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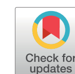
Original article

TRF1 as a major contributor for telomeres' shortening in the context of obesity



Lucas Kich Grun[a,b], Nevton da Rosa Teixeira Jr.[a,b], Lúcia von Mengden[a,c], Marco Antônio de Bastiani[a,c], Mariana Migliorini Parisi[d], Rafael Bortolin[e], Patrícia Lavandoski[a,b], Vinícius Pierdoná[a,b], Letícia Biscaino Alves[f], José Cláudio Fonseca Moreira[a,g], Cláudio Corá Mottin[f,h], Marcus Herbert Jones[h], Fábio Klamt[a,c], Alexandre Vontobel Padoin[f], Fátima Costa Rodrigues Guma[a,b,i], Florencia María Barbé-Tuana[a,b,j,*]

[a] Postgraduate Program: Biochemistry, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[b] Group of Inflammation and Cellular Senescence and Laboratory of Molecular Biology and Bioinformatics, Department of Biochemistry/ICBS, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[c] Laboratory of Cellular Biochemistry, Department of Biochemistry/ICBS, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[d] Interdisciplinary Group of Health (GIS), Center of Education and Research of the Institute of Cardiology of Cruz Alta, University of Cruz Alta (UNICRUZ), Cruz Alta, Brazil
[e] Department of Civil and Environmental Engineering, Universidad de la Costa, Barranquilla, Atlántico, Colombia
[f] Center of Obesity and Metabolic Syndrome, São Lucas Hospital at Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) and Postgraduate Program: Medicine and Health Sciences, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, RS, Brazil
[g] Laboratory of Cellular Stress, Department of Biochemistry/ICBS, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[h] Laboratory of Respiratory Physiology, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), School of Medicine, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, RS, Brazil
[i] Laboratory of Biochemistry and Cellular Biology of Lipids, Department of Biochemistry/ICBS, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[j] Postgraduate Program in Cellular and Molecular Biology, School of Sciences, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, RS, Brazil

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ABSTRACT

Obesity is a prevalent multifactorial chronic disorder characterized by metabolic dysregulation. Sustained pro-oxidative mediators trigger harmful consequences that reflect at systemic level and contribute for the establishment of a premature senescent phenotype associated with macromolecular damage (DNA, protein, and lipids). Telomeres are structures that protect chromosome ends and are associated with a six-protein complex called the shelterin complex and subject to regulation. Under pro-oxidant conditions, telomere attrition and the altered expression of the shelterin proteins are central for the establishment of many pathophysiological conditions such as obesity. Thus, considering that individuals with obesity display a systemic oxidative stress profile that may compromise the telomeres length or its regulation, the aim of this study was to investigate telomere homeostasis in patients with obesity and explore broad/systemic associations with the expression of shelterin genes and the plasma redox state. We performed a cross-sectional study in 39 patients with obesity and 27 eutrophic subjects. Telomere length (T/S ratio) and gene expression of shelterin components were performed in peripheral blood mononuclear cells by qPCR. The oxidative damage (lipid peroxidation and protein carbonylation) and non-enzymatic antioxidant system (total radical-trapping antioxidant potential/reactivity, sulfhydryl and GSH content) were evaluated in plasma. Our results demonstrate that independently of comorbidities, individuals with obesity had significantly shorter telomeres, augmented expression of negative regulators of the shelterin complex, increased lipid peroxidation and higher oxidized protein levels associated with increased non-enzymatic antioxidant defenses. Principal component analysis revealed TRF1 as a major contributor for firstly telomeres shortening. In conclusion, our study is first showing a comprehensive analysis of telomeres in the context of obesity, associated with dysregulation of the shelterin components that was partially explained by TRF1 upregulation that could not be reversed by the observed adaptive non-enzymatic antioxidant response.

* Correspondence to: School of Sciences, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, RS, Brazil.
E-mail address: florencia.tuana@pucrs.br (F.M. Barbé-Tuana).

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1. Introduction

Obesity, recognized as a multifactorial chronic disorder, is the most prevalent non-communicable disease (NCD) and a preventable condition that has tripled since 1975, affecting more than 650 million adults nowadays [1]. It constitutes a major risk factor for other comorbidities such as cardiovascular diseases, diabetes, musculoskeletal disorders, and some cancers. Obesity is associated with chronic inflammation and oxidative stress and besides contributing to the emergence of physiologic imbalances, obesity accelerates aging and reduces lifespan [2].

Aging is a biological process characterized by the progressive deterioration of physiological functions, and some proposed hallmarks include increased genomic instability and telomere attrition [3]. Aging is also a risk factor for the appearance of comorbidities and augmented susceptibility to death [4,5]. Because obesity is an important risk factor for the development of many age-related diseases associated with low-grade systemic inflammatory status and oxidative stress (OS) [6], it seems reasonable to speculate that aging and the condition of obesity share common features. The theory of free radicals associated with aging [7] postulates that aging is the result of macromolecules (DNA, protein, and lipid) damage accumulation, mainly caused by mitochondrial reactive oxygen species (ROS) production connected with the reduced ability of the organism to deal with these deleterious effects, that plays a significant role in the organism's lifespan.

Telomeres are specialized nucleoprotein structures located at the end of eukaryotic chromosomes. They are formed by a double-stranded tandemly repeated DNA sequence (TTAGGG) [8] followed by a G-rich 3'-single strand protrusion [9]. Telomeres play a critical role in the maintenance of genomic integrity [10,11]. The tridimensional structure of the telomeres is assembled in association with six telomere-specific proteins, named the shelterin complex, composed of TRF1, TRF2, TIN2, POT1, TPP1, and RAP1. The shelterin complex binds to telomeric repeats and mediates the formation of an intricate structure sequestering the single-stranded telomeric DNA [12,13]. A proper function of the complex is necessary to avoid unwanted repair events and the induction of the DNA damage response (DDR).

Because telomere length (TL) shortens at each cycle of replication [14], TL has been considered a marker for biological aging [15]. Experimental and clinical studies have already established a causal relation between inflammation, OS [16] and TL [17]. We have recently published a review with meta-analysis to critically evaluate and summarize the scientific evidence about the effect of obesity on TL among 69 different studies. Our results showed a trend toward a negative correlation between obesity and shorter TL [18]. In addition, no study has yet addressed the interconnection between inflammation, oxidative stress (OS) and the shelterin components in a single report. Thus, considering that individuals with obesity have an augmented chronic systemic oxidative profile that may compromise the homeostasis of the telomeric region, the aim of this study was to investigate TL from peripheral blood mononuclear cells (PBMC) in patients with obesity and explore its association with the expression of shelterin genes and the plasma redox state.

2. Methodology

The project was approved by the Research Ethics Committee of the Institutions under protocol number 760.537 (UFRGS) and 640.817 (PUCRS). Participants read, agreed and signed an informed consent.

We included 66 subjects with age ranging between 18 and 65 years old. Based on BMI (kg/m^2) [1], participants were divided into two groups. Individuals with severe or morbid obesity (BMI $\geq$ 35.0 kg/m^2) (n = 39) were recruited by convenience at the Center of Obesity and Metabolic Syndrome unit at São Lucas Hospital at Pontifícia Universidade Católica do Rio Grande do Sul. The control group (n = 27) was composed of healthy eutrophic individuals (18.5 kg/m^2 $\geq$ BMI $\leq$ 24.9 kg/m^2) who did not have any obesity associated comorbidity nor cancer and were not alcohol or tobacco consumers.

2.1. Samples

PBMC were purified using Histopaque®-1077 (Sigma-Aldrich, USA) from 10 mL of whole blood previously collected in tubes containing EDTA as an anticoagulant. Yield (0.8–1.0 $\times 10^6$ PBMC per 1 mL of whole blood from eutrophic patients and higher yield in the group of patients with obesity) and cell viability (> 95%) were determined by trypan blue dye exclusion (Sigma-Aldrich). Plasma samples were stored at −80 °C until use.

2.2. DNA extraction

Genomic DNA (gDNA) was extracted from 0.5 $\times$ 10^6 PBMC using UltraPure Phenol: Chloroform: Isoamyl Alcohol reagent (25:24:1, v/v, Sigma-Aldrich) and Proteinase K (2 mg/mL, Promega, USA) as previously described [19]. gDNA was stored at −20 °C until use.

2.3. Relative telomere length

Relative telomere length was assessed as we previously described [20,21] with modifications from previous work [22]. For each sample, two qPCR reactions were performed – for the amplification of a telomere sequence (T) and for the single-copy autosomal gene, encoding the ribosomal acid phosphoprotein P0 *36B4* (S) (Supplementary Table 1). Results were analyzed by the comparative cT (cycle threshold) method (ΔΔcT) [23] and expressed as relative T/S ratio.

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted from 1 $\times$ 10^6 PBMC using TRIzol® reagent according to the manufacturer's instructions (Invitrogen, USA). RNA purity was assessed spectrophotometrically by absorbance at 260/280 nm in a BioPhotometer Plus (Eppendorf, Germany). Complementary DNA (cDNA) was synthesized from 2 μg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) and stored at −20 °C until use.

2.5. Quantitative real-time polymerase chain reaction (qPCR)

All reactions were performed in triplicate using the Platinum® Taq DNA polymerase enzyme (Invitrogen) in a 96-well Real-Time PCR instrument StepOnePlus™ (Applied Biosystems). The specificity of the amplified products was confirmed by dissociation curves analyses at the end of each reaction. All plates included two different controls: a negative control, to detect possible contamination of the reagents or false-positive in the absence of sample and a random sample to monitor the interplacement variation and used to obtain a normalizing factor to compare inter-plate variation. Triplicates with standard deviation ≥ 0.3 cT were excluded and rerun.

Gene sequence information was collected (www.ensembl.org and https://www.ncbi.nlm.nih.gov/refseq/) and used to design specific primers for *TRF1, TRF2, TIN2, RAP1, TPP1, POT1, DKC1*, *TERT* or *IL-1β* and *TNF-α* using a freely available software from Integrated DNA Technologies (www.idtdna.com). PCR reactions were performed in triplicate in a final volume of 20 μL. We used 3 μL of cDNA (1:20) as a template for qPCR reactions and SYBR green as the fluorescent detector. Primers' sequence, concentration, and specific running conditions are depicted in Supplementary Table 1. Thermal cycling profile for gene expression consisted of an initial denaturation step at 94 °C for 10 min followed by 40 cycles of 15 s at 94 °C, 15 s at specified annealing temperature (Supplementary Table 1) and 15 s at 72 °C for data acquisition. Sole product amplification and absence of primer-dimer was confirmed using melting curve analysis at the end of each run. Additionally, we confirmed the amplification of a unique amplicon of the specified size

by agarose gel electrophoresis. Samples were normalized using the housekeeping gene *GNB2L1* as suggested by GeNorm (https://genorm.cmgg.be/) analysis and calibrated by the average of the ΔcT of the group. In order to analyze data by the ΔΔcT method [23], we previously confirmed similar PCR efficiencies between all studied targets (*TRF1, TRF2, RAP1, TPP1, POT1, DKC1, TERT, IL-1β* and *TNF-α)* and endogenous control (*GNB2L1*).

2.6. Lipid peroxidation

Lipid peroxidation levels in plasma samples were assessed by the non-enzymatic formation of eicosanoid levels in response to phospholipid oxidation using an 8-isoprostane (8-iso-P) competitive enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical, USA) according to the manufacturer's instructions. Lipid peroxidation was measured at 370 nm by spectrophotometry using a SpectraMax® i3 plate reader (Molecular Devices).

2.7. Protein carbonyl content

Protein carbonyl content was quantified as previously described [24]. To sum up, plasma samples were treated with 10 mM dinitrophenylhydrazine (DNPH) in 2 M HCl for 1 h at room temperature and then precipitated with 10% trichloroacetic acid (TCA). The subsequent protein pellet was washed 3 x with 1:1 ethanol/ethyl acetate mixture. Proteins were solubilized in 8 M urea and centrifuged at 16,000 × *g* for 4 min to remove any trace of insoluble material. The carbonyl content was measured at 370 nm by spectrophotometry using a SpectraMax® i3 plate reader (Molecular Devices, USA).

2.8. Total reactive antioxidant potential (TRAP) and Total antioxidant reactivity (TAR)

Total reactive antioxidant potential (TRAP) is an *in vitro* non-enzymatic method based on the action of total antioxidants on the luminescence decay of luminol-enhanced chemiluminescence generated by the reaction of luminol with a reliable and quantifiable source of alkyl peroxyl radical derived from 2,2′-azobis-(2- amidinopropane) dihydrochloride (AAPH). The thermal decomposition of these compounds in the presence of luminol produces luminescence, which is quenched by the addition of peroxyl radical scavengers [25,26]. Plasma samples were added to the reaction and the luminescence produced by the free radical reaction was quantified every 5 s using a MicroBeta TriLux 1450 liquid scintillation counter (Perkin–Elmer, USA) until the readings were stabilized. Raw data were analyzed as 1 - area under the curve (1 - AUC).

The Total Antioxidant Reactivity (TAR) was obtained from the same experiment. The TAR results were calculated as the ratio of light intensity in the absence of samples/first light intensity reading after sample addition (l_0/l_1).

2.9. Sulfhydryl groups (-SH)

Total sulfhydryl groups were measured as previously described [27]. The assay provides a colorimetric readout of total reduced thiol content of a sample, based on the capacity of thiols groups to chemically reduce 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's Reagent), which possesses a reactive disulfide bond susceptible to reduction. The reduction occurs by an exchange reaction in which a mixed disulfide and a yellow-colored 5-thio-2-nitrobenzoic acid (TNB) is formed. The intensity of the yellow color (indicating reduced thiol content) was measured at 412 nm by spectrophotometry using a SpectraMax® i3 plate reader (Molecular Devices).

2.10. Reduced glutathione (GSH) content

This method is based on the reaction of GSH with ortho-phthalaldehyde (OPA) [28]. Briefly, plasma samples were incubated with 1:1 metaphosphoric acid (MPA) in order to be deproteinized. Samples were maintained in the dark for 15 min after an addition of OPA (1 mg/mL, Sigma-Aldrich). The fluorescence was measured by excitation at 350 nm and emission at 420 nm by spectrophotometry using a SpectraMax® i3 plate reader (Molecular Devices). The results were quantified based on a GSH standard curve.

2.11. Data imputation

Missing data from each experimental procedure was imputed using the fully conditional specification approach, also known as multivariate imputation by chained equations, by supplying all other experimental data as well as demographic information. In brief, the algorithm imputes an incomplete column (the target column) by generating 'plausible' synthetic values given other columns in the data. Each incomplete column must act as a target column and has its own specific set of predictors. The default set of predictors for a given target consists of all other columns in the data. For predictors that are incomplete themselves, the most recently generated imputations are used to complete the predictors prior to the imputation of the target column. The imputation procedure was implemented using the mice package in the R statistical environment [29,30]. The mice function was employed using predictive mean matching method (PMM), 50 multiple imputations (m = 50), 10 iterations (maxit = 10) and seed = 123. The mean of the 50 computed imputations was considered the final values (expressed as black dots in the plots).

2.12. Principal component analysis (PCA)

Imputed experimental data from all procedures, except TRAP, was Box-Cox transformed. The arcsine square root transformation was employed for TRAP imputed values. After transformation, PCA computation and visualization was implemented using FactoMineR and factoextra packages in the R statistical environment [30–32].

2.13. Flow cytometry

Ex vivo protein expression of TRF1 was analyzed by flow cytometry. PBMC (1 × 10^6 cells) were stained with anti-TRF1 antibody conjugated with Alexa Fluor-647 (1:30, clone G-7, Santa Cruz Biotechnology, CA, USA) incubated for 30 min at 4 °C in the dark with the transcription factor kit (BD Pharmingen™, USA) following manufacturer's instructions. Samples (10,000 events) were acquired in the Accuri C6 Flow Cytometer (BD Biosciences, USA) and analyzed in the monocyte or lymphocyte gates with the Accuri C6 software. Results are expressed as median fluorescence intensity (MFI).

2.14. Statistical analyses

Categorical data are presented by absolute and relative frequency and continuous variables, using median and interquartile range, according to the asymmetry of the variables. Shapiro-Wilk test was performed to verify the normal distribution of samples for each variable. The comparison between groups was evaluated by unpaired *t*-test or Mann-Whitney test, depending on normal *vs*. non-normal distributed sample, respectively. To obtain data with a possible relationship between TL and age of the subjects, and among all observed variables, Spearman non-parametric correlation tests were performed. GraphPad Software Inc. version 6.01 (La Jolla, California, USA) and Statistical Package for Social Sciences (SPSS) (SPSS v.22 for Windows, IBM Corp. Armonk, New York, USA) were used in all analyses. All tests were bidirectional and the differences were considered significant at $p < 0.05$

Table 1
Baseline and demographic characteristics.

	Groups		p value
	Control (n = 27)	**Obese (n = 39)**	
Gender (male), n/total (%)	11/27 (40.7)	9/39 (23.1)	0.1742
Age (years), median (IQR)	28.0 (26.0–33.0)	36.0 (32.7–41.2)	**0.0006**
BMI, median (IQR)	22.1 (20.7–23.2)	45.0 (41.4–50.2)	< **0.0001**
Physical activity, n/total (%)	2/27 (7.4)	0/39 (0)	0.1636
Comorbidities, n/total (%)			
Dyslipidemia	0/27 (0)	24/39 (61.5)	–
Hepatic steatosis	0/27 (0)	19/39 (48.7)	–
Hypertension	0/27 (0)	21/39 (53.8)	–
Metabolic syndrome	0/27 (0)	21/39 (53.8)	–
Type 2 diabetes mellitus	0/27 (0)	11/39 (28.2)	–

IQR = Interquartile range.
Results are shown with number (percent) or median IQR (25–75%).
Bolded results indicate significant differences between groups (95% confidence interval).

(*), p < 0.01 (**), p < 0.001(***) or p < 0.0001 (****).

3. Results

3.1. Baseline and demographic characteristics

Subjects with obesity (n = 39) were older than subjects in the control (n = 27) group (p < 0.0006) and had more comorbidities (p < 0.0001). No differences regarding gender and physical activities were observed (Table 1).

3.2. Shorter telomere length in patients with obesity

Because age was different between groups (p < 0.0006) and telomeres naturally shorten with age, we compared TL after adjustment for age. Individuals with obesity had significantly shorter telomeres when compared to healthy controls (p = 0.026) (Fig. 1A). Because telomeres attrition could also be explained by the presence of comorbidities, we did a subgroup analysis. We divided the group of patients with obesity (n = 39) according to the absence (n = 7) or presence of comorbidities (n = 32). Kruskal-Wallis analysis showed differences in the T/S ratio among the groups (p = 0.0008). Post-hoc Dunns' multiple comparisons test revealed that obese patients without (p = 0.05) and with comorbidities (p = 0.01) were different from controls (Fig. 1B). When we matched eutrophic individuals (n = 7), and obese patients without (n = 7) or with comorbidities (n = 6) for age [median (IQR); 32.0 (26.0–42.0), 33.0 (25.0–47.0) and 34.5 (30.0–48.8) years], and for sex (male/n; 2/7; 2/7; 2/6), we observed comparable results shown in Fig. 1C. In this regard, we can conclude that the differences in the T/S ratio are related to the obesity condition by itself and not related to different comorbidity disturbances. As expected, we detected an inverse association between age and telomere length in the group of patients with obesity (r = -0.317, p = 0.0025) (Fig. 1D).

3.3. Upregulated gene expression of shelterin components, DKC1 and an inflammatory profile in patients with obesity

In this work, we included 66 subjects. Because there were missing data for some of the analyzed variables, we performed data imputation. Results from imputed (n = 66) and non-imputed incomplete data sets (n < 66) did not differ and maintained the same significance (Supplementary Table 2). Our results from imputed data showed increased mRNA levels for *TRF1* (p = 0.0216), *TRF2* (p = 0.0145), *RAP1* (p = 0.003), *POT1* (p = 0.0315) and the accessory protein *DKC1* (p = 0.0244) in PBMC from subjects with obesity. Shelterin component *TPP1* showed no difference (p = 0.9665) (Fig. 2). Irrespective of the group, gene expression of the telomerase catalytic subunit (*TERT*) was not detected in most of the samples (data not shown).

As expected, gene expression from *IL-1β* [detected in few controls (12/27) and in few obese patients (14/39); 0.035 (0.02–0.07) vs 56.92 (8.05–99.61), p < 0.0001] and *TNF-α* [detected in few controls (3/27) and in few obese patients (8/39); 0.48 (0.05–0.81) vs 1.65 (0.69– 4.24), p = 0.097], were upregulated in patients with obesity (data not shown).

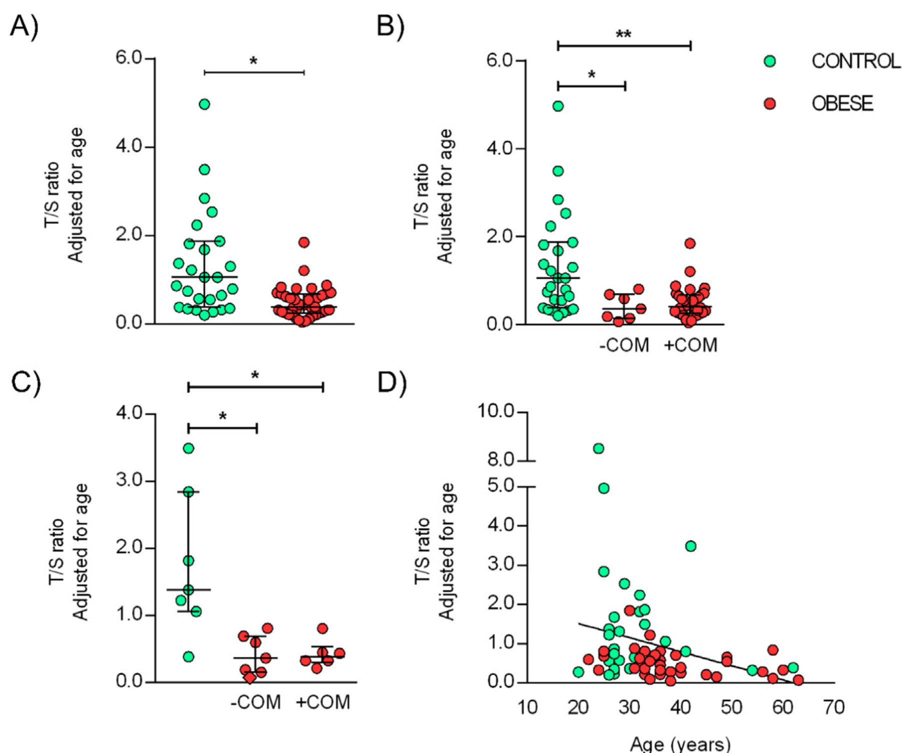

Fig. 1. Shorter telomeres among individuals with obesity. A) Individuals with obesity (n = 39) had shorter telomeres (T/S ratio) compared to healthy controls (n = 27) (p = 0.026) after adjustment for age. Differences were analyzed with Mann-Whitney *U*-test. B) Subgroup analysis of control individuals (n = 27), individuals without (n = 7) and with comorbidities (n = 32) from the obesity group show shorter telomeres' length (T/S) compared to healthy controls (p = 0.01 and p = 0.05, respectively) after adjustment for age. Differences were analyzed with Kruskal-Wallis test and Dunn's post-hoc comparisons. C) Subgroup analysis, paired for age and sex, among controls (n = 7) and individuals with obesity without (-COM, n = 7) or with comorbidities (+COM, n = 6) show shorter telomere length (T/S) compared to healthy controls (p = 0.05 and p = 0.05, respectively). Differences were analyzed with Kruskal-Wallis test and Dunn's post-hoc comparisons. D) Inverse association between age and telomere length in the group with obesity (Spearman correlation r = -0.317; p = 0.0025). Data are presented as median and IQR (25–75%). Significant differences considered when p < 0.05 (*) or p < 0.01 (**). Abbreviations: n: sample size; IQR: Interquartile range.

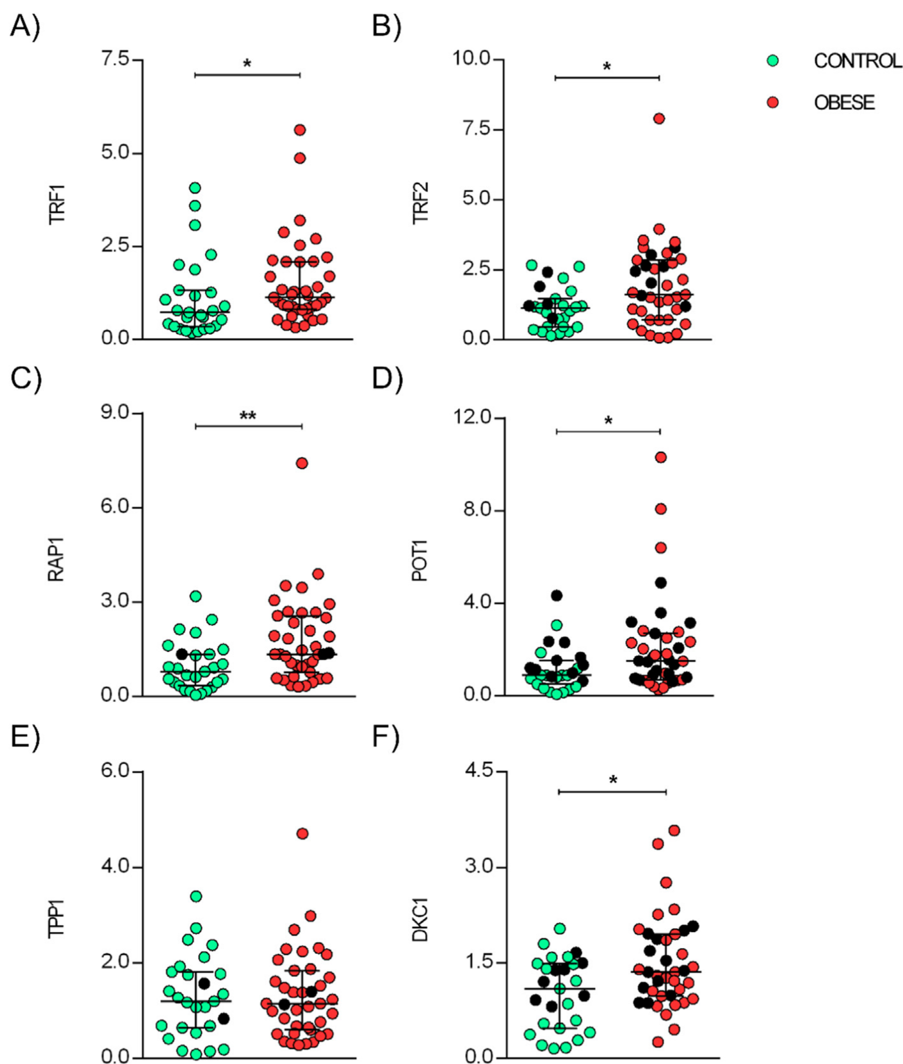

Fig. 2. Individuals with obesity showed increased gene expression levels of shelterin components. Relative gene expression of shelterin components was analyzed in PBMC from controls (n = 27) or individuals with obesity (n = 39). Samples were analyzed by the comparative method (ΔΔcT) and normalized to *GBN2L1*. A) *TRF1*, B) *TRF2*, C) *RAP1*, D) *POT1*, and F) the accessory protein *DKC1*. Shelterin component E) *TPP1* showed no difference between groups. Data are presented as median and IQR (25–75%). Differences were analyzed with Mann-Whitney *U*-test. Green dots represent individuals from the control group; red dots represent individuals with obesity and black dots represent imputed data. Significant differences considered when $p < 0.05$ (*) and $p < 0.01$ (**). Abbreviations: n: sample size; IQR: Interquartile range; TRF1: Telomeric repeat binding factor; TRF2: Telomeric repeat binding factor 2; RAP1: Repressor/activator protein 1; TPP1: Adrenocortical dysplasia protein homolog; POT1: Protection of telomeres protein 1; DKC1: Dyskerin; TERT: Catalytic subunit of the telomerase holoenzyme complex; GNB2L1: Guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1.

3.4. Augmented macromolecules' damage in patients with obesity

Patients with obesity showed increased lipid peroxidation levels, reflected as a higher amount of 8-iso-P (p = 0.0027) (Fig. 3A). We also found significantly higher oxidized protein levels (p = 0.0074) by carbonyl content measurement (Fig. 3B). These results indicate an increased damage caused by oxidative imbalance observed in plasma from patients with obesity.

3.5. Augmented non-enzymatic antioxidant defenses in obese patients

Subjects with obesity also showed total non-enzymatic antioxidant capacity (TRAP) and reactivity (TAR) significantly higher than healthy controls ($p < 0.0001$) (Fig. 3C and D), as well as higher levels of total reduced sulfhydryl groups (p = 0.0451) (Fig. 3E), with no difference in GSH content (p = 0.4127) (Fig. 3F).

3.6. TRF1 is the main factor contributing to the variance between groups

To explore additional associations, we investigated correlations among all observed variables and whether telomere shortening was correlated to any studied factor. The results demonstrated positive associations between TRF1 and many observed variables (Fig. 4). Interestingly, the T/S ratio was only inversely correlated with TRAP (non-significant correlations are depicted in Supplementary Figure 1).

Since many factors can influence telomeres' length, we performed a principal component analysis (PCA) to analyze the contribution of these analyzed factors into the shortening of telomeres as our outcome. Our analysis showed that 36.6% of the sample variance can be explained by the first principal component (PC1) (Fig. 5A), and the first two principal components (PC1 + PC2) contain 52.3% of the variance information between groups, as depicted by the plots that defined two clusters of data (Fig. 5B). In addition, the PCA allowed us to simplify the analysis and discover a hierarchical order for telomere shortening contribution to each PC. In this case, TRF1, POT1 RAP1, TPP1, and DKC1 were revealed as variables that mostly contributed to PC1 variance (Fig. 5D).

Because TRF1 appeared as a major contributor for telomere shortening, TRF1 protein expression was validated by flow cytometry in samples from newly recruited participants (Fig. 6A and B). Similar to *TRF1* gene expression (Fig. 2A), protein levels of TRF1 were higher in the group of patients with obesity (n = 10) when compared to age and sex-matched controls (n = 8) (p = 0.0002). Demographic data from this new cohort is depicted in Supplementary Table 3.

4. Discussion

Obesity is characterized as an energy imbalance-related disorder where adipose tissue expansion is central for metabolic impairment. Obesity leads to an increased risk of morbidity and reduced life expectancy [33,34]. Metabolic dysfunctions associated with obesity, such as dyslipidemia, hypertension, insulin resistance, high LDL levels, and DM2, contribute to a pro-inflammatory and pro-oxidative imbalance

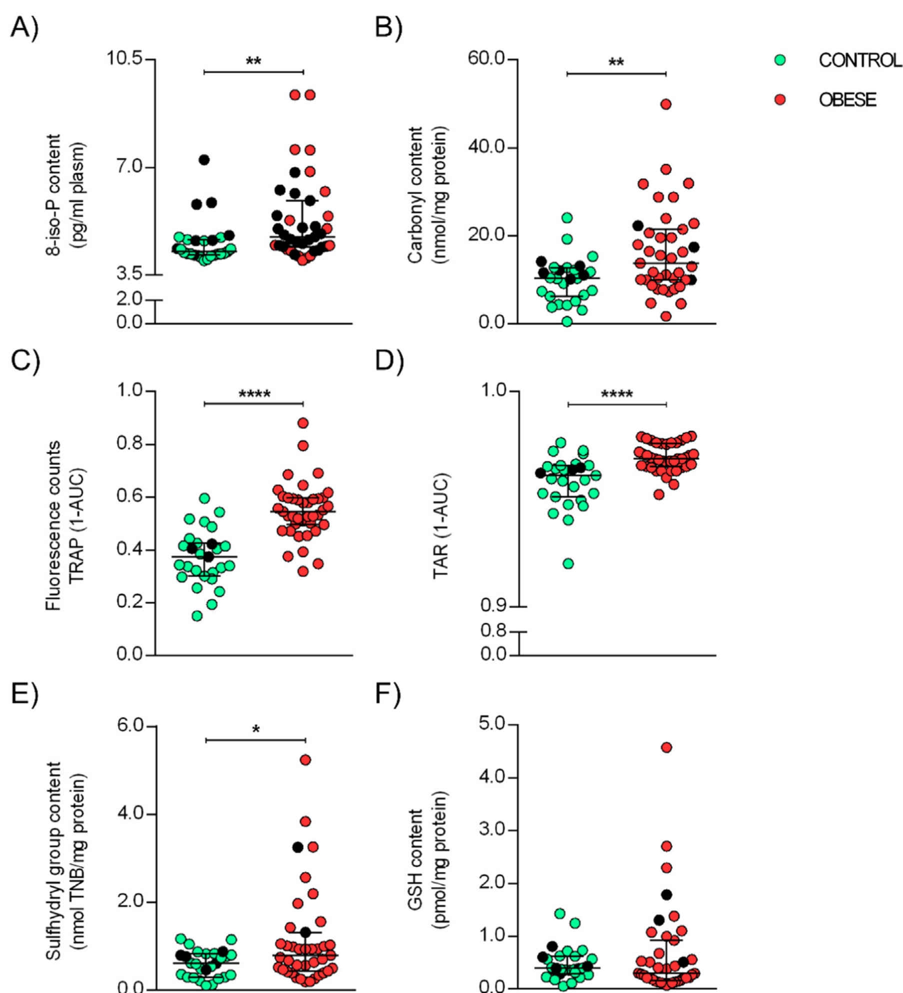

Fig. 3. Increased macromolecules' damage in plasma from subjects with obesity. Plasma from patients with obesity showed, A) increased levels of lipid peroxidation (p = 0.0027) and B) higher oxidized protein levels (p = 0.0074). C) TRAP and D) TAR were both higher in patients with obesity (p > 0.0001), as well as E) total sulfhydryl groups (p = 0.0451). F) There was no difference in GSH content (p = 0.4127). Data are presented as a median and IQR (25–75%). Black dots represent imputed data. Differences were analyzed with Mann-Whitney *U*-test. Green dots represent individuals from the control group; red dots represent individuals with obesity and black dots represent imputed data. Significant differences considered when p < 0.05 (*), p < 0.01 (**) or p < 0.0001 (****). Abbreviations: IQR: Interquartile range; TRAP: Total Reactive Antioxidant Potential; TAR: Total Antioxidant Reactivity; GSH: Reduced Glutathione.

environment [35]. Increased ROS levels play a crucial role in the metabolic syndrome onset, mostly triggering insulin resistance and dysregulating glucose homeostasis [36]. Even more interesting is the observation that all the above-mentioned obesity-related comorbidities are also associated with shortened telomeres [37–39].

From our study, three important conclusions can be drawn. First, our results demonstrate that adults with severe or morbid obesity show reduced telomeres' length in comparison with eutrophic subjects. Second, it evidences that the condition of obesity alters telomeres' homeostasis through the shelterin components, mainly TRF1, and finally, indicates that the antioxidant adaptive responses are not sufficient to counteract the oxidative stress and telomeres attrition.

One hypothesis about aging is that immediate telomeres' shortening occurs early in many diseases, suggesting that telomere length may be an important biological marker for the early development of pathologies [40]. Indeed, dysregulated telomere length has been implicated and causally related to hematological diseases, such aplastic anemia, dyskeratosis congenita and idiopathic pulmonary fibrosis [41–43] and also different types of cancers [44,45]. However, in the context of obesity, the nature of the association with shorter TL is still under debate. Obese children are at higher risk for the persistence of obesity and development of other comorbidities in adulthood [46]. Buxton et al.

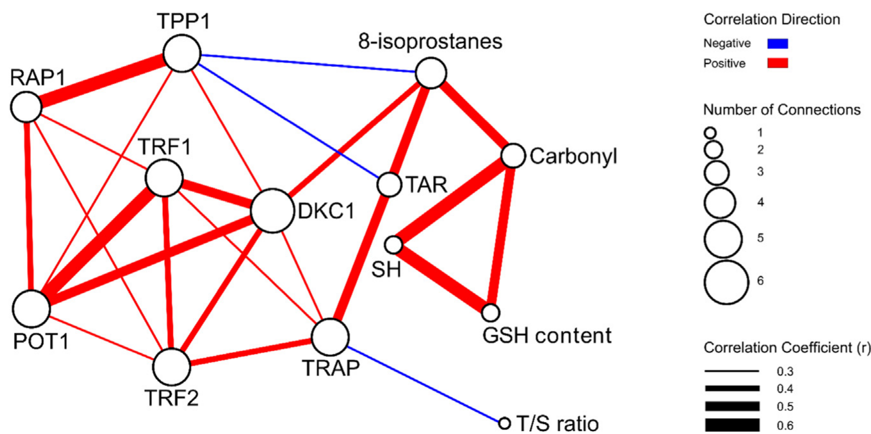

Fig. 4. Plot depicting gene interactions between significantly different (p < 0.05) variables. The number of interactions within one variable is represented by the size of the node (circle), and the magnitude (line thickness) and color (blue or red) of the connector represent the regression coefficient (based on r values) and direction (r > 0 or r < 0), respectively. The absence of significant correlation (r) and significance (p > 0.05) between variables is not represented. Abbreviations: TRF1: Telomeric repeat binding factor; TRF2: Telomeric repeat binding factor 2; RAP1: Repressor/activator protein 1; TPP1: Adrenocortical dysplasia protein homolog; POT1: Protection of telomeres protein 1; DKC1: Dyskerin; TRAP: Total Reactive Antioxidant Potential; TAR: Total Antioxidant Reactivity; GSH: Reduced Glutathione.

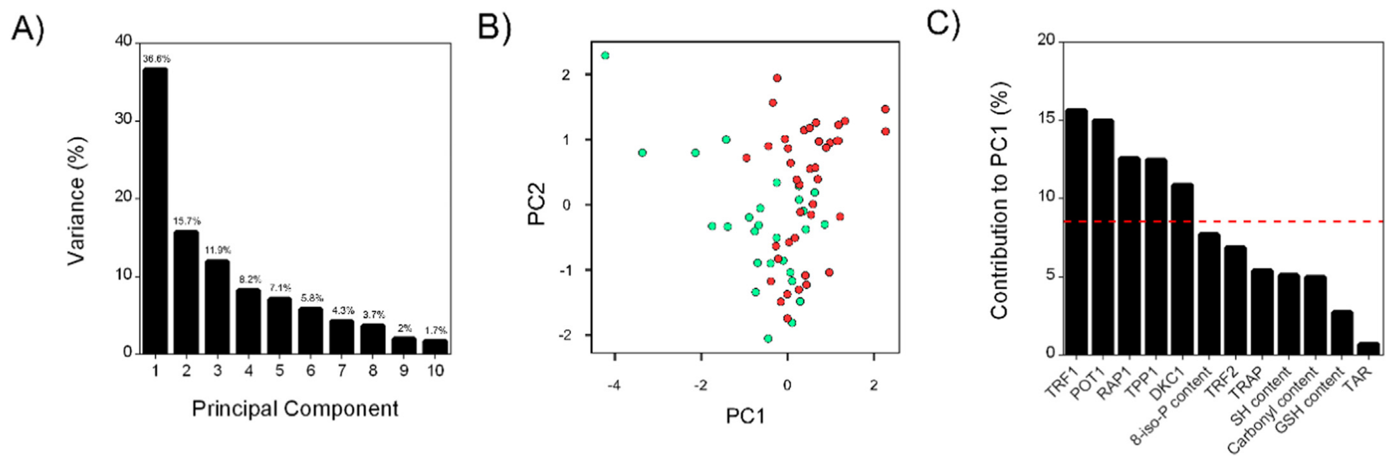

Fig. 5. TRF1 as a major contributor for clustering among the groups. Principal component analysis (PCA) showed that A) 36.6% of the sample variance can be explained by the first principal component (PC1), and B) the first two principal components (PC1 + PC2) contain 52.3% of the variance information between groups. C) Bar plot showing TRF1 as a variable that mostly contributed to PC1. The red dashed line indicates the expected average contribution (1/12 studied variables = 8,33%) of each variable. Abbreviations: TRF1: Telomeric repeat binding factor; TRF2: Telomeric repeat binding factor 2; RAP1: Repressor/activator protein 1; TPP1: Adrenocortical dysplasia protein homolog; POT1: Protection of telomeres protein 1; DKC1: Dyskerin; TRAP: Total Reactive Antioxidant Potential; TAR: Total Antioxidant Reactivity; GSH: Reduced Glutathione.

demonstrated that the impact caused by accelerated shortening of telomeres due to obesity might begin as early as childhood. Their results evidence a strong association between short leukocyte telomeres and the occurrence of obesity for both genders [47]. To our knowledge, there is only one study in adults suggesting that shorter telomeres in leukocytes may be a risk factor for increased adiposity possibly accelerating the aging process in the context of obesity [48].

Our results showing shorter TL in patients with obesity after age-adjustment are in accordance with a previous meta-analysis published by our group where we evaluated 63 original studies comprising 119,439 subjects. Although heterogeneity was high among the studies, in this analysis we observed a weak and moderate negative correlation between obesity and TL, suggesting that additional studies are still on demand [18]. Telomeres' attrition doesn't always correlate with aging in all studied groups from humans, especially in the elderly; suggesting that the argument of telomeres length as a biomarker of aging is still under debate [49,50].

Also, many confounding variables such as gender and comorbidities might influence TL and male gender is associated with shorter telomeres [51]. For example, in our first cohort (Table 1), the number of men in each group was different (40.7% and 23.1% in the control and obese group, respectively), and analyses were not adjusted for sex. However, the higher percentage of males is in the control group, suggesting that, if present, the effect of sex on the T/S ratio would bias the TL in the opposite direction: diminishing the T/S in the control group. In addition, the absence of comorbidities in the control group could be misinterpreted and super estimate the differences found in the TL from the obese group; shorter TL could be interpreted as due to the obesity condition either by itself or to the different comorbidities disturbances. Indeed, subgroup analysis, separating patients with obesity according to the absence or presence of comorbidities demonstrated differences in the T/S ratio among the groups (control *versus* obese) and no differences between obese patients without and with comorbidities. We observed comparable results when groups were paired for age and sex without and with comorbidities. In this regard, we can conclude that the observed differences in the T/S ratio are related to the obesity condition by itself and not related to different comorbidities disturbances. In line with these findings, longitudinal studies are focusing on biological mechanisms that elucidate the relationship between accelerated telomere shortening and obesity. Formichi et al. monitored 93 individuals with severe obesity after bariatric surgery and observed reduced TL in obese patients when compared to healthy individuals with no effect after 12 months of surgery [52]. Contrary, Laimer et al. observed a recovery and increase in TL post-bariatric surgery after 10 years of accompaniment [53]. Their results may indicate that telomere length restoration may be achieved by pronounced and sustained weight loss over a longer period of intervention but no mention of the possible mechanism of action was addressed.

Telomere length can be controlled at distinct levels. Cross-talk between the enzyme telomerase and the shelterin proteins is necessary to preserve telomeres homeostasis [54]. In our settings, we did not observe gene expression of the catalytically active component of the

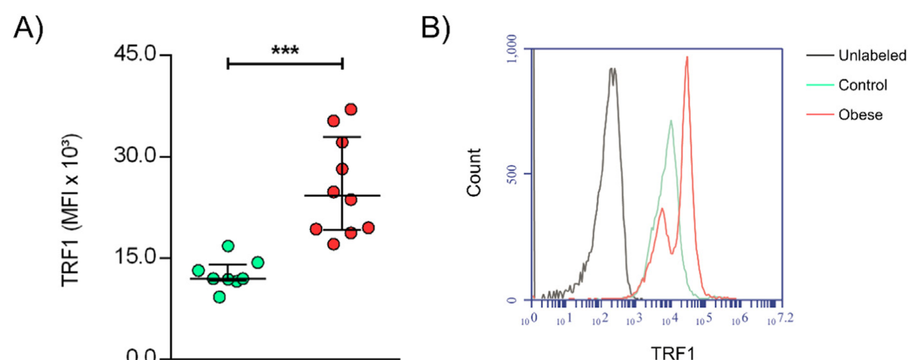

Fig. 6. Augmented TRF1 protein expression. A) TRF1 analysis showed an overexpression in PBMC of obese patients (red dots, n = 10) compared to controls (green dots, n = 8) (p = 0.0002). B) Representative histograms of TRF1 protein expression. Abbreviations: MFI: median fluorescence intensity.

telomerase enzyme (*TERT*) in PBMC. This result is expected, as circulating peripheral leukocytes are not proliferating cells and telomerase has low activity in T and B cells but not in monocytes [55]. On the other side, in this study, we observed an up-regulation of *IL-1β* gene expression in PBMC from subjects with obesity, a cytokine also synthesized by adipocytes and resident and infiltrated macrophages that might contribute to the establishment of a local and systemic inflammation environment during the expansion of the adipose tissue [56,57]. Thus, this para-inflammation state present in obesity might induce an augmented rate of proliferation of hematopoietic precursors in the bone marrow and the expansion and proliferation of leukocytes in peripheral sites driving leukocyte senescence and telomeres shortening. In agreement with these findings, we have previously shown that plasma from patients with obesity is responsible for the induction of an immunosenescent phenotype in non-related donor healthy PBMC [58].

Shelterin proteins are main regulators of telomere length metabolism. The tridimensional structure of the T and D-loop is responsible for protecting chromosomes ends from degradation, repair activities and in active-telomerase cells, of its action [12]. In the context of obesity, our study reveals gene expression up-regulation of *TRF1*, *TRF2*, *POT1* and *RAP1* transcripts in PBMC. TRF1, TRF2, and POT1 have all been described as cis-negative regulators for TL [59], although they perform different functions in telomeres' maintenance during homeostasis. We measured different variables, as gene expression from the shelterin complex, parameters of the redox system, parameters of inflammation and telomeres shortening. Because the aim of our work was to identify, which of the variables, in the context of the obesity, best explain and reflect the dynamics of our system: the observation of shortened telomeres, we performed a PCA. PCA was used as a statistical tool to systematically extract from all that measurements, the variable/variables that best reflected the dynamics of telomeres attrition [60], and the results identified TRF1 as a major negative regulator of telomeres' attrition in the context of obesity. To our knowledge, we are first to demonstrate up-regulation of TRF1 in PBMC from patients with obesity, a condition that is associated with a higher incidence of cancer [61–63]. TRF1 depletion has been associated with chromosome breakage [64] and protection but not elongation of the telomeres [65]. In contrast with these findings, we have additional data from our cohort of patients with obesity showing a higher frequency of micronucleus and karyolysis in oral mucosal epithelial cells (Parisi et al., manuscript under preparation), conditions also associated with a higher predisposition for the augmented incidence of cancer observed in the context of obesity. Although our study design did not allow us to demonstrate causal relationships but associations, our data suggest that an initial genetic damage already established is present in the context of obesity and might be interpreted as signaling for future transformation.

Similar to TRF1 and TRF2, recent data suggest that POT1 would initially inhibit the translocation of the enzyme telomerase to the telomeres and prevent its interaction with the telomeres, therefore elongation [66]. In addition, POT1's promoter has an element recognition site for the binding of NF-κB suggesting another link with inflammation. Indeed, in a macrophages lineage, POT1 action has been observed in extranuclear locations in the cytosol, pointing to extra-telomeric functions associated with the inhibition of the endocytosis mechanism and reduction of the inducible nitric oxide synthase (iNOS) enzyme [67]. In conclusion, these observations suggest that POT1 up-regulation might act as a mechanism for telomeres elongation inhibition, a link with inflammation and impairment of the innate immune system, contributing to an augmented susceptibility towards bacterial infections, as already documented in the context of obesity. Our results on PCA also revealed POT1 up-regulation as an important factor, resemblant to TRF1 and contributing to telomere shortening in the context of obesity. Indeed, POT1 is related to repress DDR by the serine/threonine kinases ataxia telangiectasia-mutated Rad3-related (ATR) - mediated pathway [68], protecting telomeric DNA from genetic abnormalities. These findings could possibly be related to an attempt to prevent the activation of DNA damage response related to dysfunctional telomeres.

TRF1 and TRF2 forms a bridge with TIN2 and connects with ssDNA through POT1 [69]. Knock out mice confirm the role of TRF2 in the assembly of the T-loop. Its overexpression is associated with augmented T-loop formation, inversely correlated with telomerase activity [70] and functions avoiding end-to-end fusions [71,72]. The presence of a single strand non-protected or uncapped DNA is a trigger for the DDR signaling and induction of senescence or apoptosis. Because TRF2 physically interacts with enzymes from the nucleotide excision repair [73], it's upregulation is also associated with chromosomal instability, suggesting again an augmented risk factor for the development of cancer, as shown in prostate cancer [61].

In this report, we showed augmented *RAP1* expression in the context of obesity. This result was at least surprisingly, as previous works done in mice suggest a protective role for RAP1 against obesity and metabolic dysfunction [74–76]. However, RAP1 is associated in the shelterin complex through the interaction with TRF2 and does not have regulatory functions for telomere length [77]. Additional non-telomeric cytoplasmic functions have been described for mammalian RAP1 through interaction with the inhibitor kappa B kinases (IKK) in the cytoplasm and the modulation of nuclear factor kappa B (NF-κB)-mediated pathways. A regulatory feedback has been ascribed for RAP1 where augmented levels are positively regulated by NF-κB, and human breast cancers with NF-κB hyperactivity show elevated levels of cytoplasmic RAP1 [78]. In agreement with these findings, breast carcinoma cell lines treated with tumor necrosis factor-alpha (TNF-α) have shown a 5 or 6-fold induction of TRF1, TIN2, and POT1. The pro-inflammatory milieu further suggests a link between shelterin proteins and inflammation.

Peroxisome proliferator-activated receptors (PPAR) are ligand-activated transcription factors that belong to the superfamily of nuclear hormone receptors and play a key role in various processes including modulation of differentiation and cell viability, biomolecules metabolism, and inflammation. However, the activities promoted by PPAR depends on the cell type and the binding isoform, to favor different cell processes in distinct outcomes [79,80]. Martinez et al. demonstrated that RAP1 knockout mouse embryonic fibroblasts (MEF) have down-regulation of PGC-1α [81] and PPARα, showing the premature development of obesity and associated comorbidities [74]. Furthermore, macrophages induce PGC-1α [82]. In this way up-regulation of *RAP1* gene expression, as observed in this work, could be involved in the regulation of the PPAR pathway.

Telomere shortening contributes to metabolic dysfunction through the impact on mitochondrial biogenesis and metabolism in a rodent model [83]. Metabolic dysfunctions such as those seen in obesity have an important role in accelerated aging development even in the absence of any other source [84]. Studies demonstrate that damage caused by ROS accumulation is related to physiological aging in both human [85,86] and rodent [87] models, although the mechanisms by which oxidative imbalance can accelerate aging is still under debate. In normal physiological functions, redox homeostasis allows maintenance of a reduced intracellular environment by ensuring properly protein structure and function and lower peroxidation levels [88]. However, an impairment of redox status promote an oxidative imbalance by an accumulation and dysregulated ROS signaling that may lead to anomalous cell dysfunction, contributing to disease progression [89]. Thus, excessive oxidative stress appears to be an important feature in numerous aging-related diseases since there is an increased level of ROS production that cannot be totally scavenged by antioxidants defenses [90].

In this regard, we also demonstrate that subjects with obesity have a plasmatic pro-oxidant environment observed by a higher protein oxidation and lipid peroxidation levels. Our results also indicate that this impairment caused by an oxidative imbalance leads to increased non-enzymatic antioxidant defenses, which appears to be an adaptive rescue response to avoid cellular dysfunction by an excessive damage caused by enhanced ROS levels.

Biomolecule damage caused by ROS in obesity is widely described in the literature. Several studies show an elevated protein carbonylation amount, both in adipose tissue [91] and plasma, with a strong association with diabetes mellitus [92]. Besides this, higher plasmatic lipid peroxidation levels are also detected [93]. Our results on antioxidant capacity are in agreement with a recent study that evaluated DM2 patients in relation to healthy controls, in which the authors showed an increased total antioxidant status in plasma samples [94]. Non-enzymatic antioxidants, composed by ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), polyphenols (flavonoids and phenolic acids), carotenoids, GSH, and others, represents the major defenses in plasma [95]. However, because of sample limitation, we were unable to analyze further enzymatic antioxidant defenses such as superoxide dismutase, glutathione peroxidase, and catalase.

In summary, our study adds valuable information to current literature and shows that PBMC telomeres' homeostasis is compromised, favoring shorter telomeres, in the context of severe and morbid obesity. Additionally, our study identifies TRF1 as a major regulator of telomeres attrition in the context of obesity. We suggest that telomeres uncapping by shelterin components might be partially explained by TRF1 upregulation, a mechanism that might accelerate the aging process associated with augmented damage of proteins and lipids and the pro-oxidant environment and might predispose cells for neoplastic transformation. The increased non-enzymatic antioxidant defenses observed in the plasma may be a cellular oxidative imbalance adaptive rescue response to evade dysfunctional signaling and cell death.

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Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.freeradbiomed.2018.09.039

References

[1] W.H. Organization, Obesity and overweight factsheet from the WHO, Health (2017).
[2] R.S. Ahima, Connecting obesity, aging and diabetes, Nat. Med. 15 (9) (2009) 996–997.
[3] C. Lopez-Otin, M.A. Blasco, L. Partridge, M. Serrano, G. Kroemer, The hallmarks of aging, Cell 153 (6) (2013) 1194–1217.
[4] G. Paradies, G. Petrosillo, V. Paradies, F.M. Ruggiero, Oxidative stress, mitochondrial bioenergetics, and cardiolipin in aging, Free Radic. Biol. Med. 48 (10) (2010) 1286–1295.
[5] C.H. Wang, S.B. Wu, Y.T. Wu, Y.H. Wei, Oxidative stress response elicited by mitochondrial dysfunction: implication in the pathophysiology of aging, Exp. Biol. Med. 238 (5) (2013) 450–460.
[6] F.M. Sohet, A.M. Neyrinck, B.D. Pachikian, F.C. de Backer, L.B. Bindels, P. Niklowitz, T. Menke, P.D. Cani, N.M. Delzenne, Coenzyme Q10 supplementation lowers hepatic oxidative stress and inflammation associated with diet-induced obesity in mice, Biochem. Pharmacol. 78 (11) (2009) 1391–1400.
[7] D. Harman, Aging: a theory based on free radical and radiation chemistry, J. Gerontol. 11 (3) (1956) 298–300.
[8] E.H. Blackburn, Structure and function of telomeres, Nature 350 (6319) (1991) 569–573.
[9] W. Palm, T. de Lange, How shelterin protects mammalian telomeres, Annu. Rev. Genet. 42 (2008) 301–334.
[10] M.A. Shammas, Telomeres, lifestyle, cancer, and aging, Curr. Opin. Clin. Nutr. Metab. Care 14 (1) (2011) 28–34.
[11] M. Lisby, T. Teixeira, E. Gilson, V. Geli, The fate of irreparable DNA double-strand breaks and eroded telomeres at the nuclear periphery, Nucleus 1 (2) (2010) 158–161.
[12] J.D. Griffith, L. Comeau, S. Rosenfield, R.M. Stansel, A. Bianchi, H. Moss, T. de Lange, Mammalian telomeres end in a large duplex loop, Cell 97 (4) (1999) 503–514.
[13] Y. Doksani, J.Y. Wu, T. de Lange, X. Zhuang, Super-resolution fluorescence imaging of telomeres reveals TRF2-dependent T-loop formation, Cell 155 (2) (2013) 345–356.
[14] J.D. Watson, Origin of concatemeric T7 DNA, Nat.: New Biol. 239 (94) (1972) 197–201.
[15] S. Bekaert, T. De Meyer, P. Van Oostveldt, Telomere attrition as ageing biomarker, Anticancer Res. 25 (4) (2005) 3011–3021.
[16] A.M. Valdes, T. Andrew, J.P. Gardner, M. Kimura, E. Oelsner, L.F. Cherkas, A. Aviv, T.D. Spector, Obesity, cigarette smoking, and telomere length in women, Lancet 366 (9486) (2005) 662–664.
[17] T. De Meyer, E.R. Rietzschel, M.L. De Buyzere, W. Van Criekinge, S. Bekaert, Telomere length and cardiovascular aging: the means to the ends? Ageing Res. Rev. 10 (2) (2011) 297–303.
[18] E. Mundstock, E.E. Sarria, H. Zatti, F. Mattos Louzada, L. Kich Grun, M. Herbert Jones, F.T. Guma, J. Mazzola In Memoriam, M. Epifanio, R.T. Stein, F.M. Barbe-Tuana, R. Mattiello, Effect of obesity on telomere length: systematic review and meta-analysis, Obesity 23 (11) (2015) 2165–2174.
[19] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162 (1) (1987) 156–159.
[20] F.M. Barbe-Tuana, M.M. Parisi, B.S. Panizzutti, G.R. Fries, L.K. Grun, F.T. Guma, F. Kapczinski, M. Berk, C.S. Gama, A.R. Rosa, Shortened telomere length in bipolar disorder: a comparison of the early and late stages of disease, Rev. Bras. De. Psiquiatr. 38 (4) (2016) 281–286.
[21] L.S. Czepielewski, R. Massuda, B. Panizzutti, E.D. da Rosa, D. de Lucena, D. Macedo, L.K. Grun, F.M. Barbe-Tuana, C.S. Gama, Telomere length in subjects with schizophrenia, their unaffected siblings and healthy controls: evidence of accelerated aging, Schizophr. Res. 174 (1–3) (2016) 39–42.
[22] R.M. Cawthon, Telomere measurement by quantitative PCR, Nucleic Acids Res. 30 (10) (2002) e47.
[23] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, Methods 25 (4) (2001) 402–408.
[24] R.L. Levine, D. Garland, C.N. Oliver, A. Amici, I. Climent, A.G. Lenz, B.W. Ahn, S. Shaltiel, E.R. Stadtman, Determination of carbonyl content in oxidatively modified proteins, Methods Enzymol. 186 (1990) 464–478.
[25] D.D. Wayner, G.W. Burton, K.U. Ingold, S. Locke, Quantitative measurement of the total, peroxyl radical-trapping antioxidant capability of human blood plasma by controlled peroxidation. The important contribution made by plasma proteins, FEBS Lett. 187 (1) (1985) 33–37.
[26] M.T. Dresch, S.B. Rossato, V.D. Kappel, R. Biegelmeyer, M.L. Hoff, P. Mayorga, J.A. Zuanazzi, A.T. Henriques, J.C. Moreira, Optimization and validation of an alternative method to evaluate total reactive antioxidant potential, Anal. Biochem. 385 (1) (2009) 107–114.
[27] J.R. Winther, C. Thorpe, Quantification of thiols and disulfides, Biochim. Biophys. Acta 1840 (2) (2014) 838–846.
[28] R.W. Browne, D. Armstrong, Reduced glutathione and glutathione disulfide, Methods Mol. Biol. 108 (1998) 347–352.
[29] S. Buuren, K. Groothuis-Oudshoorn, mice: multivariate imputation by chained equations in R, J. Stat. Softw. 45 (3) (2011).
[30] R.C. Team, R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing; 2014. R Foundation for Statistical Computing, Vienna, Austria, 2016.
[31] S. Lê, J. Josse, F. Husson, FactoMineR: an R package for multivariate analysis, J. Stat. Softw. 25 (1) (2008) 1–18.
[32] A. Kassambara, F. Mundt, Factoextra: extract and visualize the results of multivariate data analyses, R. Package Version 1 (3) (2016).
[33] G. Murdolo, M. Piroddi, F. Luchetti, C. Tortoioli, B. Canonico, C. Zerbinati, F. Galli, L. Iuliano, Oxidative stress and lipid peroxidation by-products at the crossroad between adipose organ dysregulation and obesity-linked insulin resistance, Biochimie 95 (3) (2013) 585–594.
[34] I. Savini, M.V. Catani, D. Evangelista, V. Gasperi, L. Avigliano, Obesity-associated oxidative stress: strategies finalized to improve redox state, Int. J. Mol. Sci. 14 (5) (2013) 10497–10538.
[35] L. Marseglia, S. Manti, G. D'Angelo, A. Nicotera, E. Parisi, G. Di Rosa, E. Gitto, T. Arrigo, Oxidative stress in obesity: a critical component in human diseases, Int. J. Mol. Sci. 16 (1) (2014) 378–400.
[36] N. Houstis, E.D. Rosen, E.S. Lander, Reactive oxygen species have a causal role in multiple forms of insulin resistance, Nature 440 (7086) (2006) 944–948.
[37] A. Aulinas, M.J. Ramirez, M.J. Barahona, E. Valassi, E. Resmini, E. Mato, A. Santos, I. Crespo, O. Bell, J. Surralles, S.M. Webb, Dyslipidemia and chronic inflammation markers are correlated with telomere length shortening in Cushing's syndrome, PLoS One 10 (3) (2015) e0120185.
[38] I. Strazhesko, O. Tkacheva, S. Boytsov, D. Akasheva, E. Dudinskaya, V. Vygodin, D. Skvortsov, P. Nilsson, Association of insulin resistance, arterial stiffness and telomere length in adults free of cardiovascular diseases, PLoS One 10 (8) (2015) e0136676.
[39] A.R. Bonfigli, L. Spazzafumo, F. Prattichizzo, M. Bonafe, E. Mensa, L. Micolucci, A. Giuliani, P. Fabbietti, R. Testa, M. Boemi, F. Lattanzio, F. Olivieri, Leukocyte telomere length and mortality risk in patients with type 2 diabetes, Oncotarget 7 (32) (2016) 50835–50844.
[40] H. Oeseburg, R.A. de Boer, W.H. van Gilst, P. van der Harst, Telomere biology in healthy aging and disease, Pflug. Arch.: Eur. J. Physiol. 459 (2) (2010) 259–268.
[41] P. Scheinberg, J.N. Cooper, E.M. Sloand, C.O. Wu, R.T. Calado, N.S. Young,

Association of telomere length of peripheral blood leukocytes with hematopoietic relapse, malignant transformation, and survival in severe aplastic anemia, JAMA 304 (12) (2010) 1358–1364.
[42] H. Tummala, A. Walne, L. Collopy, S. Cardoso, J. de la Fuente, S. Lawson, J. Powell, N. Cooper, A. Foster, S. Mohammed, V. Plagnol, T. Vulliamy, I. Dokal, Poly(A)-specific ribonuclease deficiency impacts telomere biology and causes dyskeratosis congenita, J. Clin. Investig. 125 (5) (2015) 2151–2160.
[43] M. Armanios, Telomerase mutations and the pulmonary fibrosis-bone marrow failure syndrome complex, New Engl. J. Med. 367 (4) (2012) 384 (author reply 384).
[44] Y. Chen, Y. Wu, X. Huang, P. Qu, G. Li, T. Jin, J. Xing, S. He, Leukocyte telomere length: a novel biomarker to predict the prognosis of glioma patients, J. Cancer Res. Clin. Oncol. 141 (10) (2015) 1739–1747.
[45] L.M. Hurwitz, C.M. Heaphy, C.E. Joshu, W.B. Isaacs, Y. Konishi, A.M. De Marzo, S.D. Isaacs, K.E. Wiley, E.A. Platz, A.K. Meeker, Telomere length as a risk factor for hereditary prostate cancer, Prostate 74 (4) (2014) 359–364.
[46] R. Lakshman, C.E. Elks, K.K. Ong, Childhood obesity, Circulation 126 (14) (2012) 1770–1779.
[47] J.L. Buxton, R.G. Walters, S. Visvikis-Siest, D. Meyre, P. Froguel, A.I. Blakemore, Childhood obesity is associated with shorter leukocyte telomere length, J. Clin. Endocrinol. Metab. 96 (5) (2011) 1500–1505.
[48] O.T. Njajou, R.M. Cawthon, E.H. Blackburn, T.B. Harris, R. Li, J.L. Sanders, A.B. Newman, M. Nalls, S.R. Cummings, W.C. Hsueh, Shorter telomeres are associated with obesity and weight gain in the elderly, Int. J. Obes. 36 (9) (2012) 1176–1179.
[49] K.A. Mather, A.F. Jorm, R.A. Parslow, H. Christensen, Is telomere length a biomarker of aging? A review, J. Gerontol. Ser. A: Biomed. Sci. Med. Sci. 66 (2) (2010) 202–213.
[50] J.L. Sanders, A.B. Newman, Telomere length in epidemiology: a biomarker of aging, age-related disease, both, or neither? Epidemiol. Rev. 35 (1) (2013) 112–131.
[51] Y.-Y. Cheng, T.-W. Kao, Y.-W. Chang, C.-J. Wu, T.-C. Peng, L.-W. Wu, H.-F. Yang, F.-Y. Liaw, W.-L. Chen, Examining the gender difference in the association between metabolic syndrome and the mean leukocyte telomere length, PLoS One 12 (7) (2017) e0180687.
[52] C. Formichi, S. Cantara, C. Ciuoli, O. Neri, F. Chiofalo, F. Selmi, A. Tirone, G. Colasanto, L. Di Cosmo, G. Vuolo, F. Pacini, Weight loss associated with bariatric surgery does not restore short telomere length of severe obese patients after 1 year, Obes. Surg. 24 (12) (2014) 2089–2093.
[53] M. Laimer, A. Melmer, C. Lamina, J. Raschenberger, P. Adamovski, J. Engl, C. Ress, A. Tschoner, C. Gelsinger, L. Mair, S. Kiechl, J. Willeit, P. Willeit, C. Stettler, H. Tilg, F. Kronenberg, C. Ebenbichler, Telomere length increase after weight loss induced by bariatric surgery: results from a 10 year prospective study, Int. J. Obes. 40 (5) (2016) 773–778.
[54] D. Hockemeyer, K. Collins, Control of telomerase action at human telomeres, Nat. Struct. Mol. Biol. 22 (11) (2015) 848–852.
[55] N.P. Weng, Telomere and adaptive immunity, Mech. Ageing Dev. 129 (1–2) (2008) 60–66.
[56] C. Lagathu, L. Yvan-Charvet, J.P. Bastard, M. Maachi, A. Quignard-Boulange, J. Capeau, M. Caron, Long-term treatment with interleukin-1beta induces insulin resistance in murine and human adipocytes, Diabetologia 49 (9) (2006) 2162–2173.
[57] H. Tilg, A.R. Moschen, Adipocytokines: mediators linking adipose tissue, inflammation and immunity, Nat. Rev. Immunol. 6 (10) (2006) 772–783.
[58] M.M. Parisi, L.K. Grun, P. Lavandoski, L.B. Alves, I.J. Bristot, R. Mattiello, C.C. Mottin, F. Klamt, M.H. Jones, A.V. Padoin, F.C.R. Guma, F.M. Barbe-Tuana, Immunosenescence induced by plasma from individuals with obesity caused cell signaling dysfunction and inflammation, Obesity 25 (9) (2017) 1523–1531.
[59] B. van Steensel, T. de Lange, Control of telomere length by the human telomeric protein TRF1, Nature 385 (6618) (1997) 740–743.
[60] J. Shlens, A Tutorial on Principal Component Analysis, 2005, Institute for Nonlinear Science, UCSD, 2010.
[61] W. Chen, Y. Wang, F. Li, W. Lin, Y. Liang, Z. Ma, Expression of telomere repeat binding factor 1 and TRF2 in prostate cancer and correlation with clinical parameters, BioMed. Res. Int. 2017 (2017) 9764752.
[62] B.K. Oh, Y.J. Kim, C. Park, Y.N. Park, Up-regulation of telomere-binding proteins, TRF1, TRF2, and TIN2 is related to telomere shortening during human multistep hepatocarcinogenesis, Am. J. Pathol. 166 (1) (2005) 73–80.
[63] N. Matsutani, H. Yokozaki, E. Tahara, H. Tahara, H. Kuniyasu, K. Haruma, K. Chayama, W. Yasui, E. Tahara, Expression of telomeric repeat binding factor 1 and 2 and TRF1-interacting nuclear protein 2 in human gastric carcinomas, Int. J. Oncol. 19 (3) (2001) 507–512.
[64] A. Sfeir, S.T. Kosiyatrakul, D. Hockemeyer, S.L. MacRae, J. Karlseder, C.L. Schildkraut, T. de Lange, Mammalian telomeres resemble fragile sites and require TRF1 for efficient replication, Cell 138 (1) (2009) 90–103.
[65] P. Martinez, M. Thanasoula, P. Munoz, C. Liao, A. Tejera, C. McNees, J.M. Flores, O. Fernandez-Capetillo, M. Tarsounas, M.A. Blasco, Increased telomere fragility and fusions resulting from TRF1 deficiency lead to degenerative pathologies and increased cancer in mice, Genes Dev. 23 (17) (2009) 2060–2075.
[66] H. Takai, E. Jenkinson, S. Kabir, R. Babul-Hirji, N. Najm-Tehrani, D.A. Chitayat, Y.J. Crow, T. de Lange, A. POT1, Mutation implicates defective telomere end fill-in and telomere truncations in Coats plus, Genes Dev. (2016).
[67] M. Hagiwara, T. Komatsu, S.-s. Sugiura, R. Isoda, H. Tada, N. Tanigawa, Y. Kato, N. Ishida, K. Kobayashi, K. Matsushita, POT1b regulates phagocytosis and NO production by modulating activity of the small GTPase Rab5, Biochem. Biophys. Res. Commun. 439 (3) (2013) 413–417.
[68] E.L. Denchi, T. de Lange, Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1, Nature 448 (7157) (2007) 1068.
[69] Y. Chen, Y. Yang, M. van Overbeek, J.R. Donigian, P. Baciu, T. de Lange, M. Lei, A shared docking motif in TRF1 and TRF2 used for differential recruitment of telomeric proteins, Science 319 (5866) (2008) 1092–1096.
[70] A. Smogorzewska, B. van Steensel, A. Bianchi, S. Oelmann, M.R. Schaefer, G. Schnapp, T. de Lange, Control of human telomere length by TRF1 and TRF2, Mol. Cell. Biol. 20 (5) (2000) 1659–1668.
[71] B. van Steensel, A. Smogorzewska, T. de Lange, TRF2 protects human telomeres from end-to-end fusions, Cell 92 (3) (1998) 401–413.
[72] R. Rai, Y. Chen, M. Lei, S. Chang, TRF2-RAP1 is required to protect telomeres from engaging in homologous recombination-mediated deletions and fusions, Nat. Commun. 7 (2016) 10881.
[73] P. Munoz, R. Blanco, G. de Carcer, S. Schoeftner, R. Benetti, J.M. Flores, M. Malumbres, M.A. Blasco, TRF1 controls telomere length and mitotic fidelity in epithelial homeostasis, Mol. Cell. Biol. 29 (6) (2009) 1608–1625.
[74] P. Martinez, G. Gomez-Lopez, F. Garcia, E. Mercken, S. Mitchell, J.M. Flores, R. de Cabo, M.A. Blasco, RAP1 protects from obesity through its extratelomeric role regulating gene expression, Cell Rep. 3 (6) (2013) 2059–2074.
[75] M.T. Duong, E. Sahin, RAP1: protector of telomeres, defender against obesity, Cell Rep. 3 (6) (2013) 1757–1758.
[76] P. Martinez, G. Gomez-Lopez, D.G. Pisano, J.M. Flores, M.A. Blasco, A genetic interaction between RAP1 and telomerase reveals an unanticipated role for RAP1 in telomere maintenance, Aging Cell (2016).
[77] S. Kabir, D. Hockemeyer, T. de Lange, TALEN gene knockouts reveal no requirement for the conserved human shelterin protein Rap1 in telomere protection and length regulation, Cell Rep. 9 (4) (2014) 1273–1280.
[78] H. Teo, S. Ghosh, H. Luesch, A. Ghosh, E.T. Wong, N. Malik, A. Orth, P. de Jesus, A.S. Perry, J.D. Oliver, N.L. Tran, L.J. Speiser, M. Wong, E. Saez, P. Schultz, S.K. Chanda, I.M. Verma, V. Tergaonkar, Telomere-independent Rap1 is an IKK adaptor and regulates NF-kappaB-dependent gene expression, Nat. Cell Biol. 12 (8) (2010) 758–767.
[79] Y. Kidani, S.J. Bensinger, Liver X receptor and peroxisome proliferator-activated receptor as integrators of lipid homeostasis and immunity, Immunol. Rev. 249 (1) (2012) 72–83.
[80] G. Derosa, A. Sahebkar, P. Maffioli, The role of various peroxisome proliferator-activated receptors and their ligands in clinical practice, J. Cell. Physiol. 233 (1) (2018) 153–161.
[81] P. Martinez, M. Thanasoula, A.R. Carlos, G. Gomez-Lopez, A.M. Tejera, S. Schoeftner, O. Dominguez, D.G. Pisano, M. Tarsounas, M.A. Blasco, Mammalian Rap1 controls telomere function and gene expression through binding to telomeric and extratelomeric sites, Nat. Cell Biol. 12 (8) (2010) 768–780.
[82] G.C. Rowe, S. Raghuram, C. Jang, J.A. Nagy, I.S. Patten, A. Goyal, M.C. Chan, L.X. Liu, A. Jiang, K.C. Spokes, D. Beeler, H. Dvorak, W.C. Aird, Z. Arany, PGC-1alpha induces SPP1 to activate macrophages and orchestrate functional angiogenesis in skeletal muscle, Circ. Res. 115 (5) (2014) 504–517.
[83] E. Sahin, S. Colla, M. Liesa, J. Moslehi, F.L. Muller, M. Guo, M. Cooper, D. Kotton, A.J. Fabian, C. Walkey, R.S. Maser, G. Tonon, F. Foerster, R. Xiong, Y.A. Wang, S.A. Shukla, M. Jaskelioff, E.S. Martin, T.P. Heffernan, A. Protopopov, E. Ivanova, J.E. Mahoney, M. Kost-Alimova, S.R. Perry, R. Bronson, R. Liao, R. Mulligan, O.S. Shirihai, L. Chin, R.A. DePinho, Telomere dysfunction induces metabolic and mitochondrial compromise, Nature 470 (7334) (2011) 359–365.
[84] V.M. Mikhel'son, I.A. Gamalei, Telomere shortening is the main mechanism of natural and radiation aging, Radiatsionnaia Biol. Radioecol. 50 (3) (2010) 269–275.
[85] D. Revesz, Y. Milaneschi, J.E. Verhoeven, B.W. Penninx, Telomere length as a marker of cellular aging is associated with prevalence and progression of metabolic syndrome, J. Clin. Endocrinol. Metab. 99 (12) (2014) 4607–4615.
[86] J.M. Starr, P.G. Shiels, S.E. Harris, A. Pattie, M.S. Pearce, C.L. Relton, I.J. Deary, Oxidative stress, telomere length and biomarkers of physical aging in a cohort aged 79 years from the 1932 Scottish Mental Survey, Mech. Ageing Dev. 129 (12) (2008) 745–751.
[87] V. Cattan, N. Mercier, J.P. Gardner, V. Regnault, C. Labat, J. Maki-Jouppila, R. Nzietchueng, A. Benetos, M. Kimura, A. Aviv, P. Lacolley, Chronic oxidative stress induces a tissue-specific reduction in telomere length in cast/Ei mice, Free Radic. Biol. Med. 44 (8) (2008) 1592–1598.
[88] P. Newsholme, V.F. Cruzat, K.N. Keane, R. Carlessi, P.I. de Bittencourt Jr., Molecular mechanisms of ROS production and oxidative stress in diabetes, Biochem. J. 473 (24) (2016) 4527–4550.
[89] D. Trachootham, W. Lu, M.A. Ogasawara, R.D. Nilsa, P. Huang, Redox regulation of cell survival, Antioxid. Redox Signal. 10 (8) (2008) 1343–1374.
[90] B. Halliwell, Free radicals and antioxidants - quo vadis? Trends Pharmacol. Sci. 32 (3) (2011) 125–130.
[91] B.I. Frohnert, A.R. Sinaiko, F.J. Serrot, R.E. Foncea, A. Moran, S. Ikramuddin, U. Choudry, D.A. Bernlohr, Increased adipose protein carbonylation in human obesity, Obesity 19 (9) (2011) 1735–1741.
[92] H. Kocak, Y. Oner-Iyidogan, F. Gurdol, P. Oner, R. Suzme, D. Esin, H. Issever, Advanced oxidation protein products in obese women: its relation to insulin resistance and resistin, Clin. Exp. Med. 7 (4) (2007) 173–178.
[93] S.O. Olusi, Obesity is an independent risk factor for plasma lipid peroxidation and depletion of erythrocyte cytoprotectic enzymes in humans, Int. J. Obes. Relat. Metab. Disord.: J. Int. Assoc. Study Obes. 26 (9) (2002) 1159–1164.
[94] A.T. Kharroubi, H.M. Darwish, M.A. Akkawi, A.A. Ashareef, Z.A. Almasri, K.A. Bader, U.M. Khammash, Total antioxidant status in type 2 diabetic patients in palestine, J. Diabetes Res. 2015 (2015) 461271.
[95] B. Poljsak, D. Suput, I. Milisav, Achieving the balance between ROS and antioxidants: when to use the synthetic antioxidants, Oxid. Med. Cell. Longev. 2013 (2013) 956792.