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Effects of novel antidepressant drugs on mesenchymal stem cell physiology

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ABSTRACT

It is known that users of psychotropic drugs often have weight gain, adverse effects on bone mineral density and osteoporosis, but the molecular basis for these side effects is poorly understood. The aim of this study is to evaluate the effects *in vitro* of duloxetine (a serotonin and norepinephrine reuptake inhibitor) and fluoxetine (a selective serotonin reuptake inhibitor) on the physiology of human adult stem cells. Adipose-derived stem cells (ADSCs) were isolated and characterized investigating phenotype morphology, expression and frequency of surface markers. Then, a non-toxic concentration of duloxetine and fluoxetine was selected to treat cells during adipogenic and osteogenic differentiation. Stemness properties and the differentiation potential of drug-treated cells were investigated by the quantification of adipogenic and osteogenic markers gene expression and histological staining. The collected data showed that the administration of a daily non-toxic dose of duloxetine and fluoxetine has not directly influenced ADSCs proliferation and their stemness properties. The treatment with duloxetine or fluoxetine did not lead to morphological alterations during adipogenic or osteogenic commitment. However, treatments with the antidepressant showed a slight difference in adipogenic gene expression timing. Furthermore, duloxetine treatment caused an advance in gene expression of early and late osteogenic markers. Fluoxetine instead caused an increase in expression of osteogenic genes compared to untreated cells. In contrast, in pre-differentiated cells, the daily treatment with duloxetine or fluoxetine did not alter the expression profile of adipogenic and osteogenic differentiation. In conclusion, a non-toxic concentration of duloxetine and fluoxetine does not alter the stemness properties of ADSCs and does not prevent the commitment of pre-differentiated ADSCs in adipocytes or osteocyte. Probably, the weight gain and osteoporotic effects associated with the use of psychotropic drugs could be closely related to the direct action of serotonin.

1. Introduction

According to data from the World Health Organization, Major Depressive Disorder has become the second most prevalent cause of illness-induced disability, affecting 350 million people worldwide. Since the US Food and Drug Administration (FDA)'s approval of fluoxetine in 1988, selective serotonin reuptake inhibitors (SSRIs) have been the most prescribed antidepressants on the market. Their action is to inhibit serotonin (5-hydroxytryptamine, 5HT) uptake provoking fewer side effects than older generation antidepressants (monoamine oxidase inhibitors and tricyclics). After the successful development of SSRIs, it was proposed that the simultaneous action of selective noradrenaline and serotonin reuptake could provide better efficacy. So the discovered serotonin and norepinephrine reuptake inhibitors (SNRIs),

Abbreviations: ADIPOQ, adiponectin; ADM, Adipogenic Differentiation Medium; ADSCs, Adipose-derived-Stem Cells; ALCAM, activated leukocyte cell adhesion molecule; ALPL, alkaline phosphatase; ARS, Alizarin Red S; BM, basal medium; BMP2, bone morphogenetic protein 2; DMEM, Dulbecco's modified Eagle's medium; FABP4, fatty acid binding protein 4; FBS, Fetal Bovine Serum; GLUT4, insulin-responsive glucose transporter type 4 (GLUT4); HBSS, Hanks' Balanced Salts Solution; ITGB1, Integrin Subunit Beta 1; OC, osteocalcin; OD, Optical Density; ODM, Osteogenic Differentiation Medium; OPN, osteopontin; ORO, Oil Red O; OSX, osterix; PBS, Phosphate-buffered Saline; PPARG, peroxisome proliferator-activated receptor gamma; PS, penicillin/streptomycin; RANKL, receptor activator of nuclear factor kappa B ligand; RUNX2, runt related transcription factor 2; SNRIs, serotonin and norepinephrine reuptake inhibitors; SSRIs, selective serotonin reuptake inhibitors; TFRC, transferrin receptor 1; THY1, Thy-1 cell surface antigen

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such as duloxetine and venlafaxine, demonstrated to be effective and to present a better profile of adverse drug reactions than tricyclics [1]. Serotonin is a regulator of mood, but also plays an important role in functions such as appetite, bone metabolism, sleep, sex, and temperature. Antidepressant treatments that act on serotonin pathways may therefore be expected to have some impact on body weight and bone mass. Weight change occurring during antidepressant treatment remains an important risk that may lead patients to discontinue the drugs and to relapse becoming a threatening public health hazard with serious consequences in chronic metabolic conditions [2,3]. Research has concentrated on central and peripheral mechanisms of appetite, direct influence of drugs on fat storage of adipocytes, modulation of hormonal signaling of ghrelin and leptin, changes in the production of cytokines, the impact of genes, but the mechanisms underlying antidepressant-induced weight gain is still unclear. Because of heterogeneity in study designs and clinical samples it is very difficult to make precise considerations on which antidepressant is most responsible to cause weight gain [4]. Nevertheless, a systematic review by Dent et al. [5] showed that venlafaxine, fluoxetine, and sertraline have minimal effects on weight gain. Instead, an analysis of ten clinical studies by Wise et al. [6] indicated that long-term use of duloxetine and paroxetine were significantly associated with weight gain. These observations were confirmed by a recent clinical study [7] in which citalopram, escitalopram, sertraline, paroxetine, venlafaxine, duloxetine, and mirtazapine, but not fluoxetine, were associated with significant weight gain. Furthermore, the meta-analysis study conducted by Serretti and Mandelli demonstrated that fluoxetine causes weight loss during the acute phase of treatment. Instead, the other antidepressants have no transient or negligible effect on body weight in the short term [8]. Since only fluoxetine has shown an anti-obesity effect, the FDA only approves the administration of fluoxetine for the treatment of bulimia nervosa [9]. But SSRIs are also prescribed for certain non-psychiatric conditions, including chronic pain, fibromyalgia and post-menopausal vasomotor symptoms, such as night sweats and hot flashes [10]. In recent years, multiple clinical studies have reported a positive association between the use of SSRIs and a decrease in bone mineral density and an increase in risk of fractures [10–12]; this association was confirmed in multiple rodent models, but the molecular basis for these phenomenon is poorly understood [13–15]. These studies mostly investigate the effect of fluoxetine on bone density, as it is the most prescribed SSRIs. On the contrary, few studies have been done to determine if SNRIs have a negative effect on bone metabolism [16,17].

Based on these considerations, the present study aims to determine the effect of duloxetine and fluoxetine on the physiology and differentiation of human mesenchymal stem cells. Firstly, we have isolated adipose-derived stem cells (ADSCs) from human fat tissue, and examined their features investigating phenotype morphology, expression and frequency of surface markers. Then, we have selected a tolerated concentration of duloxetine and fluoxetine to treat cells during adipogenic and osteogenic differentiation. Finally, we have investigated the stemness properties and the differentiation potential in drug-treated cells by quantification of gene expression and histological staining.

2. Materials and methods

2.1. Study design and setting

Native, adipogenic or osteogenic committed and pre-differentiated Adipose-derived Stem Cells (ADSCs) were treated with duloxetine and fluoxetine. In the case of native and committed ADSCs, from the first day of cell culture duloxetine or fluoxetine were added to the medium. Instead, in the case of pre-differentiated ADSCs, cells were cultured in differentiation medium for 7 days and then treated with duloxetine or fluoxetine until the fourtheenth day for adipogenic commitment and until the twenty-first day for osteogenic commitment. The experimental design is represented in Fig. 1.

2.2. ADSCs isolation

Human adult stem cells were isolated from human adipose tissues of healthy patients (age: 21–36; BMI: 30–38) undergoing cosmetic surgery procedures, following the guidelines of the University of Padova's Plastic Surgery Clinic. As previously described elsewhere [18–20], the adipose tissues were digested with 0.075% collagenase type II (Sigma Aldrich, Saint Louis, Missouri, USA) in Hanks' Balanced Salts Solution (HBSS, Euroclone, Milano, Italy) for 2 h at room temperature. Cells from the stromal-vascular fraction were pelleted and rinsed with Phosphate-buffered Saline (PBS, EuroClone). Red blood cells contamination were deleted by a step in Red blood cells lysis buffer (Sigma Aldrich) run for 10 min at room temperature. The resulting viable cells were counted using the trypan blue exclusion assay and seeded at a density of 5×10^4 cells/cm^2 in Basal Medium (BM) consisting of Dulbecco's modified Eagle's medium (DMEM, Euroclone) supplemented with 10% Fetal Bovine Serum (FBS, EuroClone), and 1% penicillin/streptomycin (PS, EuroClone). ADSCs were maintained at 37 °C and 5% CO_2, and culture medium was changed twice a week.

2.3. ADSCs characterization

ADSCs within 3–5 passages were harvested by trypsin treatment (trypsin/EDTA, EuroClone), then counted under Bürker Chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany).

For immunofluorescence staining, 2×10^4 cells/cm^2 were seeded on glass coverslips put into 24-well plates and cultured in BM. The following day, cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 10 min, then permeabilized for 10 min in 0.5% Triton X-100 (Sigma-Aldrich). After three washing, cells were incubated in 1% bovine serum albumin (BSA; Sigma-Aldrich) solution in PBS for 30 min at RT. The cells were then stained with Alexa Fluor™ 555 Phalloidin (Thermo Fisher Scientific) for 20 min at RT. Nuclear staining was performed with NucBlue™ Fixed Cell Ready Probes™ Reagent (DAPI; Thermo Fisher Scientific). The cells were observed with the Upright Microscope ECLIPSE Ni Series (Nikon).

For flow cytometry, cells were dissociated and resuspended in flow cytometry staining buffer (R&D Systems, Minneapolis, MN, USA) at a final cell concentration of 1×10^6 cells/mL. Cells were incubated with the following fluorescent monoclonal mouse anti-human antibodies (eBioscience™, Thermo Fisher Scientific): CD14 R-PE; CD34 FITC; CD44 FITC; CD45 APC; CD73 APC; CD90 R-PE; CD105 R-PE; HLA-DR FITC. Cells were washed twice with 2 mL of flow cytometry staining buffer and resuspended in 500 µL of flow cytometry staining buffer. Fluorescence was evaluated by flow cytometry in Attune NxT flow cytometer (Thermo Fisher Scientific). Data were analyzed using Attune NxT software (Thermo Fisher Scientific).

2.4. ADSCs culture and drug-treatment

In order to identify a concentration of duloxetine and fluoxetine tolerated by ADSCs, cells were seeded at a density of 2×10^4 cells/cm^2 in 24-well plates, and treated with different concentrations of drugs (1, 2,5, 5, 10, 25, 50, 100 µM) in BM for 24 h. Then, cell viability was calculated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) analysis. Afterwards, a single concentration was chosen to test the effects of duloxetine e fluoxetine on adipogenic and osteogenic commitment. The cell cultures were incubated in BM (control condition), Adipogenic Differentiation Medium (ADM) or Osteogenic Differentiation Medium (ODM) up to 14 or 21 days. ADM was made of DMEM supplemented with 10% FBS, 1% PS, 10 µg/mL insulin (Sigma-Aldrich), 0.5 mM isobutylmethylxanthine (Sigma-Aldrich), 0.1 mM indomethacin (Sigma-Aldrich), and 1 µM dexamethasone (Sigma-Aldrich). ODM was composed of DMEM supplemented with 10% FBS, 1% PS, 10 ng/mL Fibroblast Growth Factor 2 (ProSpec, East Brunswick, NJ, USA), 10 mM beta-glycerophosphate (Sigma-Aldrich), and 10 nM

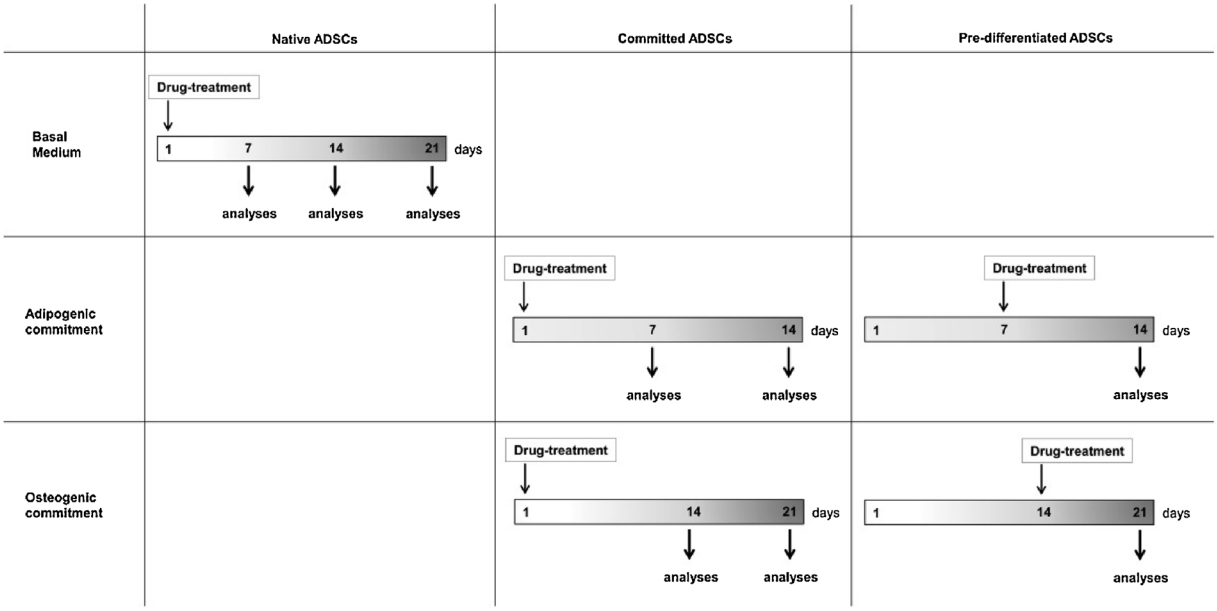

Fig. 1. Schematic representation of experimental setting. The drug-treatment with duloxetine or fluoxetine in native and committed ADSCs starts at the first day of cell culture. In pre-differentiated ADSCs drug-treatment starts after 7 days of culture for adipogenic commitment, and after 14 days for osteogenic commitment.

Table 1
Human primer sequences.

Gene	Sequences (5'- 3')	Amplicon (pb)
ADIPOQ	GTTGTGTGCCTGTTTCTGACC GCATCTATCATCCACTCTCCTATTTCTG	153
ALCAM	GTGGAGAAGTGACTAGACAGATTGG CAGTTTCGCAGACATAGTTTCCAG	168
ALPL	GGCTTCTTCTTGCTGGTGGA CAAATGTGAAGACGTGGGAATGG	181
BMP2	CCACTAATCATGCCATTGTTCAGAC CTGTACTAGCGACACCCACAA	181
CD44	CATCTACCCCAGCAACCCTA CTGTCTGTGCTGTCGGTGAT	153
FABP4	TGACCTGGACTGAAGTTCGC AAGCACAATGAATACATCATTACATCACC	193
GLUT4	CCAGTATGTTGCGGAGGCTA TCAAGTTCTGTGCTGGGTTTCA	189
ITGB1	TGCAACAGCTCTCACCTACG GTGAAACAAGATGGGCAACTCA	100
OC	GCAGCGAGGTAGTGAAGAGAC AGCAGAGCGACACCCTA	193
OPN	TGGAAAGCGAGGAGTTGAATGG GCTCATTGCTCTCATCATTGGC	192
OSX	TCAGAATCTCAGTTGATAGGGTTTCTC GGGTACATTCCAGTCCTTCTCC	183
PPARG	CAGGAGATCACAGAGTATGCCAA TCCCTTGTCATGAAGCCTTGG	173
RANKL	TCAGCATCGAGGTCTCCAAC CCATGCCTCTTAGTAGTCTCACA	194
RUNX2	AGCCTTACCAAACAACACAACAG CCATATGTCCTCTCAGCTCAGC	175
TFRC	TGTTTGTCATAGGGCAGTTGGAA ACACCCGAACCAGGAATCTC	222
THY1	CAGCATTCTCAGCCACAACC CCTCATCCTTTACCTCCTTCTCCA	154

dexamethasone [21,22]. Drug treatments were performed daily with duloxetine or fluoxetine at the final concentration of 5 μM.

2.5. ADSCs viability assay

To assess the ADSCs viability, the MTT assay was performed on native cells treated with different concentration of drugs, according to the method described by Denizot and Lang with minor modifications [23]. After removing the culture medium, cells were incubated in 1 mL of 0.5 mg/mL MTT in PBS for 3 h at 37 °C. The MTT solution was then removed, and each sample was extracted with 0.5 mL of 10% dimethyl sulfoxide in isopropanol for 30 min at 37 °C. For each sample, Optical Density (OD) values, at 570 nm, were recorded in duplicate on 200 μL aliquots using a multilabel plate reader (Victor 3, Perkin Elmer, Milan, Italy).

2.6. Real-time PCR

Total RNA was extracted with Total RNA Purification Plus Kit (Norgen Biotek Corporation, Ontario, Canada), according to the manufacture procedures. The RNA purified from genomic DNA contamination were stored at −80 °C until the use. 500 ng of total RNA of each sample was reverse transcribed with SensiFAST™ cDNA Synthesis kit (Bioline GmbH, Germany) in a LifePro Thermal Cycler (Bioer Technology, China) following the manufacture conditions: annealing at 25 °C for 10 min, reverse transcription at 42 °C for 45 min, and inactivation at 85 °C for 5 min. The resultant cDNA samples were stored at −20 °C until the next use. Real-time PCR of genes involved in adipogenic and osteogenic differentiation were investigated. Human primers were selected for each target gene with Primer 3 software (Table 1). Real-time PCR was carried out using the designed primers at a concentration of 400 nM and SensiFAST™ SYBR No-ROX mix (Bioline GmbH) on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Thermal cycling conditions were as follows: denaturation at 95 °C for 2 min; followed by 40 cycles of denaturation at 95 °C for 5 s; annealing at 60 °C for 10 s; and elongation at 72 °C for 20 s. Data analysis was performed using the classic, well-established, and widely adopted 2ΔΔCt method [24]. Ct values of target genes were normalized to that of housekeeping gene (TFRC: transferrin receptor 1). The relative gene expression between control group (ADSCs in BM) and test group (committed ADSCs in ADM/ODM; committed ADSCs in ADM/ODM plus duloxetine/fluoxetine; pre-differentiated ADSCs in ADM/ODM plus duloxetine/fluoxetine) was calculated. Results were reported as fold regulation of target genes in test group compared with ADSCs in BM. Fold regulation values greater than 2 indicate increased gene expression, fold-regulation values less than -2 indicate decreased gene expression, and fold-regulation values between -2 and 2 indicate indifferently expressed genes. Experiments were performed with 3

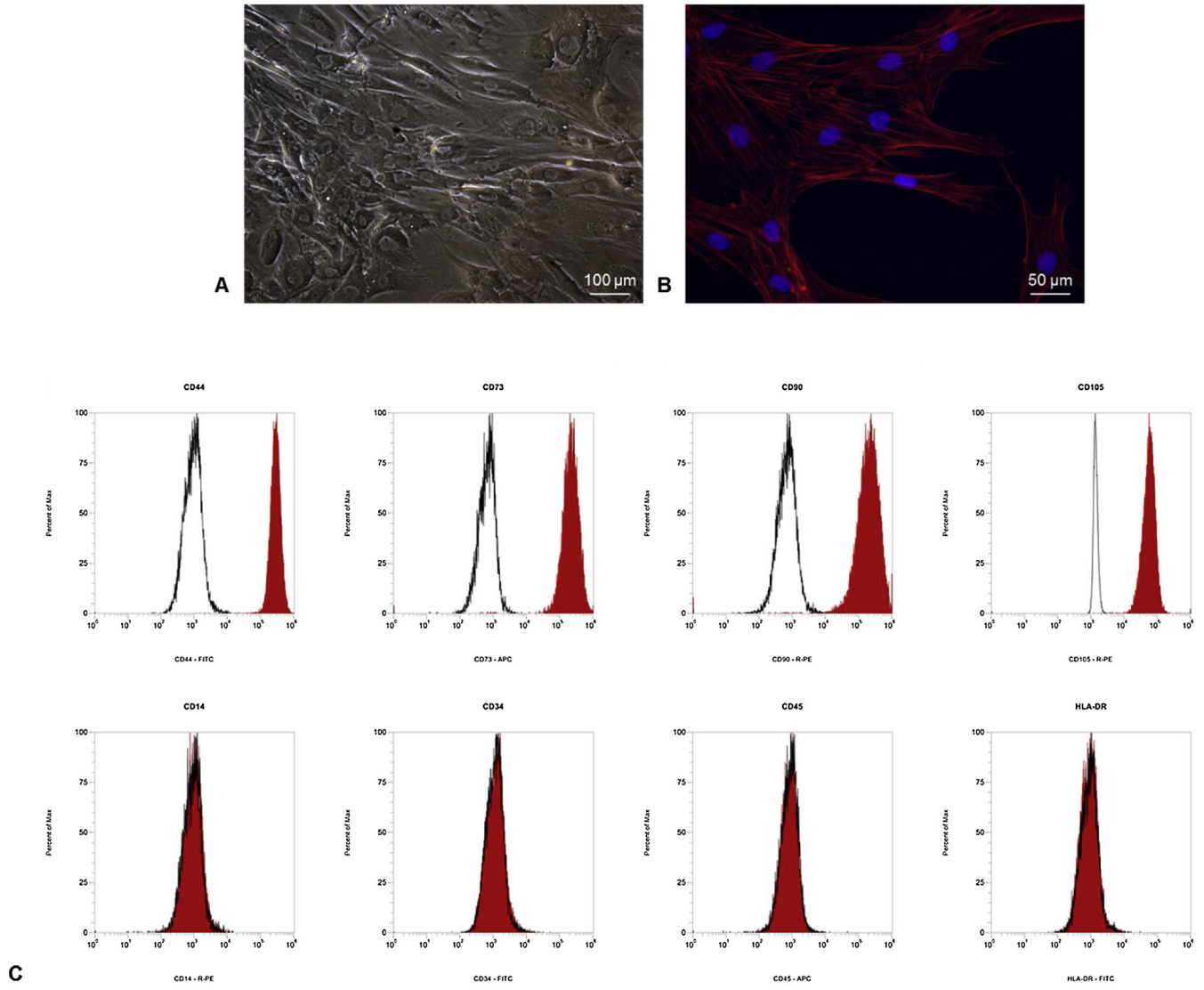

Fig. 2. Characterization of adipose-derived stem cells (ADSCs). (A) Phase-contrast microscopy image of ADSCs showing the adhesion of cells to cell culture plastic (magnification 20×). (B) Immunofluorescent image showing the spindle-shaped fibroblast-like morphology of ADSCs (magnification 40×): the actin filaments are stained with phalloidin (in red) and cell nuclei are counterstained with DAPI (in blue). (C) Detection of cell surface markers in ADSCs by flow cytometry: ADSCs are positive to CD44, CD73, CD90, and CD105, and negative to CD14, CD34, CD45, and HLA-DR (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 2
Cell surface marker expression of isolated ADSCs.

Surface Marker	% expression	Surface Marker	% expression
CD44	99,932 ± 0,365	**CD14**	0,052 ± 0,084
CD73	99,817 ± 0,282	**CD34**	0,127 ± 0,022
CD90	99,645 ± 0,272	**CD45**	0,000 ± 0,022
CD105	99,799 ± 0,286	**HLA-DR**	0,013 ± 0,011

Data are displayed as percentages expressed as mean ± standard deviation (SD).

different cell preparations and repeated at least 3 times.

2.7. Oil Red O staining and quantification

The detection of intracellular lipid droplets was performed by Oil Red O (ORO) staining [25]. An ORO (Sigma Aldrich) stock solution was made dissolving the powder in isopropanol at the concentration of 3.5 mg/mL, then an ORO working solution was prepared adding 3 parts of ORO stock solution to 2 parts of distilled water. Cells were stained with 0.5 mL of fresh ORO working solution for 15 min at room temperature. After 4 washes with distilled water, phase-contrast images were taken and ORO staining was extracted with 0.25 mL 100% isopropanol. For each sample, OD values at 490 nm were measured by a multilabel plate reader (Victor 3 Perkin Elmer).

2.8. Alizarin Red S staining and quantification

The detection of extracellular mineral deposits were performed by Alizarin Red S (ARS) staining [25]. Cells were stained with 40 mM ARS solution pH 4.2 (Sigma Aldrich) for 10 min at room temperature with gentle shaking. After 4 washes with distillated water, phase-contrast images were taken. 0.5 mL of 10% cetylpyridinium chloride (CPC, Sigma Aldrich) in 10 mM sodium phosphate solution was used to extract ARS staining. For each sample, OD values at 570 nm were measured with a plate reader (Victor 3 Perkin Elmer).

2.9. Statistical analysis

Each cell type was plated in triplicates with appropriate controls.

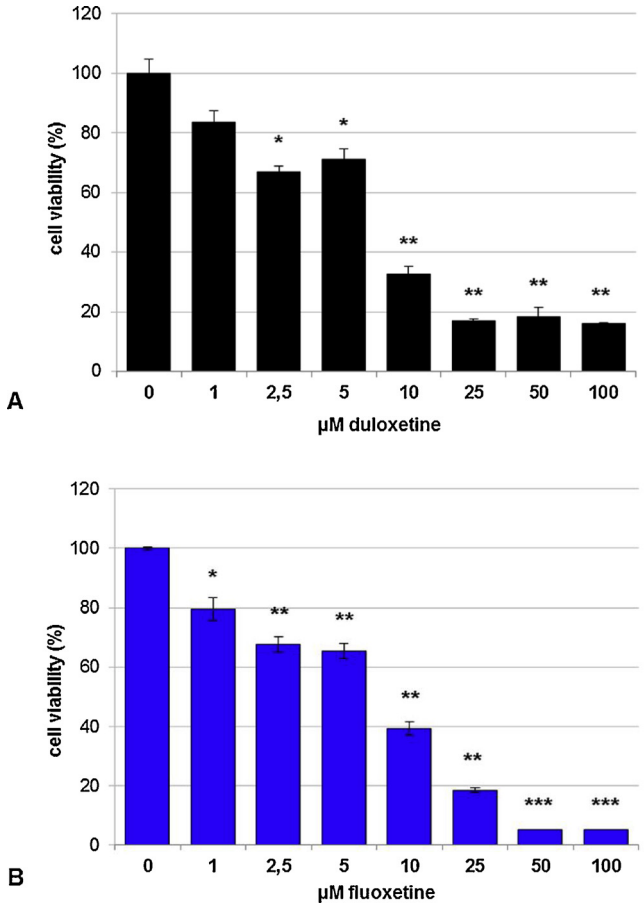

Fig. 3. Cell viability after 24 h of treatment with different drug concentrations from 0 to 100 µM. (A) Cell viability of native ADSCs treated with duloxetine; (B) Cell viability of native ADSCs treated with fluoxetine. The cell viability was calculated by MTT assays. Data are presented as mean ± standard error (3 independent experiments) * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Each experiment was performed independently three times. Results were expressed as mean ± standard deviation (SD). Comparative analysis was performed by two-way analysis of variance followed by post-hoc Bonferroni test. Student's *t*-test was performed to determine the statistical significance between two groups. Real-time PCR data were presented as fold regulation relative to control (cells in BM). The statistical level of significance was set at 0.05; different labels indicate * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3. Results

3.1. Isolation and characterization of ADSCs

Adipose derived stem cells (ADSCs) were isolated from human fat tissue by enzymatic digestion and plated under basal conditions in cell culture flasks. Cells have adhered to flask's plastic to form a monolayer of cells with a spindle-shaped fibroblast-like morphology (Fig. 2A). The cellular morphology was detected by immunostaining the actin filaments with phalloidin (Fig. 2B). The characterization of cell surface antigens by flow cytometry has shown that ADSCs were positive to the mesenchymal stem cells markers CD44, CD73, CD90, and CD105, and negative for the hematopoietic markers CD14, CD34, CD45, and HLA-DR (Fig. 2C). The percentages of isolated ADSCs expressing cell surface markers are reported in Table 2.

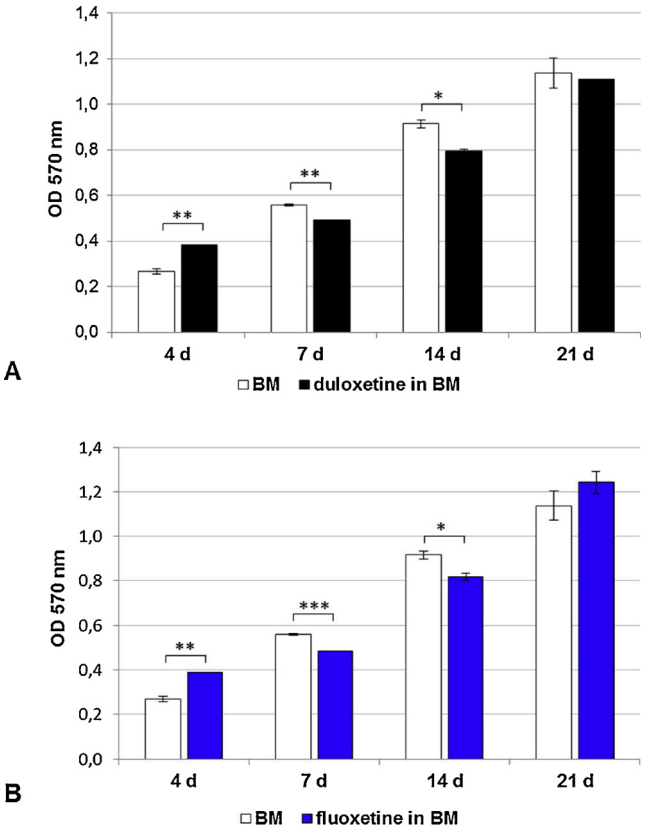

Fig. 4. Cell viability after 4, 7, 14, and 21 days of drug-treatment. (A) MTT assay of ADSCs cultured in BM plus 5µM duloxetine (black bars); (B) MTT assay of ADSCs cultured in BM plus 5µM fluoxetine (blue bars). ADSCs cultured in BM (white bars) represents the control condition. Data presented as mean ± standard error (3 measurements) * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.2. Effects of drug-treatment on stem cell proliferation and stemness properties

In order to identify a drug concentration tolerated by cells, ADSCs were treated with various concentrations of duloxetine or fluoxetine, ranged from 0 to 100 µM. Cells were incubated for 24 h, then the cell viability was calculated by MTT analysis (Fig. 3). As shown in Fig. 3A, with the increasing of duloxetine concentration, a progressive reduction in cell vitality is occurred. In particular, concentration of duloxetine greater than 5 µM determined a cell viability lower than 50% compared to the control condition (0 µM duloxetine). Also, concentrations of fluoxetine > 5 µM resulted in a 50% lower cell viability compared to the untreated control (Fig. 3B).

Additionally, ADSCs were treated daily with 5µM duloxetine or 5µM fluoxetine up to 21 days. Cell viability after 4, 7, 14, and 21 days by MTT assays was evaluated (Fig. 4). The proliferation rate of drug-treated cells revealed a similar trend in both cases. Compared to the control condition, duloxetine treatment caused a significant augmentation ($p < 0.01$) in cell proliferation on the fourth day, followed by a significant reduction after 7 and 14 days of treatment ($p < 0.01$ and $p < 0.05$, respectively). After 21 days of treatment no significant difference compared to control were detected (Fig. 4A). Also fluoxetine-treated cells (compared to no drug-treated cells) showed a significant augmentation ($p < 0.01$) in proliferation at the first time point, and then a significant reduction on the seventh and fourteenth day ($p < 0.001$ and $p < 0.05$, respectively), followed by a slight augmentation after 21 days of treatment (Fig. 4B).

To assess drug-treatment effects on stemness properties of human

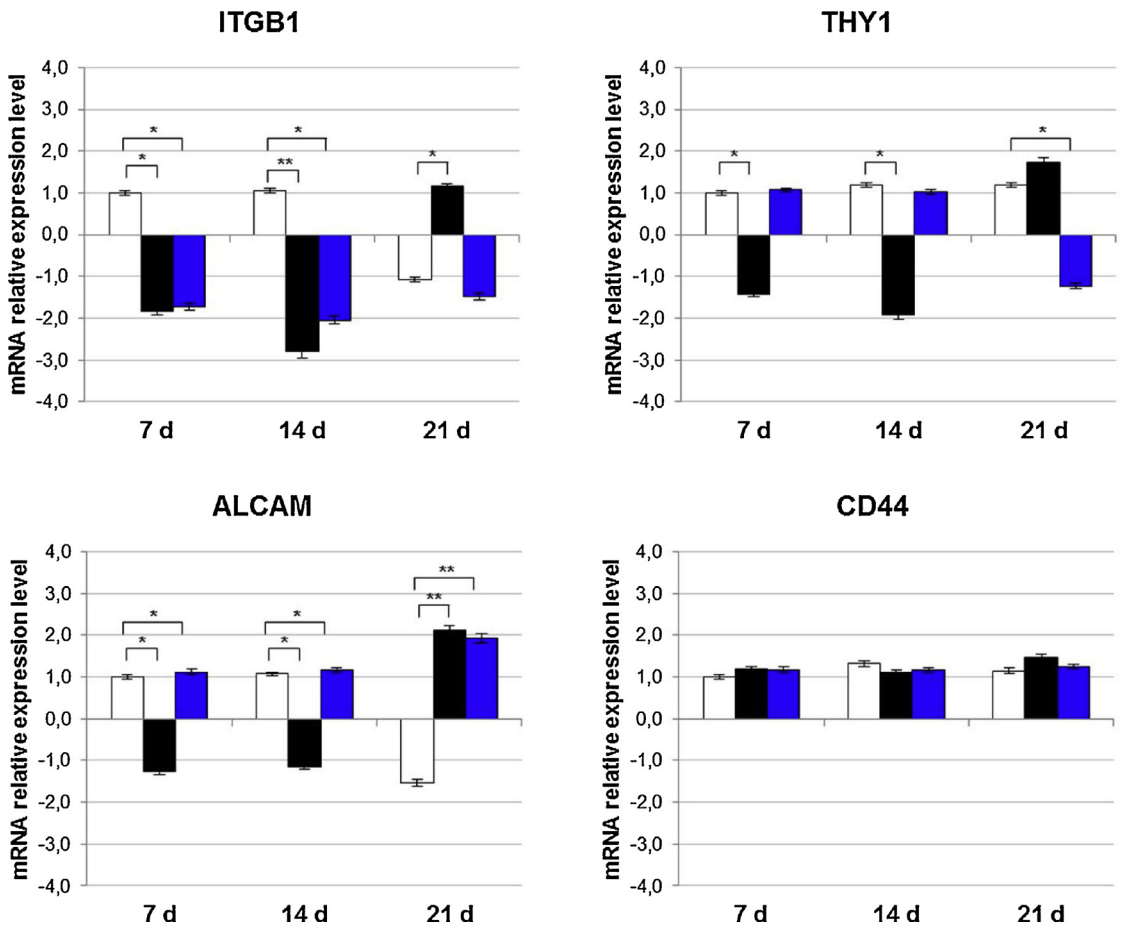

Fig. 5. Gene expression of mesenchymal stromal cell markers in human ADSCs maintained in BM (white bars), BM plus 5μM duloxetine (black bars), or BM plus 5μM fluoxetine (blue bars) for 7, 14, and 21 days. The results are reported as fold regulation respect to the mRNA expression of ADSCs in BM for 7 days * $p < 0.05$; ** $p < 0.01$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

ADSCs, a gene expression analysis of mesenchymal stromal cell markers was performed. In particular, we tested the expression of Integrin Subunit Beta 1 (ITGB1, alias CD29), Thy-1 cell surface antigen (THY1, alias CD90), activated leukocyte cell adhesion molecule (ALCAM), and CD44 in ADSCs maintained in BM supplemented with 5μM duloxetine or 5μM fluoxetine for 7, 14, and 21 days (Fig. 5). The expression of the above-mentioned markers showed an oscillation with respect to the reference condition (ADSCs in BM for 7 days) at the three selected time points, but only the ITGB1 gene in cells treated with duloxetine for 14 days showed a regulation of the fold < -2.

3.3. Effects of drug-treatment on adipogenic commitment

Human ADSCs were maintained in ADM (committed-ADM ADSCs) and daily drug-treated from the first day or the seventh day (pre-differentiated-ADM ADSCs) up to 14 days. Adipogenic commitment was assessed by both intracellular lipid staining and gene expression analysis of adipogenic markers at day 7 and 14. The ORO staining confirmed the intracellular lipid drops accumulation in ADSCs cultured in ADM for 7 and 14 days (Fig. 6A), with a significant augmentation on fourteenth day ($p < 0.01$) (Fig. 6B). The same trend was observed in committed-ADM ADSCs treated with 5 μM duloxetine (Fig. 6C) or 5μM fluoxetine (Fig. 6F): intracellular lipid content at the fourteenth day were significantly higher ($p < 0.05$)(data not shown) than that of the seventh day (duloxetine-treatment in Fig. 6E, fluoxetine-treatment in Fig. 6H). Lipid drops were also stained in pre-differentiated-ADM ADSCs treated with duloxetine (Fig. 6D) or fluoxetine (Fig. 6G). Comparing pre-differentiated-ADM ADSCs with seven-day treated committed-ADM ADSCs, a significant increase ($p < 0.01$) in lipid accumulation was observed (duloxetine-treatment in Fig. 6E, fluoxetine-treatment in Fig. 6H data not shown). Only in duloxetine treatment, lipid accumulation in pre-differentiated-ADM ADSCs was significantly higher ($p < 0.05$) than those 14-day treated committed-ADM ADSCs (Fig. 6E); in fluoxetine treatment no significant difference in lipid accumulation was calculated (Fig. 6H). Interestingly, the treatment with duloxetine decreased the accumulation of lipid drops at 14 days compared with committed-ADM cells ($p < 0,01$) (Fig. 6E).

The gene expression profile of peroxisome proliferator-activated receptor gamma (PPARG), insulin-responsive glucose transporter type 4 (GLUT4), adiponectin (ADIPOQ), and fatty acid binding protein 4 (FABP4) was analyzed to assess adipogenic commitment in stem cells cultured in ADM. The mRNA expression in committed-ADM or pre-differentiated-ADM ADSCs treated or not with duloxetine or fluoxetine was compared with that of native ADSCs. Human ADSCs cultured in ADM compared with those maintained in BM showed a significant increase in the expression of all examined markers at both 7 days and 14 days (white bars in Fig. 7). On the contrary, gene expression of these adipogenic markers showed an alteration when duloxetine or fluoxetine were added to ADM (black, blue, and striped bars in Fig. 7). The gene expression of PPARG in duloxetine treated committed-ADM ADSCs (duloxetine-ADM) (black bar) was significantly lower ($p < 0.01$) than that in ADM both at 7 and 14 days. In fluoxetine treated committed-ADM ADSCs (fluoxetine-ADM) (blue bar) PPARG shown a significant reduction ($p < 0.01$) only on the seventh day. PPARG mRNA in pre-differentiated-ADM ADSCs (striped bar) did not show significant differences compared to that in committed-ADM ADSCs on the fourteenth

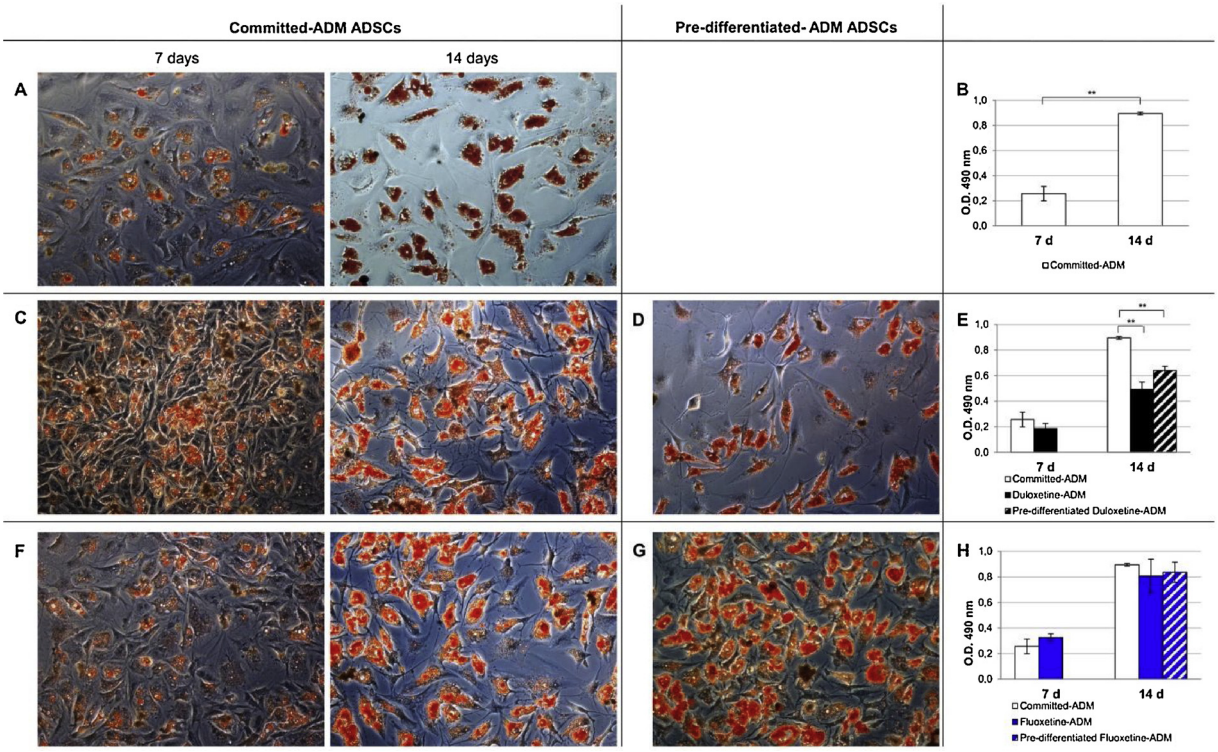

Fig. 6. Intracellular lipid content in committed and pre-differentiated ADSCs treated or not with duloxetine or fluoxetine. (A) ORO staining on committed ADSCs in ADM for 7 and 14 days; (B) ORO quantification in committed ADSCs in ADM; (C) ORO staining on committed-ADM ADSCs plus duloxetine for 7 and 14 days; (D) ORO staining on pre-differentiated ADSCs treated with duloxetine for 7 days; (E) ORO quantification in duloxetine-treated ADSCs; (F) ORO staining on committed ADSCs in ADM plus fluoxetine for 7 and 14 days; (G) ORO staining on pre-differentiated ADSCs treated with fluoxetine for 7 days; (H) ORO quantification in fluoxetine-treated ADSCs. Images at 20x magnification. Data presented as mean ± standard error (3 measurements) ** $p < 0.01$.

day. GLUT4 gene expression in drug-treated committed-ADM ADSCs was significantly lower than that in non-treated committed-ADM ADSCs both at 7 ($p < 0.05$) and 14 ($p < 0.01$) days. Only in duloxetine-treatment, GLUT4 mRNA in pre-differentiated ADSCs was significantly higher ($p < 0.01$) than that in committed-ADM ADSCs at day 14. ADIPOQ gene expression showed important alteration in committed-ADM ADSCs after 14 days of duloxetine-treatment: the ADIPOQ mRNA level in duloxetine-ADM was significantly lower ($p < 0.01$) than that not treated. On the contrary, the fluoxetine treatment showed effects on ADIPOQ expression on the seventh day: the treatment significantly lowered ($p < 0.01$) ADIPOQ mRNA level. Furthermore, the ADIPOQ expression in pre-differentiated-ADM ADSCs treated with fluoxetine was significantly higher ($p < 0.05$) than that in committed-ADM ADSCs on the fourteenth day. The gene expression of FABP4 in duloxetine-ADM was significantly lower ($p < 0.01$) than that in ADM on the seventh day, but significantly higher ($p < 0.01$) than that in ADM on day 14. On the contrary, fluoxetine-treatment did not lead to significant alterations in FABP4 expression profile.

3.4. Effects of drug-treatment on osteogenic commitment

Human ADSCs were maintained in ODM (committed-ODM ADSCs) and daily drug-treated from the first day or the fourteenth day (pre-differentiated-ODM ADSCs) up to 21 days. Osteogenic commitment was assess by both extracellular mineral deposit staining and gene expression analysis of osteogenic markers at day 14 and 21.

The ARS staining shown the extracellular mineral deposition in committed-ODM ADSCs on 21th day (Fig. 8A): ARS quantification at 21 day was significantly higher ($p < 0.01$) than that on 14th day (Fig. 8B). The same trend was observed in committed-ODM ADSCs treated with 5 µM duloxetine (duloxetine-ODM) or 5µM fluoxetine (fluoxetine-ODM) (Fig. 8C–F): ARS quantification at twenty-first day was significantly higher ($p < 0.001$) (data not shown) than that at fourteenth day (Fig. 8E–H). Also ARS quantification in pre-differentiated-ODM ADSCs (Fig. 8D-G) was significantly higher ($p < 0.01$ in duloxetine-treatment, $p < 0.001$ in fluoxetine-treatment) (data not shown) than that in duloxetine- or fluoxetine-ODM ADSCs at fourteenth day (Fig. 8E–H). Instead, extracellular mineral matrix deposition in pre-differentiated-ODM ADSCs compared with that in committed-ODM ADSCs at twenty-first day did not show significant differences (Fig. 8E–H). In duloxetine-ODM ADSCs after 21 days of treatment mineral deposition resulted higher compared to that in committed-ODM ADSCs at the same time ($p < 0,05$) (Fig. 8E); it did not happened for cells treated with fluoxetine.

The osteogenic commitment of ADSCs was evaluated by the determination of mRNA levels of genes involved the early stages of the osteogenic differentiation, such as, bone morphogenetic protein 2 (BMP2), alkaline phosphatase (ALPL), and runt related transcription factor 2 (RUNX2) (Fig. 9). But also late markers of osteogenic differentiation such as, osterix (OSX), receptor activator of nuclear factor kappa B ligand (RANKL), osteocalcin (OC), and osteopontin (OPN) were investigated (Fig. 10). The mRNA expression in native or pre-differentiated-ODM ADSCs treated with duloxetine or fluoxetine was compared with that of native ADSCs. The expression profile of early osteogenic markers in duloxetine-ODM and fluoxetine-ODM shown an opposite trend. In detail, at day 14 the expression of BMP2, RUNX2, and ALPL in duloxetine-ODM (black bar) was significantly greater ($p < 0.01$) than that in ADSCs in ODM. On the contrary, no significant variations in the expression profile of these genes was observed in case of treatment with fluoxetine (blue bar) at the same time point. At day 21 the expression of BMP2 and RUNX2 in duloxetine-ODM was significantly lower ($p < 0.01$) than that in committed-ODM ADSCs. On the contrary, the expression of these genes in fluoxetine-ODM was significantly higher ($p < 0.01$) than that in ADSCs in ODM. Duloxetine

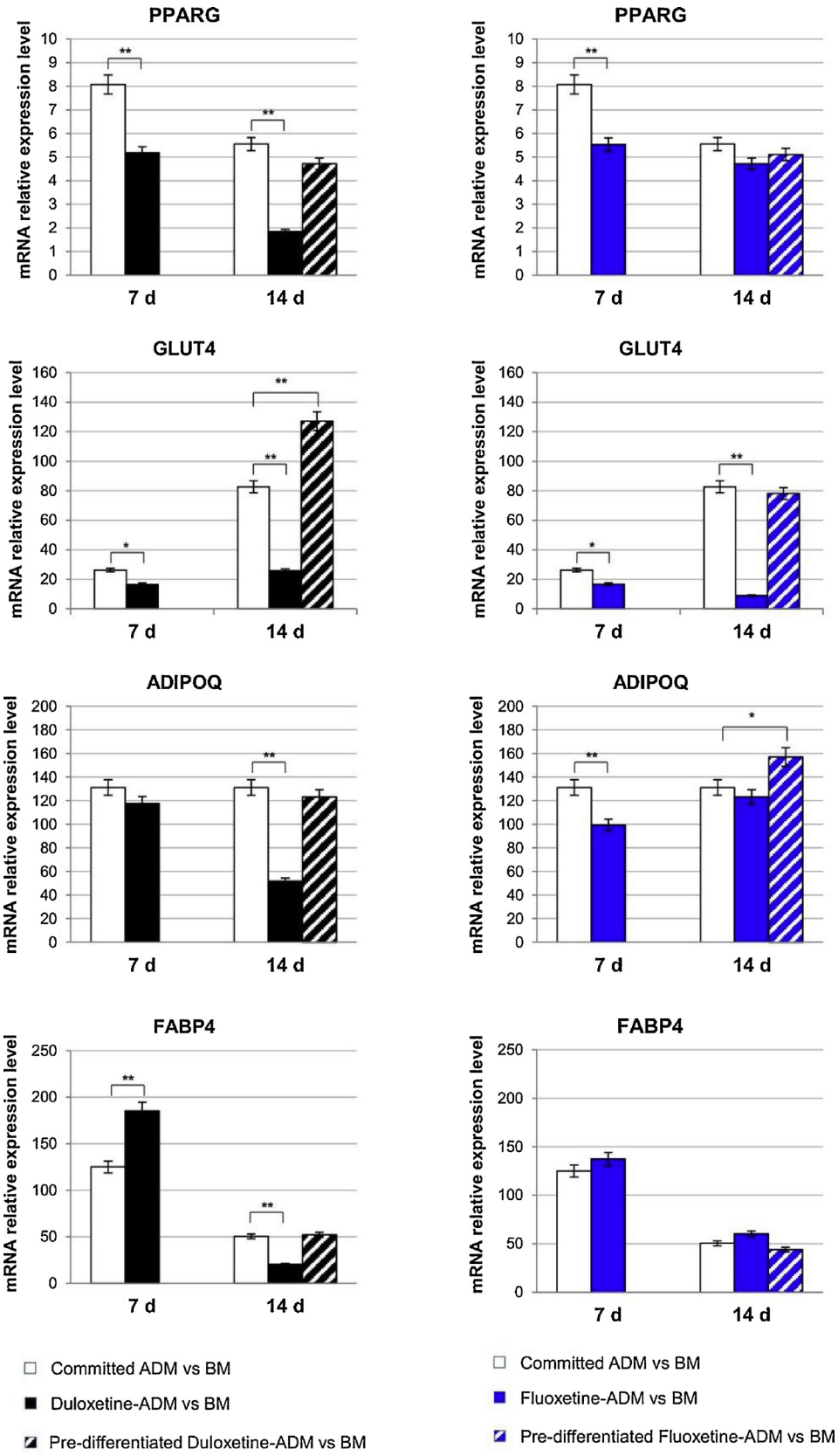

Fig. 7. Gene expression of adipogenic markers in committed and pre-differentiated ADSCs in ADM at 7 and 14 days. The results are reported as ratio with respect to the mRNA expression of native ADSCs in BM: white bars are committed ADSCs in ADM, black bars are committed ADSCs in ADM plus 5μM duloxetine, blue bars are committed ADSC in ADM plus 5μM fluoxetine, black striped bars are pre-differentiated ADSCs in ADM plus 5μM duloxetine, and blue striped bars are pre-differentiated ADSC in ADM plus 5μM fluoxetine. Data presented as mean ± standard error (3 measurements) * $p < 0.05$; ** $p < 0.01$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

and fluoxetine treatments did not lead to significant alterations in ALPL expression profile at 21th day. By compering gene expression in pre-differentiated-ODM ADSCs (striped bar) with that in committed-ODM ADSCs on 21th day only BMP2 gene shown a significant augmentation ($p < 0.05$) in duloxetine-treatment.

Also the expression profile of late osteogenic markers in duloxetine-ODM compared to those fluoxetine-treated shown an opposite trend (Fig. 10). At day 14 the expression of OSX in duloxetine-ODM ADSCs

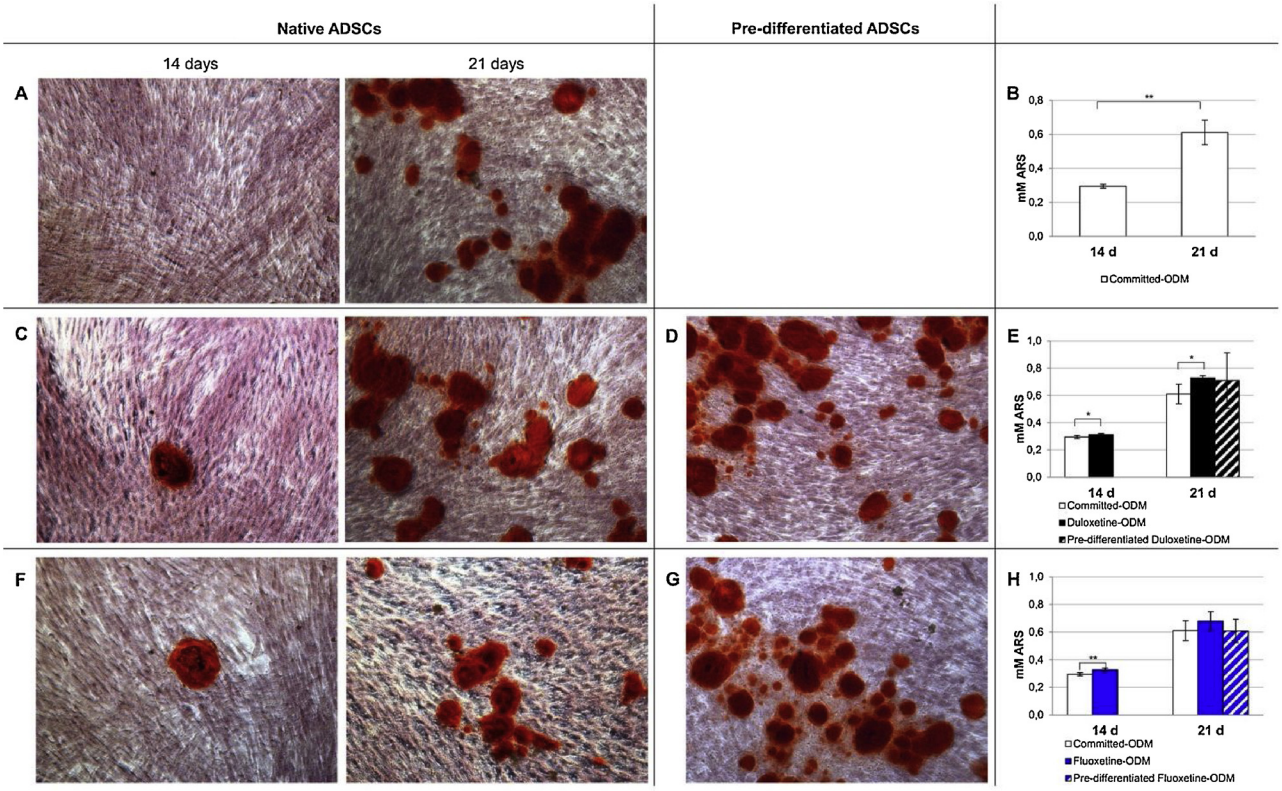

Fig. 8. Extracellular mineral deposits in committed and pre-differentiated ADSCs treated or not with duloxetine or fluoxetine. (A) ARS staining on committed ADSCs in ODM for 14 and 21 days; (B) ARS quantification in committed ADSCs in ODM; (C) ARS staining on committed ADSCs in ODM plus duloxetine for 14 and 21 days; (D) ARS staining on pre-differentiated ADSCs treated with duloxetine for 7 days; (E) ARS quantification in duloxetine-treated ADSCs; (F) ARS staining on committed ADSCs in ODM plus fluoxetine for 14 and 21 days; (G) ARS staining on pre-differentiated ADSCs treated with fluoxetine for 7 days; (H) ARS quantification in fluoxetine-treated ADSCs. Images at 20x magnification. Data presented as mean ± standard error (3 measurements) * $p < 0.05$; ** $p < 0.01$.

(black bar) was significantly higher ($p < 0.001$) than that in committed-ODM ADSCs, while on the twenty-first day a significant reduction ($p < 0.01$) was observed in duloxetine-ODM ADSCs compared to committed-ODM ADSCs. Conversely fluoxetine treatment (blue bar) has no significant effect after 14 days, but it resulted in a significant increase ($p < 0.001$) in OSX expression on the twenty-first day. The treatment with duloxetine compared with committed-ODM resulted in a significant increase ($p < 0.01$) in RANKL expression only after 21 days of treatment. Instead, fluoxetine-ODM caused a significant increase ($p < 0.01$) at 14 days and a significant reduction ($p < 0.01$) at 21 days. Only on 14th day OC expression was significantly higher ($p < 0.001$) in the duloxetine-ODM ADSCs compared with committed-ODM ADSCs. After 21 days no significant changes were recorded. In contrast, fluoxetine-ODM did not change OC expression after 14 days, but resulted in a significant increase ($p < 0.01$) in OC expression after 21 days. In ADSCs treated with duloxetine compared with those in ODM the OPN expression were significantly higher ($p < 0.001$) at 14 day and significantly lower ($p < 0.01$) at 21 day. In the case of fluoxetine-treatment, a significant increase ($p < 0.01$) was recorded only at 21 days. The expression of all late osteogenic markers in pre-differentiated-ODM ADSCs compared to that in ADSCs in 21-day ODM does not change (striped bars in Fig. 10).

4. Discussion

The majority of evidence suggests that antidepressant medication can have adverse effects on body weight and bone mineral density. The pharmacological mechanism behind the antidepressant effects of serotonin reuptake inhibitors is the negative allosteric regulation of serotonin reuptake pump, increasing serotonin concentration in specific regions of brain which impacts other physiological functions like appetite, sleep and sexual function [26]. However, serotonin plays a role also outside the central nervous system. In periphery, serotonin is mainly produced in enterochromaffin cells of the gut, in lung endothelium and platelets. It is responsible for gastrointestinal function and vasoconstriction. It has been shown to play different roles in the mammary gland, liver, and bone [27]. In addition, serotonin has an inhibitory effect on osteoblastogenesis in vitro. Interestingly, gut-derived serotonin has also been shown to regulate osteoblastogenesis and bone formation in vivo [28]. In contrast, the direct effect of antidepressant on adipogenic and osteogenic differentiation of stem cells remains unexplored. In previous works we had investigated the effects of antipsychotic drugs on the metabolism of stem cells. Like antidepressant drugs, even antipsychotic drugs are associated with weight gain and metabolic side effects. In vitro experiments on human adipose-derived stem cells and rat muscle-derived stem cells have shown that antipsychotic drugs such as clozapine, olanzapine, quetiapine, risperidone, and aripiprazole promote adipogenic commitment through PKCβ activation [29–31]. A subsequent study showed that the combination of a PKCβ inhibitor with antipsychotic drugs is able to counteract weight gain in mice [32].

The aim of the present work was to investigate the direct effects of two specific antidepressant drugs, duloxetine (SNRI) and fluoxetine (SSRI), on the physiology and metabolism of mesenchymal stem cells. Fluoxetine is the most prescribed SSRI, as it is well tolerated by patients, it is not associated with weight gain, but with weight loss during the acute phase of treatment [7,8]. Moreover, fluoxetine is the best option for the pharmacological treatment of major depressive disorder in children and adolescents [33], and it's the only treatment approved by FDA to cure bulimia nervosa [9]. Nevertheless, studies have reported a positive association between the use of fluoxetine and a decrease in bone mineral density [10–12]. On the contrary, duloxetine is associate

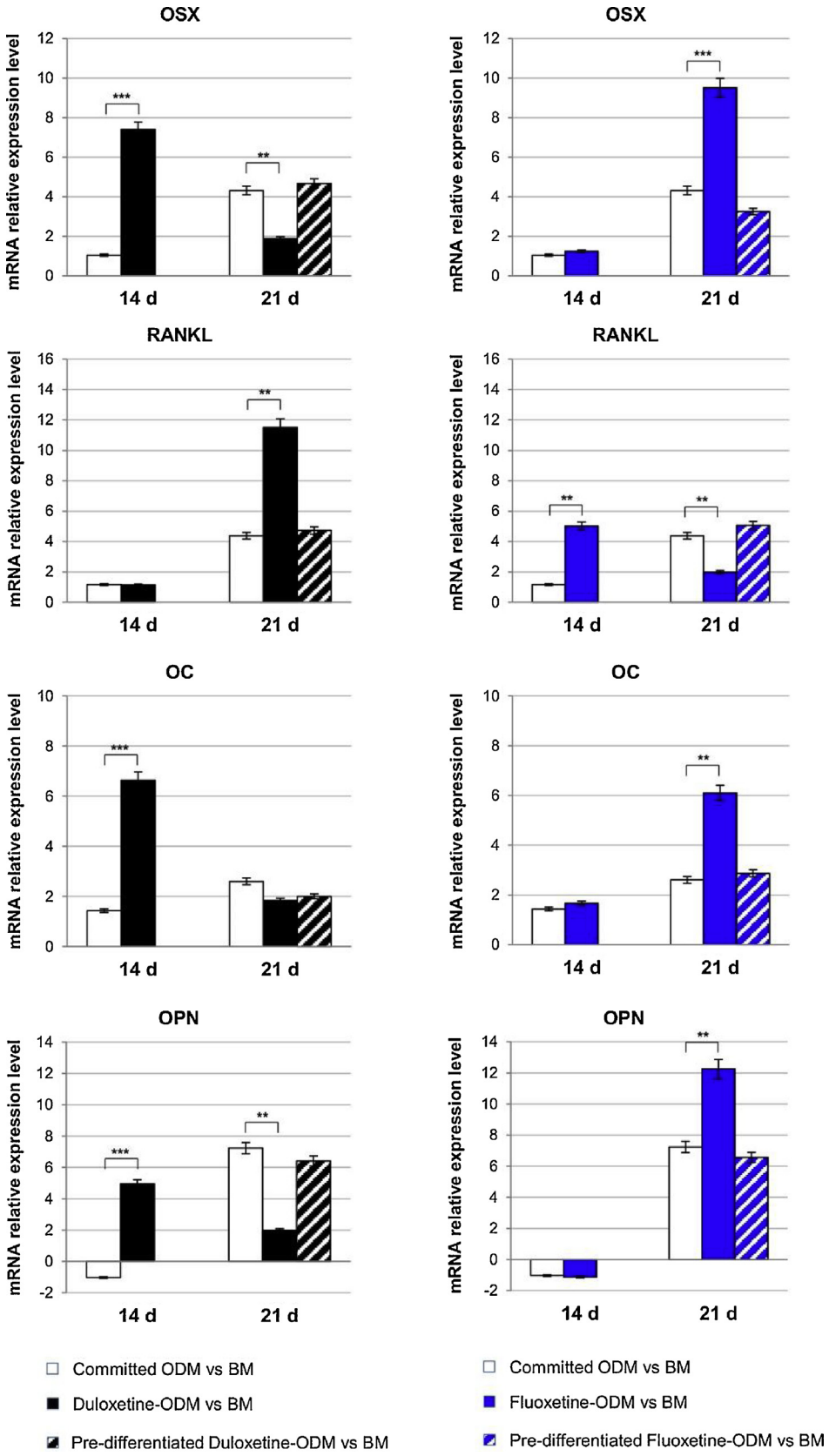

Fig. 9. Gene expression of early osteogenic markers in committed and pre-differentiated ADSCs in ODM at 14 and 21 days. The results are reported as ratio with respect to the mRNA expression of native ADSCs in BM: white bars are committed ADSCs in ODM, black bars are committed ADSCs in ODM plus 5μM duloxetine, blue bars are committed ADSC in ODM plus 5μM fluoxetine, black striped bars are pre-differentiated ADSCs in ODM plus 5μM duloxetine, and blue striped bars are pre-differentiated ADSC in ODM plus 5μM fluoxetine. Data presented as mean ± standard error (3 measurements) * $p < 0.05$; ** $p < 0.01$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

with weight gain [6,7], and there is no data on its effect on bone density. In this study, we tested the direct effects of duloxetine and fluoxetine on in vitro cultures of human mesenchymal stem cells. We have isolated cells from human adipose tissues, then we have characterized cell morphology and the surface antigen phenotype. The isolated ADSCs were plastic-adherent under standard culture conditions, expressed surface markers such as CD44, CD73, CD90, and CD105 and lacked of expression of CD14, CD34, CD45, and HLA-DR,

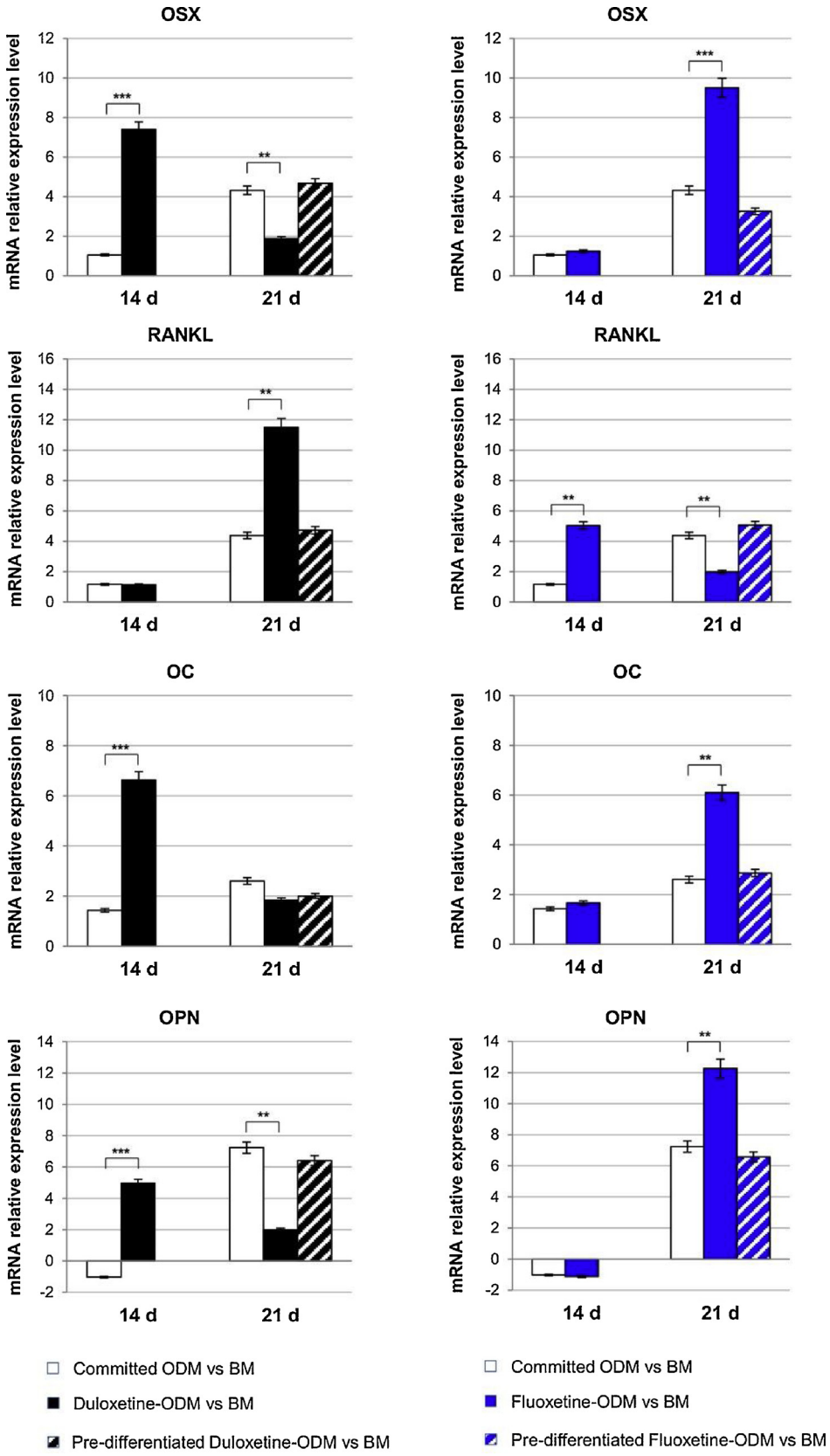

Fig. 10. Gene expression of late osteogenic markers in committed and pre-differentiated ADSCs in ODM at 14 and 21 days. The results are reported as ratio with respect to the mRNA expression of native ADSCs in BM: white bars are committed ADSCs in ODM, black bars are committed ADSCs in ODM plus 5μM duloxetine, blue bars are committed ADSC in ODM plus 5μM fluoxetine, black striped bars are pre-differentiated ADSCs in ADM plus 5μM duloxetine, and blue striped bars are pre-differentiated ADSC in ODM plus 5μM fluoxetine. Data presented as mean ± standard error (3 measurements); ** $p < 0.01$; *** $p < 0.001$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

and had multilineage differentiation potential in vitro. Thus, the isolated cells respected the minimal criteria for defining multipotent mesenchymal stem cells established by the International Society for Cellular Therapy [34]. Various concentrations of duloxetine and fluoxetine were tested on ADSCs under basal culture conditions. After 24 h, concentrations greater than 5 μM have resulted in a 50% reduction in cell viability. Then, cells were treated daily with 5 μM duloxetine or 5 μM fluoxetine up to 21 days.

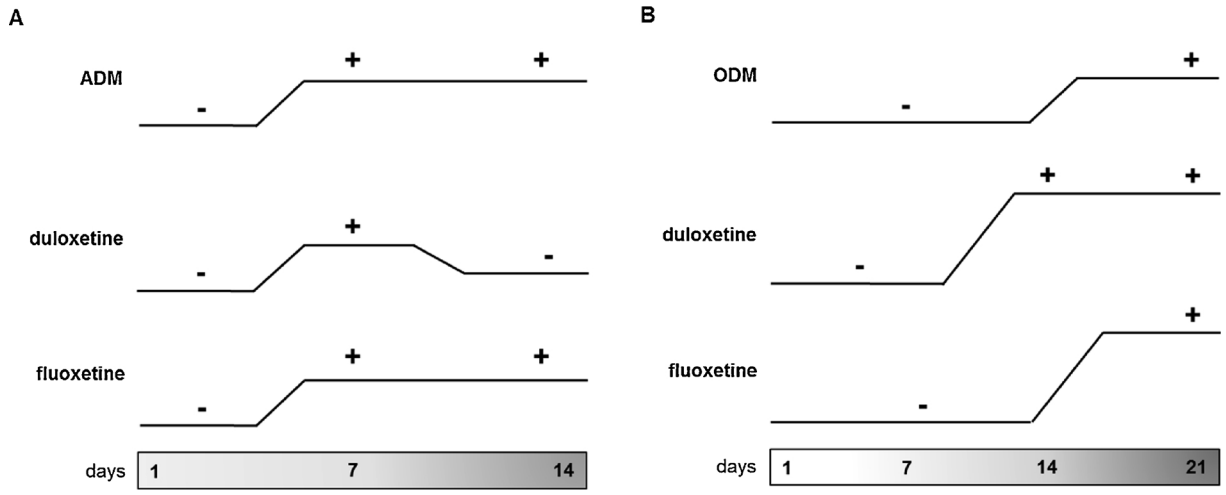

Fig. 11. Schematic representation of gene expression timing during (A) adipogenic and (B) osteogenic commitment.

Stem cell proliferation seemeds to be slightly influenced by the presence of duloxetine or fluoxetine when cultured in basal medium. During the observation interval (at 4, 7, and 14 days) significant fluctuations were observed with respect to the control condition. This effect disappeared at the twenty-first day, when the presence of duloxetine or fluoxetine did not affect stem cell proliferation that resulted comparable to the control one. Therefore, we chose the 5 μM concentration as the maximum non-toxic concentration to test the effect of duloxetine and fluoxetine on stemness properties, and adipogenic and osteogenic differentiation capabilities of ADSCs. As demonstrated by the gene expression analysis of mesenchymal stromal cell markers, ADSCs daily treated with duloxetine or fluoxetine shown the same gene expression profile of those in basal medium. Only the ITGB1 gene in cells treated with duloxetine showed a down regulation on 14th day which is annulled on the 21 st day. During the adipogenic commitment a progressive accumulation of intracellular lipid drops was recorded, likewise in the presence of duloxetine or fluoxetine; interestingly, only the treatment with duloxetine seemed to slow down lipid accumulation both in committed and pre-differentiated cells compared to non-treated committed-ADM ADSCs. It is in contrast with weight gain registered in long time treated patients [6,7]. Although the drug-treated cells showed a phenotype similar to the control cells, the adipogenic gene expression analysis indicated a slight difference in gene expression timing. On the other hand, drug-treatments did not influence the adipogenic gene expression of the pre-differentiated cells (Fig. 11A).

During the osteogenic commitment an increase in extracellular mineral deposition in drug-treated cells was observed, a significantly higher deposition was observed in duloxetine treated ADSCs at the twenty-first day. It could be correlated with the osteogenic gene expression analysis that showed an advance in gene expression of early and late osteogenic markers in stem cells treated with duloxetine compare with cells control. Instead, fluoxetine-treatment did not seem to have the same effect: mineral deposition is comparable with that of non-treated cells, however a significant increase in expression of osteogenic genes was observed at 21 days compared to untreated cells. The osteogenic gene expression of pre-differentiated cells was not influenced by the both drug-treatments (Fig. 11B). These findings about fluoxetine treatment are consistent with those of Ortuño [13], who identified in mice a dual role of fluoxetine on bone remodeling. A 3-week treatment with fluoxetine resulted in a local anti-resorptive response that increased bone mass, directly impairing osteoclast differentiation. Instead, a chronic treatment (6 weeks) induced a central serotonin-dependent increase in sympathetic output flow, which results in increased bone resorption sufficient to counteract the local anti-resorptive effects, leading to a decreased bone formation and bone loss [13]. Battaglino et al. [34] also reported positive effects on bone cells. They demonstrated that treatment of mice with fluoxetine at a dose of 10 mg/kg/d may stimulate bone formation in the femur and lumbar vertebrae [34]. Similarly, Mortazavi et al [35] reported an increase in bone formation following treatment with fluoxetine in rats with calvarial small-size bone defects.

5. Conclusion

In conclusion, these results demonstrate that a non-toxic concentration of duloxetine or fluoxetine have time-dependent effects on mesenchymal stem cells. In particular, they influence proliferation and stemness properties of non-committed ADSCs in short term, indeed, after 21 days of daily drug-treatments both cell proliferation and mesenchymal stromal cell markers expression are equal to the control condition. The treatment with fluoxetine do not lead to morphological alterations during adipogenic or osteogenic commitment while the one with duloxetine seems to decrease lipid accumulation and to increase mineral deposition.

Nevertheless, both drug treatments influence the gene expression timing of adipogenic genes in committed cells. Instead, in osteogenic commitment, duloxetine determines an anticipation of early and late osteogenic markers gene expression, and fluoxetine causes a significant increase in osteogenic genes expression. Our study suggest that, despite affecting mesenchymal stem cells commitment, the effects of duloxetine and fluoxetine on body weight and bone metabolism probably not correlate directly with mesenchymal stem cells differentiation; perhaps the effects observed on patients could be closely related to the action of the drugs on serotonin reuptake. For these reason further studies that deeply investigate the mechanism related to anti-depressant side effects will be necessary.

Since the use of antidepressants in clinical practice has dramatically increased in recent years, understanding how these drugs are associated with weight gain, osteoporosis and fracture risk is pivotal and this may influence prescribing practices as knowledge increases.

Conflict of interest

The authors declare no conflicts of interest.

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LF, CG, VV, CP and BZ conceived and designed the experiments. LF performed the experiments and wrote the paper. LF, CG, and BZ analyzed and interpreted data, LF and GB revised the manuscript. All authors read and approved the final manuscript.

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