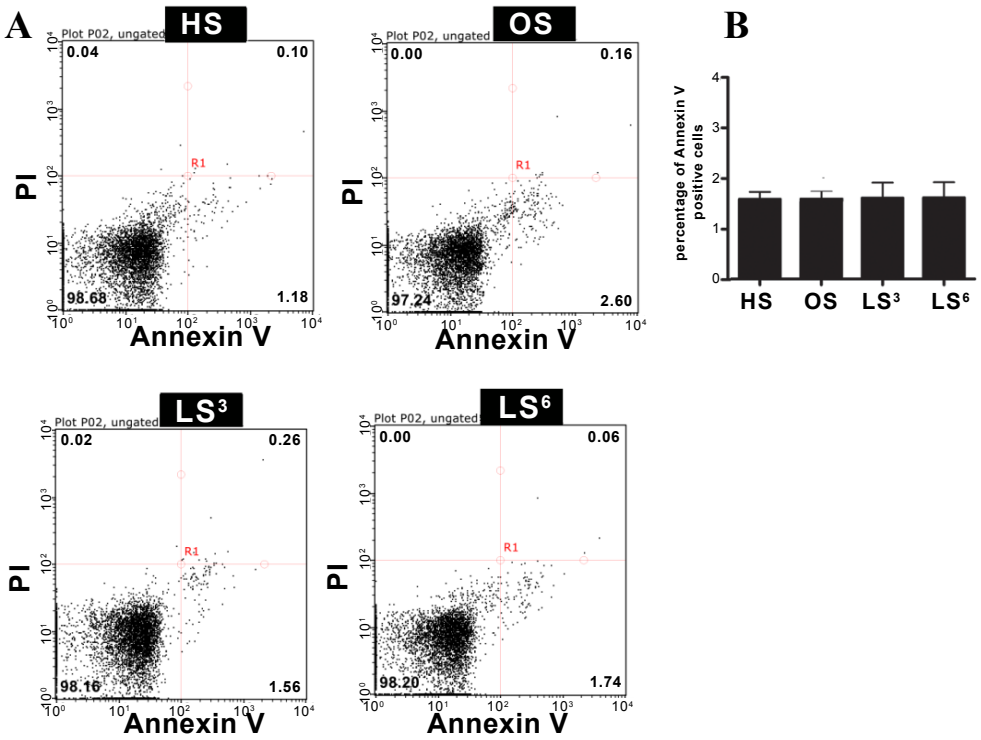

Figure 3: Analysis of apoptosis. Panel A: Flow cytometry analysis of apoptosis with Annexin assay. The assay allows the identication of early (Annexin V + and 7ADD-) and late apoptosis (Annexin V + and 7ADD +). Nevertheless, apoptosis is a continuous process and we calculated the percentage of apoptosis as the sum of early and late apoptotic cells, to avoid a discretional separation between early and late apoptosis. Panel B: The graph shows mean percentage value of apoptotic cells (± SD, n = 3). HS: Healthy Weight Serum; OS: Overweight Serum; LS^3 and LS^6: Sera from weight loss patients three and six months after first medical examination, respectively.

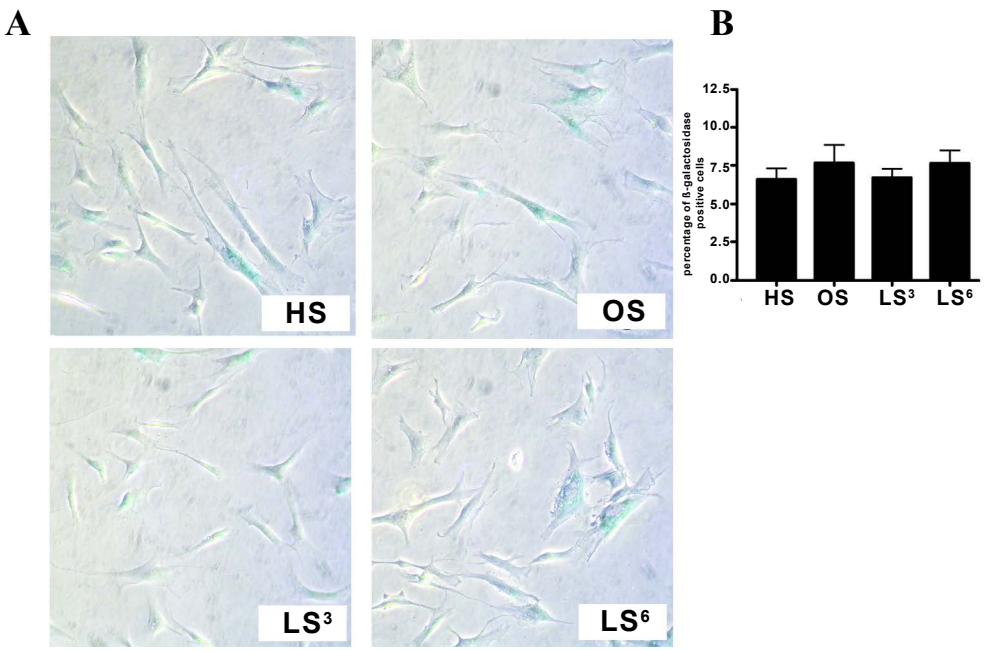

Figure 4: Senescence in MSC cultures. Panel A: Representative microscopic fields of acid beta-galactosidase positive cells (blue) in HS, OS, LS^3 and LS^6 treated cultures. Panel B: The graph shows mean percentage value of senescent cells (± SD, n = 3). HS: Healthy Weight Serum; OS: Overweight Serum; LS^3 and LS^6: Sera from weight loss patients three and six months after first medical examination, respectively.

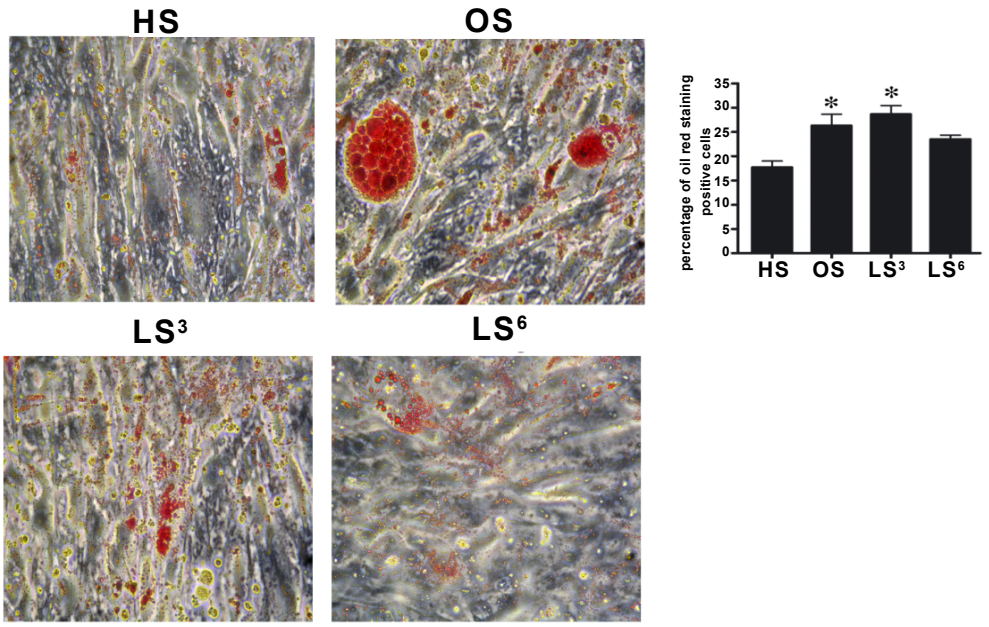

Figure 5: Adipogenic differentiation analysis. The graph shows the percentage of Oil Red O positive cells treated with HS, OS, LS^3 and LS^6 sera then induced to differentiate into adipocytes. The percentage of Oil Red O positive cells was calculated by counting at least 500 cells in different microscope fields. Data are expressed as mean values with standard deviations. Significant differ- ences (Student's t-test, $p<0.05$) in relative gene expression between OS, LS^3 and LS^6 respect to HS are marked by *. The picture shows a representative field of Oil-red positive cells. HS: Healthy Weight Serum; OS: Overweight Serum; LS^3 and LS^6: Sera from weight loss patients three and six months after first medical examination, respectively.

a previous paper, we investigated the effect of OS serum on MSCs. OS treatment induced a higher percentage of differentiated adipocytes than HS. We aimed to evaluate whether this negative effect persists after a weight loss.

Indeed, the increase in percentage of differentiated adipocytes remained also following incubation with sera of weight loss individuals compared with healthy sera treatment (Figure 5). Alizarin red staining did not exhibit significant differences in the osteogenesis process of MSCs incubated with HS, OS, LS3, and LS6, as shown in Figure 6. To gain further insights into osteocyte differentiation, we performed RT-

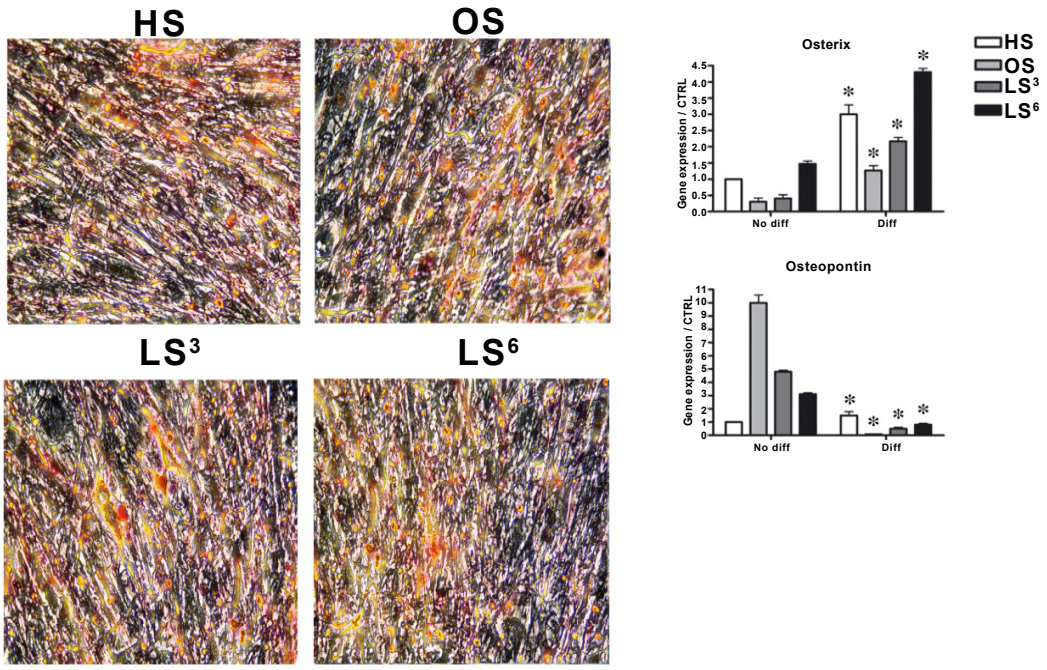

Figure 6: Osteogenic differentiation analysis. The graph represents the expression levels of Osterix and Osteopontin during osteocytes differentiation of MSCs treated with HS, OS, LS^3 and LS^6 sera. mRNA levels, expressed as arbitrary units, were normalised with respect to GAPDH which was chosen as an internal control. Significant differences (Student's t-test, $p<0.05$) in relative gene expression between differentiated and no differentiated MSCs are marked by *. The picture shows Alizarin red staining of MSCs treated with HS, OS, LS^3 and LS^6 sera and then induced to differentiate into osteocytes. HS: Healthy Weight Serum; OS: Overweight Serum; LS^3 and LS^6: Sera from weight loss patients three and six months after first medical examination, respectively.

qPCR expression analysis of early and late osteocyte differentiation markers [13-19]. This analysis showed that incubation of sera from overweight individuals altered the correct osteogenic differentiation of MSCs. Indeed, as expected, MSCs incubated with HS serum and then induced to differentiate into osteocytes showed an upregulation of Osterix and Osteopontin mRNAs, which are early and late maturation markers, respectively (Figure 6). Upregulation of Osterix levels was observed also in MSCs treated with OS, LS3, and LS6. In contrast, Osteopontin levels were elevated in undifferentiated MSCs treated with OS, LS3, and LS6, but declined to almost undetectable levels after induction of differentiation. This suggests that late differentiation is impaired.

Analysis of cytokine expression profiles in weight loss sera

Adipose tissue is an endocrine organ with a complex function in total body homeostasis with a key role in metabolism, food intake, and immunomodulation. Adipokines and cytokines are the signalling factors released from this tissue and play a role in maintaining health, but can contribute to pathologies associated with obesity.

We used the Human Obesity Antibody Array C1 (RayBiotech, Norcross, GA USA) to accurately profile the expression of the most cytokines involved in obesity. The expression levels of some cytokines did not differ significantly between the HS, OS, LS3, and LS6 samples. Others were expressed at very low levels in all experimental conditions and this did not allow their quantification. Some others showed significant changes in OS, LS3, and LS6 compared with HS serum. In order to better organize changes in expression profiles, we grouped cytokines in four classes, as shown in Table 2.

Class A contains cytokines that increased in OS compared to HS, then declined three months after first medical inspections before finally increasing again at six months. Class B presents cytokines that increased in OS serum and then declined progressively at LS3 and LS6. Class C has factors that increased in OS, then declined at LS3 without further decrease at LS6. Finally, Class D contains cytokines with variable expression patterns.

Some cytokine expression trends are in good agreement with physiological changes expected in overweight individuals that follow a diet regimen to lose weight. Others are at odds with this hypothesis as argued in detail in the discussion section.

Discussion

Adipose tissue behaves as an endocrine organ able to produce a variety of adipokines as well as proinflammatory chemokines and cytokines. Consequently, obesity is characterized from a condition of low-grade chronic inflammation to strikingly elevated concentrations of pro-inflammatory cytokines [13,14].

We previously showed that sera from overweight individuals might alter the differentiation potential of MSCs with a bias toward adipocytes differentiation. This was surprising since the analysed individuals did not show frank obese status and did not have any obese-related pathology, such as diabetes or cardiovascular diseases. Our data suggested that overweight status is not a mere aesthetic issue but may have consequences for patient health.

We aimed to evaluate if the "vicious circle" based on adipogenic promoting factors present in sera of overweight individuals, which in turn induces the release of other pro-adipogenic factors that may be arrested with diet regimen loading to weight loss. We then evaluated the effect of sera from previously overweight subjects on the differentiation potential of the MSC population. This research represents a pilot study due to the small number of evaluated cases.

	Cytokine	HS	OS	LS3	LS6
Class A	ADIPSIN ✚	163 ± 24.45	185 ± 18.5	76.5 ± 11.47 *	113 ± 12.43
	IGFBP2	143.5 ± 17.22	139 ± 20.85	74 ± 9.62 *	105.5 ± 11.60
	MSPα	578.5 ± 69.42	703 ± 98.42	236.5 ± 28.38 *	1376 ± 178.88 *
	RESIST✚	1299.5 ± 181.93	106 ± 11.66 *	0 ± 0	0 ± 0
	TIMP1	124±12.4	99.5 ± 12.93	25 ± 3.25 *	42 ± 5.04 *
Class B	ADIPONECTIN ✚	2569±256.9	3023.5 ± 393.05	1541 ± 184.92 *	494.5 ± 74.17 *
	PDGFAA	0 ± 0	178 ± 26.7 *	74 ± 10.36 *	13.5 ± 2.02 *
	LEPTIN ✚	59.5 ± 5.95	199±27.86 *	43.5 ± 5.65	0 ± 0
	TIMP2	130.5 ± 15.66	301.5±45.22 *	45.5 ± 5.0 *	0 ± 0
Class C	PDGFBB	160 ± 20.8	214 ± 32.1	20 ± 2.2 *	22.5 ± 3.37 *
	PDGFAB	180 ± 25.2	234 ± 25.74	49 ± 5.88 *	53 ± 97.42 *
	IL-6	196.5 ± 21.61	147 ± 19.11	87.5 ± 10.5 *	85 ± 8.5 *
	MCP1	152 ± 19.76	136.5 ± 19.11	0 ± 0	0 ± 0
	RANTES	169.5 ± 20.34	980.5 ± 107.85 *	150.5 ± 19.56	168 ± 9.65
Class D	PAI	87.5 ± 11.37	59.2 ± 8.88	34.5 ± 4.83 *	52 ± 7.8
	IGFBP1	32 ± 4.48	26.5 ± 3.18	0 ± 0	0 ± 0
	IL-8	34 ± 3.74	91 ± 14.56 *	244 ± 31.72 *	87 ± 9.57 *

* p <0.05 compared to healthy people.

Table 2: Cytokines detection in sera. The table shows the cytokines on the Human Obesity Antibody Array C1 (RayBiotech, Norcross, GA USA) that were detected in HS, OS, LS^3 and LS^6 sera and grouped in four classes. Array signals were acquired using the Chemidoc system (Bio-Rad) and the associated software QuantityOne. Data are expressed as arbitrary units ((± SD, number of experiment replicates: three). HS: healthy weight serum; OS: overweight serum; LS^3 and LS^6: sera from weight loss patients three and six months respectively after first medical examination. ✚ Cytokines related to metabolism; v Cytokines related to proliferation, survival and cellular differentiation.

To our surprise, sera from weight loss individuals also promoted adipogenesis and impaired osteocyte differentiation process. This effect was cell-commitment and cell differentiation specific, since cell cycle status, apoptosis, and senescence were not affected.

This finding may suggest that overweight status may induce epigenetic changes [15], which persist even after weight loss. It should be evaluated whether this "memory" can persist for periods longer than three and six months, which were our time points.

It remains to define:

i) The type of epigenetic modifications and other permanent physiological events;

ii) Which cell types undergo these persistent alterations; and

iii) The consequences on body physiology of these modifications.

We started to address this last issue by analysing changes in the levels of obesity-related circulating cytokines in overweight and weight loss subjects compared to healthy weight people.

To create a clearer picture of occurring changes, cytokines were classified into three groups: pro-inflammatory cytokines, cytokines related to metabolism, and cytokines relative to proliferation, survival, and cellular differentiation. The observed changes allowed a further classification into four classes (A, B, C and D) with different expression level patterns (see results).

Cytokines related to inflammation

RANTES, IL-6, and MCP-1 levels were lower at LS3 and remained constant at LS6 compared to OS. This conforms with a reduction of inflammation status. It should be noted that MCP-1 and IL-6 were not significantly increased in overweight people compared to controls as described for obese patients. It is unclear why MCP-1 and IL-6 levels decreased more in weight loss individuals than in the controls. Further investigation is necessary.

According to Helmersson-Karlqvist et al., TIMP1 and TIMP2 have important roles in tissue remodelling and are implicated in a number of diseases related to inflammation [16]. Therefore, the decrease of their levels during weight loss is in agreement with a reduction of inflammatory status.

Results showed a down regulation of PAI levels, a protein that is associated with cardiovascular diseases [17]. This data is in good agreement with a reduction of inflammatory status.

Changes in the expression profile of other inflammatory cytokines such as MSP-α and IL-8 cannot be directly linked to a reduction of inflammation as hypothesised that it may occur in people undergoing weight loss [18-20]. Further studies are needed.

Cytokines related to metabolism

We obtained conflicting data on the cytokines that modulate metabolism. While changes in leptin and adipsin correlate with the literature, RESISTIN and ADIPONECTIN showed more complex expression patterns.

Leptin is an important hormone that shapes appetite and hunger signals and it is secreted primarily in fat cells, as well as the stomach, heart, placenta, and skeletal muscles. Leptin decreases hunger and usually also correlates to fat mass, as the more fat is present the more leptin is produced [21]. In this way, our data evidenced an increase of leptin in overweight patients compared to control and its decrease during weight loss.

Complement plays important roles in host immune defences, and recent studies suggest that adipose tissue is an important site of production for some complement proteins like Adipsin (Factor D), a rate-limiting enzyme in the alternative complement activation pathway. Levels of alternative pathway complement components are determined in part by factors that influence body weight and by weight modifications, possibly due to changes in production in adipose tissue or at other sites [22]. Indeed, body weight reduction connotes lower levels of Adipsin at LS3 and LS6.

Resistin (or 'resistance to insulin') has been suggested to be an

important link between obesity and metabolic syndrome. Nevertheless, its role in human obesity remains unclear. Indeed, studies in humans are controversial: in several investigations, serum resistin was reported to be related to fat mass [23] while other reports reveal no correlations to BMI and per cent body fat [24].

During weight loss, we observed a decrease in resistin as if its level was associated with fat mass. Nevertheless, resistin level was very high in controls compared to overweight, LS3, and LS6.

Only a few studies have examined changes in adiponectin levels with body weight reduction in humans [25]. In these investigations, large losses of body weight resulted in significant increases in plasma adiponectin concentrations. However, whether smaller losses of body weight elicit a change in adiponectin concentrations is unclear. In LS3 and LS6 sera, contrarily, we observed a decrease of adiponectin values.

Cytokines related to proliferation, survival, and cellular differentiation

Endogenous PDGFs (PDGFAA, PDGFBB, and PDGFAB) have the ability to influence the proliferation and differentiation of human adipose derived stem cells and progenitors. Down regulation of PDGFs levels in LS3 and LS6 suggests that weight loss is associated with a reduced proliferation stimulus to adipocytes [26].

In humans, there are six IGFBP genes encoding a family of highly conserved proteins (including IGFBP1 and IGFBP-2) that have high affinity binding for the insulin-like growth factors (IGFs). Secretion of IGFBP-1 and IGFBP-2 is suppressed by insulin and diminished with increasing obesity. Whereas our data are in contrast with the literature [27,28] further investigation will be needed to understand the decline in LS subjects.

Conclusion

The findings of this study indicate that increase in fat mass induces physiological changes that negatively affect the functions of stem cells involved in adipogenesis and osteogenesis. This phenomenon occurs in overweight people and persists for several months after weight loss. On this premise, physicians should suggest that overweight patients be cautious about their health even if they do not exhibit any obesity-related diseases.

Suggestions to improve lifestyle and weight loss are even more compelling considering the significant changes in obesity related cytokines we detected.

To gain further insights on the complex phenomena documented here, we aim to prosecute this pilot study with a larger cohort of patients.

Acknowledgments

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