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Filamin B and CD13 Are Components of Senescent Secretomes That May Be Involved in Primary (Stress Induced) and Paracrine Senescence of Mesenchymal Stromal Cells

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ABSTRACT

Objective: In the present study we aim to evaluate whether some of the proteins previously identified in the secretome of senescent bone marrow (BM)- and adipose (A)-mesenchymal stromal cells (MSCs) could be involved in regulation of senescence phenomena.

Materials and Methods: Among the identified proteins, we selected Filamin B (FLNB) and Aminopeptidase N (CD13) that were exclusively found in the secretome of senescent cells. We silenced their mRNA expression in BM-MSCs by means of RNA interference technology and induced acute senescence by hydrogen peroxide treatment. Our goal was to evaluate if FLNB or CD13 silencing may affect onset of senescence.

Results: Our preliminary data showed that CD13 protein could be a key factor involved in the regulation and determination of both primary and paracrine induced senescence in human BM-MSCs. On the other hand, Filamin B seems not to be involved in the determination of primary senescence whereas its presence could be part of the mechanism that regulates the senescence induction in human BM-MSCs by paracrine signaling.

Conclusion: Our study provides a useful base for identifying the complex extracellular protein networks involved in the regulation of MSC cellular senescence.

Keywords: Mesenchymal stromal cells, senescence, SASP, Filamin B, CD13

INTRODUCTION

Cellular senescence is the permanent cell cycle arrest evoked in response to a variety of stressors (1). This physiological response to stress has a pleiotropic effect: i) beneficial, since senescence can counteract tumor growth by blocking the proliferation of transformed cells, ii) detrimental by contributing to organismal aging (2–4). Recently, it has been demonstrated that senescence may also contribute to organism development and tissue repair processes (5). To date, a deep understanding of mechanisms regulating senescence induction is lacking since we do not have a clear definition of "senescent cell". Currently, senescent cells are identified as cells showing enlarged and flattened morphology, altered gene expression patterns, high β-galactosidase enzyme activity, telomere dysfunction-induced foci (TIF), presence of DNA segments with chromatin alterations reinforcing senescence (DNA-Scars), senescence-associated heterochromatin foci (SAFH), and senescence-associated secretory phenotype (SASP) (3, 6).

Recent studies have showed that SASP may contribute to cell cycle arrest through autocrine/paracrine pathways. Actually, the pool of molecules secreted by senescent cells may represent a danger signal that sensitizes normal neighboring cells to enter senescence (7, 8). SASP also determine detrimental consequences to whole organism. It can threaten organ and tissue functionality and contribute to aging of the organism (3, 8). On the other hands, cancer cells can profit of signaling pathway induced by secreted inflammatory cytokines, growth factors and proteases for their growth (9).

Senescence of stem cells, such as mesenchymal stromal cells (MSCs) could be very deleterious for the body's physiology, since it can greatly impair tissue homeostasis and repair. MSCs contain a subpopulation of multipotent stem cells able to differentiate in bone, fat and cartilage and can also support hematopoiesis and contribute to homeostasis of several organs and tissues (10, 11). In addition, MSCs have a significant therapeutic potential for the treatment of several human diseases, where they exhibit repair and regenerative properties (12). The beneficial functions of MSCs have been attributed to their autocrine and paracrine activity (13). Therefore, senescence of MSCs may have a great impact on body's health given that it can alters the composition of their secretome and can impair the key MSC biological functions (3, 4, 14).

In a previous research by means of LC-MS/MS analyses, we evaluated the SASP composition of bone marrow (BM) and adipose (A) MSCs following different genotoxic stress treatment. These analyses allowed us to identify

eleven proteins that were solely present in all the senescent phenotypes and absent in healthy MSC secretomes (3).

In the current study we aim to determine whether Filamin 3 (FLNB) and aminopeptidase N (CD13), two of the eleven proteins previously identified in the secretome of senescent BM- and A-MSCs, could be involved in the key functions of senescent cells.

Our preliminary study may provide a solid basis to identify the complex extracellular protein networks involved in the senescence of MSCs.

MATERIALS and METHODS

BM-MSC Cultures

MSCs were isolated from bone marrow of three healthy subjects (6–10 years) after they provided an informed consent. The cells were separate on Ficoll density gradient (GE Healthcare, Italy). Then, we collected the mononuclear fraction. The cells (1.0–2.5 x 105 cells/cm^2) were seeded in α-MEM, 10% FBS and β-FGF. Following 72 hours, we eliminated non-adherent cells and cultivated the adherent ones to confluency. We further propagated the cells in order to use them for the assays reported below. We ensured that BM-MSCs met the minimum criteria stated for defining MSCs by International Society for Cellular Therapy (ISCT) (15) (data not shown). All reagents were purchased from Euroclone S.p.A., Italy unless otherwise specified.

Filamin B and Aminopeptidase N Silencing

siRNAs targeting the human Filamin B (Filamin B siRNA: sc-60641; Santa Cruz, CA, USA) the Aminopeptidase N (CD13 siRNA: sc-29960; Santa Cruz), and a scrambled (siRNA-A control: sc-37007; Santa Cruz) mRNAs, were used to transfected BM-MSC cultures by means of Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocol. We verified the efficiency of gene silencing by quantitative reverse transcription polymerase chain reaction (RT-qPCR), as reported below.

Hydrogen Peroxide (H_2O_2) Treatment

We incubated MSCs with 300 μM H_2O_2 in complete medium for 30 minutes. Subsequently, we discarded the medium, and incubated the MSCs in a fresh medium for 24 hours. To confirm that H_2O_2 treatment induced senescence in MSC cultures, we performed an in situ senescence-associated β-galactosidase assay (reported below).

Preparation of Conditioned Medium (CM) From Naïve and Senescent MSCs

CM were collected from H_2O_2 treated MSCs (acutely senescent cells) following transfection with Filamin 3 siRNA or CD13 siRNA or control siRNA, respectively. As control, we collected CM from naïve MSCs (healthy cells) following transfection with control siRNA.

Treatment with CM

In order to analyze the effect of CMs on the behavior of young functional MSCs, we incubated exponentially growing cells in their respective media supplemented with 50% CM from acutely senescent MSCs following transfection with Filamin 3 siRNA or CD13 siRNA or control siRNA, respectively. We also incubated young MSCs in their respective media supplemented with 50% CM from naïve healthy MSCs following transfection with control siRNA. Following 48 hours, we performed the beta-galactosidase assay as described below.

In Situ Senescence-Associated Beta-Galactosidase Assay

We fixed MSCs with 2% formaldehyde and 0.2% glutaraldehyde. Subsequently, we washed cells with PBS and then incubated them at 37°C with the following staining solution (citric acid/phosphate buffer (pH 6), K4Fe(CN)6, K3Fe(CN)6, NaCl, MgCl2, and XGal) for at least 2 hours. We calculated the percentage of b-galactosidase-positive cells by counting the number of blue cells out of at least 500 cells in different microscope fields (16). All reagents were purchased from Sigma-Aldrich, MO, USA.

RNA Extraction and RT-qPCR

We extracted total RNA using EuroGOLD Trifast (EuroClone S.p.A., Italy) according to the manufacturer's protocol. We analyzed mRNA expression levels of Filamin 3 and Aminopeptidase N by RT-qPCR as previously reported (17). Primer pairs were designed by means of Primer Express software (Primer Express, Thermo Fisher Scientific, MA, USA). GAPDH mRNA expression levels were used as internal controls. Each reactions was repeated at least 3 times (18). Primer sequences are the following: FLNB (F) 5'-GTGTCCGTGTTAATGTAT-3'; FLNB (R) 5'-CTGAGAAGTGTAAGAAGG-3'; CD13 (F) 5'-CATCATCAG CATTACCAACAAC-3'; CD13(R) 5'AACGAGCCACCACCATAA 3'; GAPDH (F) 5'-GGAGTCAACGGATTTGGTCGT- 3'; GAPDH (R) 5'-ACGGTGCCATGGAATTTGC 3'.

Statistical Analysis

A one-way analysis of variance followed by Student's t and Bonferroni's tests was performed. We used the mixed-model variance to analyze data with continuous outcomes. All analysis were carried out by means of GraphPad Prism statistical software 5.01 (GraphPad, CA, USA).

RESULTS

Identification of MSC Senescent-Specific Phenotype Proteins

In a previous study we collected the conditioned media from BM-MSCs and A-MSCs at early passages and following different stress-inducing agent treatment (i.e. hydrogen peroxide, doxorubicin, low and high X-ray irradiation, and replicative exhaustion, respectively) to identify a set of proteins exclusively expressed in senescent secretomes. To this purpose, we performed the LC-MS/MS analyses. By means of high-resolution MS, we identified several hundred proteins present in all senescence induction conditions in both BM- and A-MSCs (the number of proteins fell into the following range: from 279 in BM-MSC to 876 in A-MSC doxorubicin treated). In order to obtain biological information, high-resolution MS data were systematically organized by Gene Ontology (GO) that is able to perform an enrichment analysis to obtain the relative frequency of biological functions present in the proteomic profile analyzed. GO database is based on three biological functions (ontological terms) that are molecular functions, biological processes, and cellular components (3). We focused the GO analysis on two ontological terms like "molecular functions" and "biological processes". We conducted an enrichment analysis by PANTHER gene ontology which allowed to match all our data onto ontology terms of reference. Then, we

Table 1. Proteins exclusively expressed in the MSC senescent phenotypes

	Name of protein	UniProt ID
Extracellular matrix/cytoskeleton/cell junctions	Filamin B	E7EN95
	Tubulin alpha 1C chain	F5H5D3
	Tubulin beta chain	Q5JP53
Ox-redox factors	Peroxiredoxin 6	P30041
	Protein deglycase DJ-1(PARK7)	Q99497
	TXNDC5 (ERP46)	I3L3M7
Regulators of gene expression	Major vault protein	Q14764
	14-3-3 protein epsilon	P62258
	Proteasome subunit beta type 4	P28070
Miscellaneous	Aminopeptidase N (CD13)	P15144
	Cathepsin D	P07339

Venn analysis allowed the identification of 11 proteins that were shared and exclusively expressed in all the different senescent phenotypes and absent in the secretomes of healthy mesenchymal stromal cells (MSCs). The proteins were divided into Gene Ontology groups plus a miscellaneous collection. The table has been modified from Ozcan et al. (3)

identified the ontology terms frequently present in our datasets. In senescent secretome we identified four common enriched ontologies that were: metabolic processes; extracellular matrix/cytoskeleton/cell junctions; regulators of gene expression, and ox-redox factors. Then, the Venn diagram allowed to identify proteins present in all the senescent phenotype and absent in the control MSC secretomes. We found eleven proteins solely present in all the analyzed senescent phenotypes and absent in the healthy MSC secretomes (Table 1). For detailed results of this study see also Ozcan et al. (3).

BM-MSC Silencing

In order to evaluate whether the previously identified proteins in the BM- and A-MSC senescent phenotypes could be involved in the key functions of senescent cells we decided to silence their expression in BM-MSCs by means of RNA interference technology. Among the 11 identified proteins, we selected Filamin 3 (FLNB) and aminopeptidase N (CD13) belonging to different Gene Ontology groups (i.e. extracellular matrix/cytoskeleton/cell junction class and miscellaneous class, respectively) (Table 1).

BM-MSC cultures were tested for FLNB and CD13 silencing. We marked as MSC siFLNB and siCD13 the BM-MSC silenced for FLNB or CD13 genes, respectively. MSCs silenced with a scrambled siRNA were marked as MSC siCTRL. As showed by RT-qPCR analysis, FLNB and CD13 siRNAs were able in decreasing target mRNAs in BM-MSCs. We observed a 70% decrease of Filamin3 mRNA and a 75% decrease of CD13 mRNAs compared to control MSCs, as reported in Figure 1.

Validation of FLNB and CD13 Proteins in the Regulation of Senescence

We induced acute senescence by H_2O_2 treatment in healthy functional MSCs following the FLNB or CD13 gene silencing. This experiment allowed us to evaluate the role of the two selected proteins in senescence. Before and following senescence induction, we evaluated the percentage of senescent cells by beta-galactosidase assay. In healthy MSCs the FLNB or CD13 gene silencing did not determine significant changes in the percentage of senescent

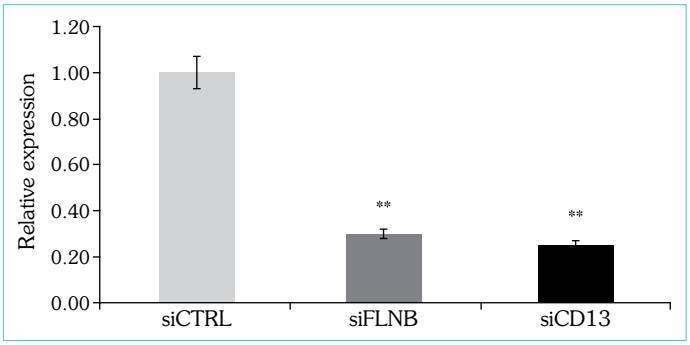

Figure 1. FLNB and CD13 silencing

RT-qPCR analysis: mRNA levels were normalized with respect to GAPDH as the internal control. The histogram shows the mean expression values (±SD, n=3). Changes in the mRNA levels of siFLNB and siCD13 BM-MSC were compared with that of siCTRL cells, as reference. We used the comparative cycle threshold method to quantify expression levels. (**p<0.01)

cells (Fig. 2a, b). Conversely, H_2O_2 treatment produced a lower number of senescent cells in MSC cultures having silenced CD13, compared with wild type controls (11,7% ±2,3 versus 42% ±4,6), (Fig. 2c, d). The silencing of FLNB did not affect the primary senescence process induced by H_2O_2 treatment (Fig. 2c, d).

CM From FLNB or CD13 Silenced MSCs Negatively Regulate Senescence

It has been widely demonstrated that a plethora of molecules secreted by senescent cells may contribute to cell cycle arrest in normal cells trough autocrine/paracrine pathways. These secreted molecules can regulate the senescence process and may represent a signal that sensitizes functional neighboring cells to senesce (7–9, 19). We cultivated young functional MSCs in the presence of CM from acute senescent MSC transfected with siFLNB or siCD13 or siCTRL. This experiment aimed to determine whether SASP from H_2O_2 treated MSCs having silenced FLNB or CD13 were still able to induce senescence in healthy cells by paracrine mechanism. As control, we cultivated healthy functional MSCs with CM from siCTRL MSCs. As expected, we observed a significant increase of senescent cells in young MSC cultivated in the presence of CM from acute senescent

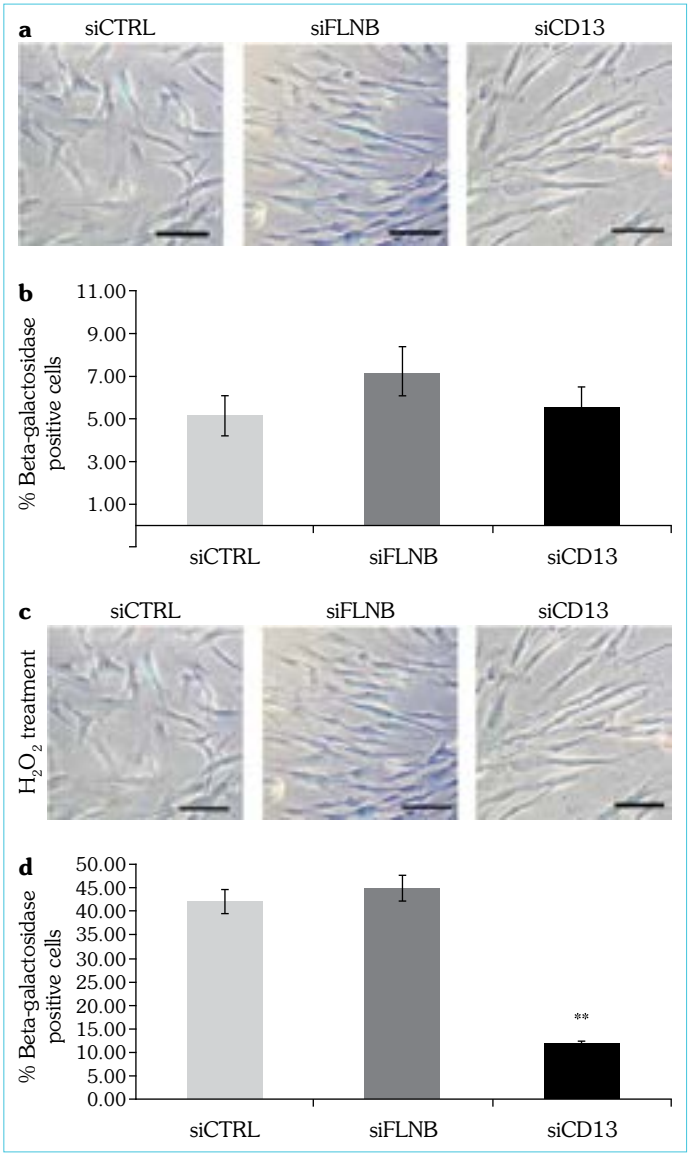

Figure 2. a–d. Senescence analysis following silencing

(a) Representative microscopic fields of acid beta-galactosidase positive senescent cells (blue) in BM-MSC transfected with siFLNB, siCD13, and siCTRL are shown. The black bars equal 100 µm. The histogram in (b) shows the mean percentage value of senescent cells. (c) Representative microscopic fields of acid beta-galactosidase positive senescent cells (blue) in H_2O_2 treated BM-MSC transfected with siFLNB, siCD13, and siCTRL. The histogram in (d) shows the mean percentage value of senescent cells. (±SD, n=3, **p<0.01)

(H_2O_2 treated) siCTRL MSCs compared to young cells treated with CM from siCTRL MSCs (24,2% ±2.9 versus 8,6% ±1,9) (Fig. 3a, b). Interestingly, the treatment of young cells with CM from acute senescent MSC transfected with siFLNB or siCD13 showed a significant decrease in the percentage of beta-galactosidase positive cells respect to control cells (13,4% ±1,8 versus 24,2% ±2,9 and 10,1% ±1,7 versus 24,2% ±2.9, respectively) (Fig. 3a, b).

DISCUSSION

Senescence, a stress response associated with the permanent cell cycle arrest, is strictly related to organism aging and aging-associated disorders (19). It is known that the extracellular microenvironment has a fundamental role in regulating the cellular response to senescence (19, 20). Recent studies showed that senescent cells secrete a pool of molecules (SASP) such as inflammatory cytokines, chemokines, growth factors, and matrix metalloproteinases that can regulate and determine the senescence response. Moreover, these secreted factors may sensitize functional neighboring cells to senesce (19).

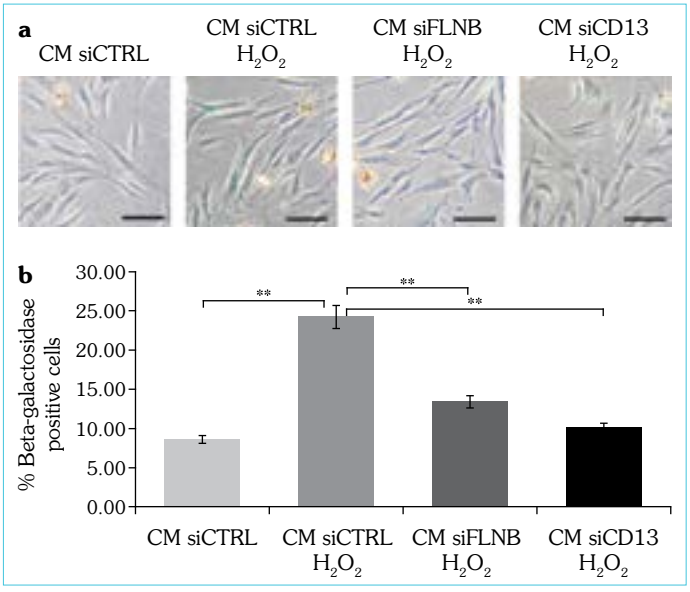

Figure 3. a, b. Senescence analysis following conditioned media treatment

(a) Representative microscopic fields of acid beta-galactosidase positive senescent cells (blue) in young functional BM-MSCs incubated with conditioned media (CM) obtained from H_2O_2 treated MSCs. The H_2O_2 treated MSCs were transfected with siFLNB, siCD13, and siCTRL. The black bars equal 100 µm. The histogram in (B) shows the mean percentage value of senescent cells. (±SD, n=3, **p<0.01). The square brackets indicate the comparison analyses we performed

Senescence of stem cells, such as those present in MSCs, could be very deleterious for the body's physiology, since it can greatly impair tissue homeostasis and repair (12).

Although many studies highlighted the importance of the SASP in regulating cellular response to senescence signaling, the identification of secreted factors is still a challenging issue, since the typology of secreted proteins strictly depends on genotoxic stress and cell type (2–4). To date, a comprehensive analysis of the secretome profile of senescent MSCs is still lacking.

In trying to identify the common features for MSC SASPs, we previously analyzed the senescent secretome composition of BM- and A-MSCs following different genotoxic stress treatment by means of LC-MS/MS analyses. These analyses allowed us to identify eleven proteins that were solely expressed in all the senescent phenotypes and absent in the healthy MSC secretomes (Table 1) (3). Among the eleven identified proteins, we selected the Filamin B and CD13 to evaluate whether they could be involved in the key functions of senescent BM-MSCs. To this purpose we decided to silence their expression in BM-MSCs by means of RNA interference technology. Our results showed that the partial CD13 silencing in H_2O_2 treated MSCs determine a remarkable decrease of senescent cells compared with respective control cells (Fig. 2c, d). Interestingly, the treatment of normal functional cells with CM from senescent-

induced MSC transfected with siCD13 also showed a significant decrease in the percentage of senescent cells positive cells respect to control ones (Fig. 3a, b). These preliminary data let us hypothesize that CD13 protein could be one of the key factors involved in the regulation and determination of both primary (stress induced) and paracrine senescence in human BM-MSCs.

CD13 is a ubiquitous enzyme present in many human tissues and cell types. In addition to enzymatic activity, it is also associated to other functions, such as to be a receptor for some human viruses and participate in the antigen presentation. CD13 is involved in the regulation of several aspects of normal (hematopoiesis and myeloid cell functions) as well as malignant cell development (i.e. proliferation, differentiation, proliferation, apoptosis, invasion, secretion, motility and angiogenesis) (21, 22). An intriguing study conducted by Iaffaldano et al. suggested that CD13 could contribute to obesity programming in the fetus and that high maternal serum CD13 represent an obesity risk marker (23). Interestingly, it has been widely demonstrated that obesity is associated with an increase of senescent cells since it can contribute to create an environment that accelerates senescence within the tissues of the organism (24, 25).

With regard to Filamin 3, we observed that its partial silencing in H_2O_2 treated MSCs did not determine significant changes in the percentage of senescent cells respect to control MSCs (Fig. 2c, d). Conversely, the treatment of normal functional cells with CM from senescent-induced MSC transfected with siFLNB showed a significant decrease in the percentage of senescent cells respect to control ones (Fig. 3a, b). These results suggest that Filamin B is not involved in the determination of primary (stress induced) senescence whereas its presence could be part of the mechanism that regulate the paracrine senescence induction in human BM-MSCs.

Filamin B is an actin-binding protein which crosslinks actin cytoskeleton filaments into a dynamic structure. It has a key role in skeletal development and its pathogenic mutations are the solely cause of skeletal deformities (26). Recently, Tsui et al. reported that differential expression of Filamin B splicing variants may play a key role in proliferation and differentiation of giant cell tumor of bone (27). Another intriguing study identified Filamin B in human sera as a potential biomarker for prostate cancer (28). The role of Filamin B in enhancing the invasiveness of cancer cells is also reported (29). In contrast, Bandaru et al. demonstrated that Filamin B negatively regulates tumor progression by suppressing the local growth, angiogenesis and metastasis (30). To our knowledge there are no literature data exploring the role of Filamin B in senescence process.

Although our preliminary data suggest a key role in regulating senescence in human BM-MSCs for both proteins, we are currently planning further experiments to validate these interesting data.

In the near future, we will also analyze the role of the other nine proteins that were solely expressed in all the senescent phenotypes and absent in the healthy MSC secretomes (Table 1) in regulating and determining cellular senescence. Finally, all the experiments will be validated also in human A-MSCs.

CONCLUSION

In conclusion, our preliminary study provides a useful base for identifying the complex extracellular protein networks involved in the regulation of MSC cellular senescence. The importance of our study also resides on the consideration that senescence may significantly affect the therapeutic potential of MSCs. As a whole, our data and further investigations may allow to modify the currently used in vitro expansion protocols for MSC therapeutic applications in order to prevent or reduce deleterious senescence-related effects.

Ethics Committee Approval: Ethics committee approval of July 14, 2008; University of Campania "Luigi Vanvitelli" former Second University of Naples; Naples, Italy.

Informed Consent: Informed consent was obtained from the parents of the participants.

Peer-review: Externally peer-reviewed.

Author Contributions: Conceived and designed the experiments: TS, SO, UG. Performed the experiments: TS, SO, NA, MBA. Analyzed the data: GDB, MABM, GF. Wrote the paper: TS, SO, UG. All authors have read and approved the final manuscript.

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