Article

J. Biol. Regul. Homeost. Agents. 2022; 36(5): 1311–1319
https://doi.org/10.23812/j.biol.regul.homeost.agents.20223605.141

Effectiveness of a Fermented Functional Food on Telomere Dynamics and miRNAs in Middle-Aged/Elderly Healthy Individuals: A 2-Year Randomized, Double-Blind, Controlled Clinical Trial

Marotta Francesco[1,*], Zerbinati Nicola[2], Lorenzetti Aldo[1], Ayala Antonio[3], Barbagallo Mario[4], Munoz Mario[3], Rasulova Saida[1], Osato Maki[5]

[1]ReGenera R&D International for Aging Intervention, 20154 Milano, Italy
[2]Department of Medicine and Surgery, University of Insubria, 21100 Varese, Italy
[3]Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of Seville, 41004 Seville, Spain
[4]Geriatric Unit, Department of Internal Medicine and Geriatrics, University of Palermo, 90128 Palermo, Italy
[5]Redox Molecular Biology Laboratory, ORI Research Institute, 800-8005 Gifu, Japan
*Correspondence: fmarchimede@libero.it (Marotta Francesco)
Published: 20 October 2022

Background: A specific fermented papaya preparation (ORI-FPP®), has been well established with non clinical and clinical validation in modulating redox and immune systems as well as mitochondrial efficiency. In addition, recent data from the Italian NIH (National Institute of Health) have shown that this compound was able to increase telomeres length and telomerase activity in an aged mouse model.
Objective: The purpose of the study was to test the clinical effect of ORI-FPP® in healthy middle-aged/elderly subjects in terms of telomere dynamics and aging-related miRNAs (micro ribonucleic acids), as compared to an antioxidant formulation.
Materials: The study population comprised 107 healthy non smoker middle-aged/elderly subjects (58 women and 49 men, mean age 56.6 ± 13.8 years, age range 44–74 years) free from medications and with a mean body mass index (BMI) of 24.7 ± 2.6. Subjects were double-blinded and randomly allocated to 2 groups: (A) ORI-FPP® 4.5 g plus 1 cp of cellulose and (B) flavoured sugar 4.5 g plus 1 cp containing a mix of antioxidants. Leucocyte telomere length (TL), gene expression related to telomere maintenance genes (*TERT* (telomerase reverse transcriptase) and *Wrap53*) and telomerase activity (TRAP) were assessed by quantitative reverse transcriptase-polymerase chain reaction after both nutraceutical interventions. This was also associated with gene expression evaluation of: SOD (superoxide dismutase), CAT (catalase), *GPx1* (glutathione peroxidase-1) and *hOGG1* (human 8-oxoguanine DNA glycosylase 1) as well as *miRNA-146a* and *miRNA-181a*. Dietary and psychological (10-item Cohen Perceived Stress Scale) questionnaires were administered at the start and throughout the two-year study.
Results: TL was unaffected by treatments but a significant TL increase was observed at the end of the study in the 60–74 years old group treated with FPP®. Gene expression of *TERT* and *WRAP53* showed a significant upregulation (168% and 57%, respectively) in group A as compared with group B starting from the 6th month observation ($p < 0.01$ vs group B), together with TRAP ($p < 0.05$ vs group B). CAT, SOD1 and *GPx1* were comparably upregulated by both treatments. However, only FPP® upregulated *hOGG1*, *miRNA-146a* and *miRNA-181a*. *TERT* and *WRAP53* gene expressions were significantly correlated to *GPx*, *hOGG1*, *miRNA-146a* and *miRNA-181a* gene expression.
Conclusions: These data suggest that ORI-FPP®, unlike the antioxidant control formulation, could significantly benefit TL dynamics. This phenomenon seemed correlated to a concomitant antioxidant and, namely, DNA (deoxyribonucleic acid)-protecting genes upregulation and aging-related miRNAs.

Keywords: telomere length; telomerase; fermented papaya; FPP®; telomerase genes; miRNAs

Copyright: © 2022 The Author(s). Published by Biolife Sas. This is an open access article under the CC BY 4.0 license.
Note: *J. Biol. Regul. Homeost. Agents.* stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Journal of BIOLOGICAL REGULATORS and Homeostatic Agents

Introduction

Several pieces of evidence support that chronic systemic inflammation aggravates reactive oxygen species (ROS)—mediated telomere dysfunction, decreasing regenerative potential in multiple tissues and accelerating cellular aging in the absence of any other genetic or environmental factors [1–3]. The association of these detrimental factors has been dramatically come to the spotlights during COVID-19 pandemic [4,5]. The length of telomeres, i.e., the tandem-repeated hexamers at the terminal of mammalian chromosomes, can be regarded as a biomarker of aging and age-related morbidity and mortality, the telomeric loss being a sort of cellular aging clock [6,7]. The 4 to 15 kilobases-long telomere tends to gradually shorten by 20–40 bases per year. Several recent studies suggest the crucial role for the telomere biology system in the maintenance of DNA (deoxyribonucleic acid) and cellular integrity, with important implications for health and disease risk across a wide range of age-related disorders and in some cancer survival [8–11]. Indeed, to prevent premature telomere decay there is a dynamic physiological process involving also partial counteractions from telomerase, a nuclear regulator of telomere elongation and also mitochondrial reactive oxygen species [12,13]. Moreover, telomere length and telomerase activity are intimately linked with immune function due to affecting lymphocyte development, differentiation and replicative ability [14]. Aging is characterized by a progressive shortening and morpho-functional derangement of telomeres, their richness in guanine content being highly exposed to endogenous and exogenous oxi-inflammatory damage [15]. Given the relevant implications of the telomere dysfunction in the development and progression of many age-related diseases [16], a growing interest is being devoted to the quest of slowing down the rate of physiological aging by several natural moieties, mostly belonging to flavonoids, terpenoids, saponins, and polysaccharides [17,18]. For instance, polysaccharides have been shown to enhance telomerase activity while reducing p16 protein expression [19]. As a matter of fact the main components of astragalus membranaceus, which is endowed by the capacity to increase telomerase activity, are polysaccharides, flavonoids, and saponins [20]. However, preliminary positive clinical data with a proprietary astragalus formulation in a clinical setting were obtained from a study lacking an antioxidant positive control and with limited nutrigenomic studies [21]. However, the same compound showed also a paradoxical telomere loss effect in an animal setting [22]. Other natural compounds have been screened and compared on telomere dynamics but this has taken place either on human peripheral blood mononuclear cells [23] or is still limited to mouse models [24]. Recent data from an Italian NIH (National Institute of Health) research group has shown that a specific fermented papaya preparation (ORI-FPP®), endowed with basic science and clinical validation as for its effectiveness on redox and immune system modulatory as well as on mitochondrial efficiency [25–28], was able to beneficially affect telomeres length and telomerase activity in an aged mouse model [29]. Further elements to consider in this context is the finding that the knockdown of *WRAP53β* determines a poor DNA (deoxyribonucleic acid) double-strand break repair and the consequent build-up of genetic debris impairs the recovery mechanisms after DNA insults such as shown by Henriksson *et al.* [30] in experimentally-induced cell cycle arrest. Indeed, the WD40 domain-containing protein WD40 encoding RNA (ribonucleic acids) antisense to p53, *WRAP53* represents a scaffold protein involved in telomere elongation and DNA repair. As a matter of fact, the functional loss of *WRAP53* has been implicated both in carcinogenesis process and in premature aging-associated diseases [31].

The aim of the present study was to test a patented functional food, i.e., Fermented Papaya Preparation (ORI-FPP®) (patent number: 6401792; Osato Research Institute, Gifu, Japan) in telomere dynamics and miRNA (micro ribonucleic acids) in healthy middle-aged/elderly subjects as compared to an antioxidant formulation. The ORI-FPP® is obtained from Carica papaya L, grown in selected non-GMO (genetically modified organism) and organically-treated Hawaiian crops. The properly cleaned fruit undergoes a patented 10 months yeast fermentation process with pharmaceutical-grade batch-to-batch control by electron spin resonance (ESR). We preferred to rely on this compound, having several other fermented papaya extracts failed to exert significant antioxidant effect in biological systems as checked by ESR (Mantello P., ORI Oxidative Stress Research Lab, Gifu, Japan. In-house data).

Materials and Methods

Study Population and Methods

The selected population comprised 107 healthy non smoker middle-age/elderly subjects (58 women and 49 men, mean age 56.6 $\pm$ 13.8 years, age range 44–74 years and free from medications. The mean body mass index (BMI) was 24.7 $\pm$ 2.6.

The exclusion criteria for the study were: Significant present or past mood disorders; Vigorous physical training, relevant drinking ($>$7 alcoholic drinks/week), history of cancer; Cardiovascular disease and people reporting sleep-apnea or consistent sleep disorders. Dietary and psychological (10-item Cohen Perceived Stress Scale) questionnaire were administered at the entrance and once bi-monthly. A detailed dietary quali-quantitative questionnaire with peculiar attention to antioxidant food content was followed and participants were asked to fill out a daily food consumption report on a digital support. Such food frequency questionnaire had been internally validated against a 7 days diet record in a pilot study. The use of food supplements or strenuous physical activity discouraged. Subjects were

asked to report immediately any medication intake for concurrent disease/discomfort which was considered when deciding if participation in the trial should continue.

The study was conducted under double-blind fashion and codes were cracked at the end with group A and B designed on the two different treatments which were as follows: Group A was given FPP® 4.5 g, 1 sachet two times a day and the one in the morning having a placebo capsule added (vegan cellulose); Group B was given a fruit-flavoured sugar 4.5 g, 1 sachet 2 times a day the one in the morning including a capsule containing an antioxidant mixture (50 mg EGCG (epigallo cathechin gallat), 20 mg anthocyanidins, 200 mg trans-resveratrol, 80 mg ubiquinol, 5 mg zinc, 100 mg Centella asiatica extract and 200 IU (international units) vitamin E).

Each subject recruited for the study was fully informed in compliance with the guidelines of the Declaration of Helsinki. Participants were recorded by a code number to protect their privacy. To avoid circadian biological effects, all samples were withdrawn between 8:00 AM and 9:00 AM using EDTA (etheylenediaminetetraacetic acid)-added tubes following a regular overnight fast. The participants were required to avoid any late hours work or social life activity and no caffeine or alcohol was allowed for 2 days preceeding the blood test. A 40 mL sample of venous blood was retrieved from each participant. Peripheral blood mononuclear cell (PBMC) were isolated in Vacutainer Cell Preparation Tubes (Vacutainer® CPT; Bekton Dickinson, Franklin Lakes, NJ, USA), treated with the anticoagulant K3-ethylenediaminetetraacetic acid by gradient centrifugation (2000× rpm/20 minutes) using Lymphoprep (ELITech-Group, Benelux, Luxenbourg). Biochemical and gene expression tests were carried out at the entry and at 1, 3, 6, 12 and 24 months afterward.

Telomere Length Measurement

PBMCs were isolated by entity gradient centrifugation of venous peripheral blood from patients and cryopreserved. Later on, DNA was purified from total nucleic acid using Ambion RNase I kit (Thermo Fisher Scientific, Milano, Italy). Relative mean DNA telomere length was assayed by qPCR (quantitative polymerase chain reaction) test and confirmed by Southern blot TRF (telomere restriction fragment) comparative analysis. Relative TL was calculated by dividing the normalized telomere sequence (T) by normalized single copy gene sequence (S). The T/S ratio was then calculated for each sample by applying the formula (T/S ratio 1 = 538 bp), to measure telomere length in kb for all samples and related to human genomic DNA reference sample. By doing so, an ICC (intra class correlation) >0.97 intra- and inter-plate repeatability could be confirmed each time. This parameter was tested at entry and at 3, 6 and 24 months.

Telomerase Activity Assay

Out of 1 mL of cryopreserved sample, a total of 1×10^6 viable PBMCs were analysed. Telomerase activity was assayed by real-time telomeric repeat amplification protocol (TRAP). All patients' samples were run on the same plate in triplicate together with controls and TRAP buffer dilutions mixed to a 50 μL of reaction mixture. This yielded a correlation coefficients versus the standard curves above 0.98 and overall efficiency of 96–100%. A Bio-Rad CFX96/C1000 qPCR was employed with the following steps: An initial denaturation at 95 °C/2 minutes followed by 37 cycles of 95 °C/35 seconds, 50 °C/35 seconds and 72 °C/90 seconds. The coefficients of variation (CV) of intraassay Ct values for the standard dilutions was 1.3% and inter-assay was 0.43%. The PCR (polymerase chain reaction) products were used for the ELISA (enzyme-linked immunosorbent assay) assay using a multiplate reader set at 450 nm absorbance reference wavelength of 690 nm.

Telomerase and Anti-Oxidant Gene Expression Measurement

Reverse transcription was carried out with random hexamers using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. Human *hTERT* (human telomerase reverse transcriptase) TaqMan Assay (Applied Biosystem) was employed to assess telomerase gene expression.

Relative gene expression was also measured as follows. Total qPCR reaction of 20 μL, contained 5 μL cDNA, 10 μL TaqMan Multiplex Master Mix, 1 μL *TERT* (telomerase reverse transcriptase) TaqMan Assay and 1 μL GAPDH (glyceraldehyde-3-phosphate dehydrogenase) TaqMan Assay. Each 20 μL qPCR reaction contained, 5 μL cDNA, 10 μL TaqMan Multiplex Master Mix (Applied Biosystem) and 1 μL GAPDH Assay at 95 °C for 20 seconds, followed by 45 thermal cycles (95 °C for 1 second and 60 °C for 20 seconds). The threshold cycle values were extrapolated and retrieved via the Expression Suite for QuantStudio Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, CA, USA). The software calculates the relative individual threshold cycle so to calculate the corrected efficiency of each reaction. The comparative threshold (CT) cycle was normalized according to the formula ΔCT = CT (tested gene) – CT (housekeeping gene) (Table 1). Then, the relative gene expression value was extrapolated by the formula: $\Delta\Delta$CT = ΔCT sample – ΔCT calibrator and the obtained value was expressed as fold change (FC) = $2^{-\Delta\Delta Ct}$. The quality of RNA was confirmed by an Automated Electrophoresis Station (Bio Rad, Hercules, CA, USA).

miRNA Analysis

For this purpose, 200 μL of plasma was collected in aliquots into RNase (recombinant ribonuclease)/DNase

Journal of BIOLOGICAL REGULATORS and Homeostatic Agents

Table 1. Primer sequences used for analyzing.

Gene	Forwards primer	Reverse primer	Rs
SOD1	AGCGAGTTATGGCGACGAAG	CAGCCTGCTGTATTATCTCCA	1800668
CAT	CAGCCTGCTGTATTATCTCCA	CCTCACAGATTTGCCTTCTCC	7943316
GPx-1	CCCGTGCAACCAGTTTGG	TGAGGGAATTCAGAATCTCTTCGT	1800668
SIRT-1	TGTGGTAGAGCTTGCATTGATCTT	GGCCTGTTGCTCTCCTCAT	7895833
FOXO3	CTCCCGTCAGCCAGTCTAT	AGTCACTGGGGAACTTGTCG	2802292
h-hOGG1	ACTGTCACTAGTCTCACCAG	AGGGGAAGGTGCTTGGGGGAA	1052133

SOD1, superoxide dismutase; CAT, catalase; *GPx-1*, glutathione peroxidase-1; *Sirt-1*, sirtuin 1; *FOXO3*, forkhead box O3; *hOGG1*, human 8-oxoguanine DNA glycosylase 1.

(deoxyribonuclease)-free tubes and stored at –80 °C for further analysis. All samples were extracted and tested in a single batch in order to minimize unnecessary repeated freeze and thaw handling. Total RNA was purified using the mirVANA Isolation kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. In each case, a spectrophotometer-based analysis (NanoDrop Lite; Thermo Fisher Scientific, Milano, Italy) confirmed the, quali-quantitative sample characteristics. Then, samples were processed for hybridization by Agilent Human miRNA array yielding a further robust quality control before proceeding to analysis. Sample-to-sample variation in miRNA quantification was normalised by adding a mixture of 50 pmol/L of 5 fmol/μL Caenorhabditis elegans miRNA-39 and 0.7 μL of RNA, MS2 (Roche, Indianapolis, IN, USA) to each plasma sample during extraction. All the samples were eluted with 100 μL of RNase-free water. The amplification was followed by using Real-Time PCR System, version 4.0 (Illumina, San Diego, CA, USA), according to the manufacturer's instructions, with denaturation at 50 °C/2 minutes and at 95 °C/10 minutes followed by cycling at 95 °C/15 seconds and at 60 °C/1 minute for 50 cycles. From our prior in-house miRNA analysis in an aged population, two miRNAs were analysed in duplicate (*miR181a* and *miR146a*) using the TaqMan microRNA reverse transcription (RT). For each assay, a high-density scan (600 fields of view) was performed. *miR-16* was selected as an internal control and the expression level of the selected miRNAs was calculated using comparative cycle threshold CT method. This was carried out as described above with fold miRNA expression change measurement as the $\Delta\Delta$Ct method. Experiments were performed in duplicate. PCR reactions were as follows: Denaturation at 95 °C for 15 minutes, followed by 50 amplification cycles of 94 °C for 15 seconds, 55 °C for 30 seconds, and 70 °C for 30 seconds. Tests were done at entry, 3, 6, 12 and 24 months afterwards.

Statistical Analysis

All tests were performed by SPSS version 22 (IBM Inc., Armonk, NY, USA). Results are presented with means $\pm$ standard deviations (SD) for continuous variables. Whenever the "antioxidant"-control group didn't show any significant difference per sè during the study, we used also baseline value of FPP® to analyze its time-course changes of significance. No separation into groups based on physical activity was performed, given the overall lack of any significant workload among participants. Differences between study participants were assessed by *t*-tests following Gaussian distribution, and Chi2-tests and a critical alpha level of 0.05 was used for all analyses. When analysing the 60–74 years old subgroups, because of the limited sample size, Mann-Whitney U tests and chi-square tests were used to study intergroup differences.

Results

All subjects completed the study and no drop out or substantial protocol violation occurred. No adverse effect connected to any of the treatments employed were reported by participants and no significant change occurred in the routine blood chemistry or stress score which remained within baseline normal limits (data not shown).

Telomere Length Measurement

Telomere length values recorded before and along the treatments showed a picture of scattered values distribution with a non-significant trend increase in the group treated with FPP® (Fig. 1). However, this trend reached a statistically significant value from the 6th month onwards in the 60–74 years age group supplemented with FPP® when analysed as age-adjusted differences TL z score ($p < 0.05$ vs group B and vs baseline, Fig. 2.) The telomere length either when considered as a whole and when listed within each specific age-cluster group, showed no significant correlation with BMI, gender or stress score.

Telomerase Activity

When testing the telomerase activity at the end of the study, as compared to pre-supplementation values, it appeared that FPP but not antioxidant mixture yielded a robust enzymatic enhancement (over 50%, $p < 0.01$, Fig. 3). Such an effect significantly occurred from the sixth month observation (data not shown).

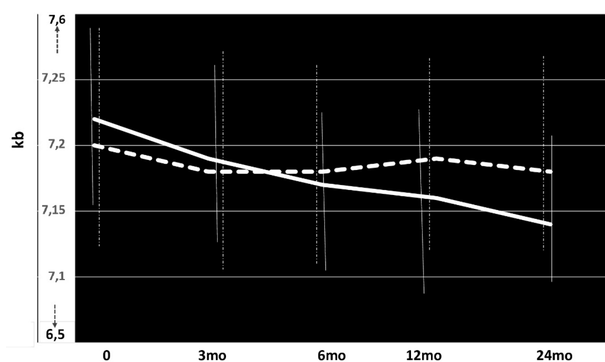

Fig. 1. Time course Telomerase length profile during 24 months treatments in all patients, irrespective of the age. No significant difference appeared as a whole between the two different nutraceutical interventions when applied to the whole group. Straight lines: Antioxidant cocktail; Dotted lines: FPP®.

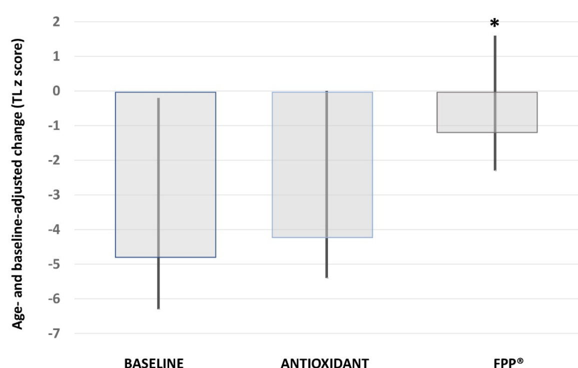

Fig. 2. Age- and baseline-adjusted differences changes in TL z score at the end of antioxidant cocktail and FPP® intervention period. $^{*}p < 0.05$ vs antioxidant-supplemented group.

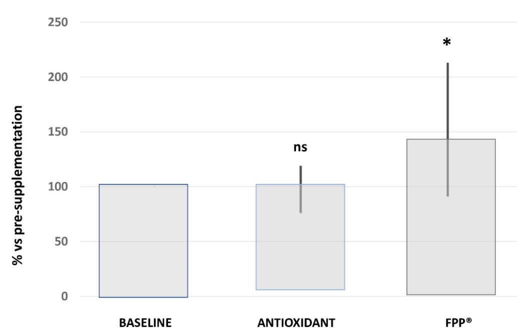

Fig. 3. Telomerase activity: Effect of nutraceutical supplementations at 24 months. NS, Not statistically significant. $^{*}p < 0.01$ vs antioxidant-supplemented group (over 50% increase).

Telomerase Maintenance Genes

Human telomerase reverse transcriptase (*hTERT*) was unaffected by the antioxidant cocktail administration (data not shown) whereas FPP® consumption determined a statistically significant increase starting already at the third month observation and the same occurred with *WRAP53* ($p < 0.05$, Fig. 4). No correlation with either BMI, age and stress score was noted.

Anti-Oxidant, FOXO3 and Sirtuins Gene Expression Measurement

Both treatment groups brought about a comparable statistically significant upregulation of SOD1 (superoxide dismutase) and CAT (catalase) gene expression ($p < 0.05$ vs baseline, Fig. 5). Unlike the group supplemented with an antioxidant cocktail, showing an upregulation of *GPx-1* (glutathione peroxidase-1), the FPP®-administered group showed a significantly stronger upregulation of *GPx-1* and also of *hOGG1* (human 8-oxoguanine DNA glycosylase 1) ($p < 0.01$ vs group B and 0.01 vs baseline). This effect appeared at the third month observation and kept a steady state throughout the whole observation period. On the other hand, the other gene expressions tested (*Sirt1* (sirtuin 1), *FOXO3* (forkhead box O3), the latter showing a not significant trend upregulation) did not significantly vary following any of the two investigational treatments (data not shown). *hTERT* and *WRAP53* gene expressions were significantly and directly correlated to *GPx1* and *hOGG1* gene expression in the FPP®-treated group (r: 0.76, $p < 0.05$, Fig. 4) but not to the other gene expressions tested.

miRNA Analysis

Irrespective of the age group and unrelated to BMI and gender, FPP® supplementation yielded a statistically significant upregulation of *miR181a* and *miR146a* (fold increase: +1.7 and +1.8, respectively, $p < 0.01$ vs baseline, Fig. 6) starting from the third month's observation.

When plotting these two miRNAs against *hTERT* at the end of the study period in the FPP®-supplemented group, a robust positive correlation appeared (r: 0.86, $p < 0.01$, Fig. 7).

Conclusions

Telomere shortening or attrition can follow several genetic and environmental modifiers (nutritional abnormalities, redox system imbalance etc) [32–35]. These detrimental telomere events have been advocated for as substantial factors leading to a damage of DNA checkpoint response with irreversible growth arrest as the senescence endpoint of p53-growth inhibitory mechanisms activation [36,37]. Given the overall good physical with lack of stress-related health conditions and of our study population, it was not surprising to note that the average telomere length was grossly comparable to a younger healthy control (data in house). However, by retrospectively applying the age-related analysis, a significant shortening was indeed observed. Although we haven't measured inflammatory markers, one cannot rule out that such older subjects

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

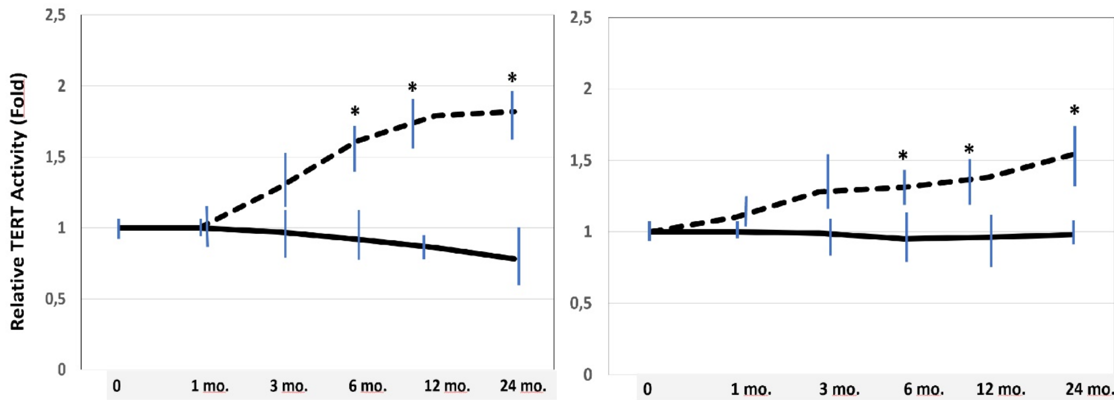

Fig. 4. Time-course of relative activity of telomerase maintenance genes *TERT* and *WRAP53* expressed as fold variation. $^{*}p <$ 0.05 vs baseline.

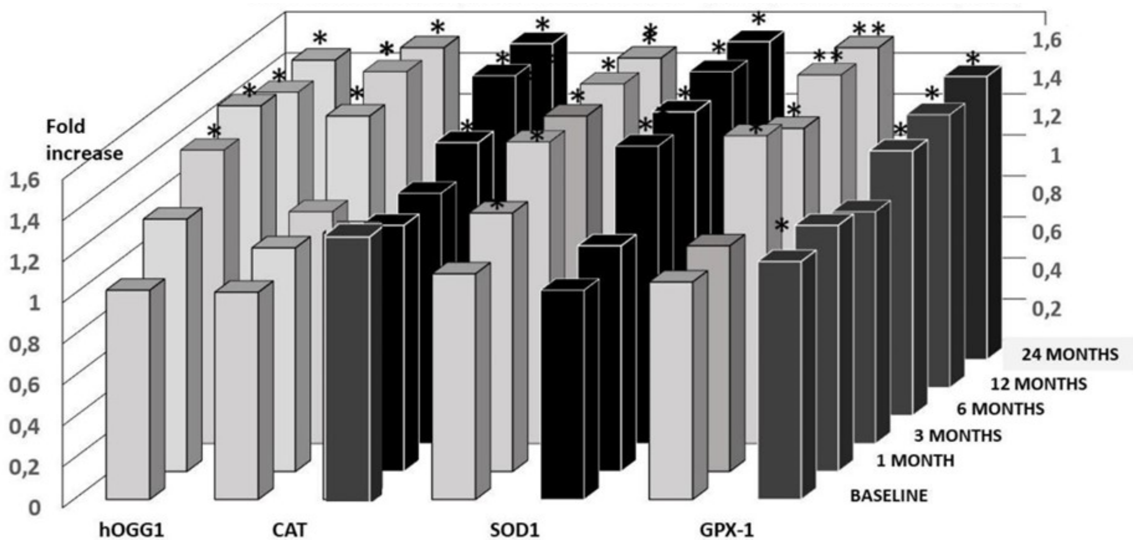

Fig. 5. Time-course gene expression: Effect of FPP® (white) and antioxidant (black). $^{*}p < 0.05$ vs baseline; $^{**}p < 0.05$ vs antioxidant cocktail-supplemented group. All other antioxidant-supplemented related values were not statistically different from baseline and are not shown.

had a co-existent low grade inflammation. The relevance of these two factors, i.e., aging and inflammation, conjuring up in affecting telomere health has been shown in recent years [38]. On the other hand, data dating back over ten years has shown that this fermented supplement yielded a significant curbing action on pro-inflammatory gene expression, as we were the first worldwide to report it [39,40]. Hence, it was of interest to note that, although both tested supplements upregulated the SOD1 and CAT redox balance genes, only the functional food supplement FPP® enabled also a telomere elongation counterpart in the older subjects. Concomitantly, FPP® but not the antioxidant cocktail positively affected telomerase activity, i.e., the enzyme responsible for the telomere maintenance, in all subjects, irrespective of age. Similar effects have been reported by an astragalus-based food supplement in one independent study but addressing patients with metabolic syndrome and not generally healthy individuals, besides the study had a far limited follow up [41,42]. Similarly, the "walnut study" [32] addressed a large population of elderly with a variety of concurrent chronic diseases/bad lifestyle habits (hypertension, diabetes, dyslipidemia, smoking etc). Thus, the present study is the first independent study in healthy, non-obese, middle-aged/elderly subjects in whom a nutraceutical intervention proved to have beneficial effect on telomere dynamics. The peculiarity of action of FPP®, as compared to a conventional antioxidant cocktail but also to prior natural telomerase activators, also emerged in its upregulation of *Wrap53* which is a natural p53 antisense transcript controlling p53 induction upon DNA damage. This data was in-

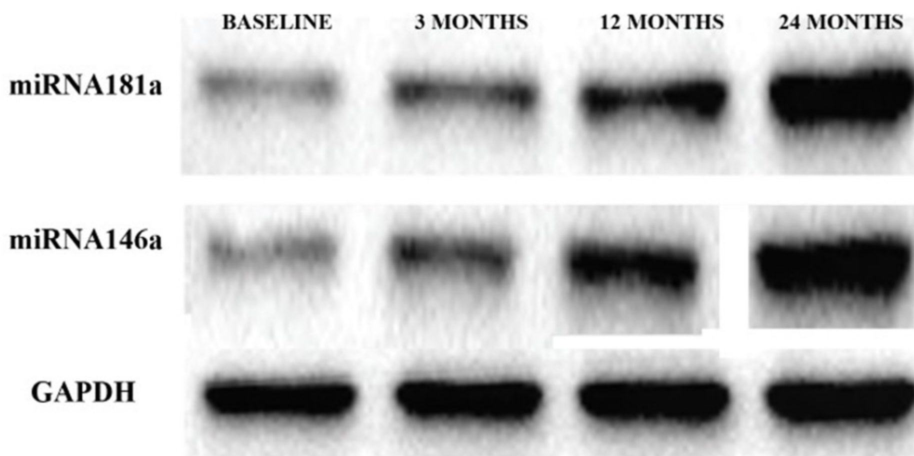

Fig. 6. Time-course effect of FPP® supplementation on miRNA146a and miRNA181a gene expression. The control antioxidant formulation did not significantly affect the above miRNAs either when analyzing the whole group or selecting the 60–74 years group (data not shown). The original picture was elaborated but not over-run/modified in order to yield background cleansing with some sharpness limitations.

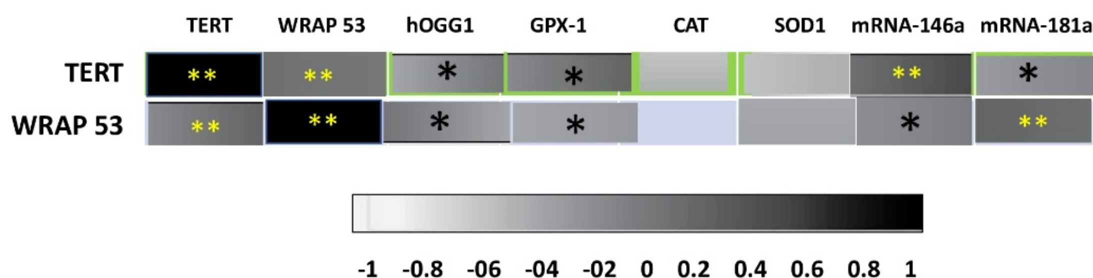

Fig. 7. Pearson correlation between *TERT* and *WRAP53* genes expression with DNA repair gene, redox genes and selected miRNAs genes expression. $^{*}p < 0.05$; $^{**}p < 0.01$.

deed in accordance with the upregulation of a typical DNA repair gene, i.e., *hOGG1* [43] as observed in the FPP®-treated group. Along these functional genomic unfolding efforts, we also investigated into some miRNAs since these non-coding molecules play a crucial gene expression regulatory role by binding to specific target messenger RNAs to halt its translation or fostering its degradation [44]. We focused our attention to two specific miRNAs, *miRNA-181a* and *miRNA-146a*, which have been reported to be associated to aging process per sè [45–48]. These miRNAs were indeed highly correlated to telomere dynamics, *GPx-1* and *hOGG1* gene expression. One limitation of our study was the unfeasibility to compare these data with other miRNAs trials. This is because, like in our case, most works in this area have availed themselves of relative expression method which generate arbitrary units which are not amenable to robustly discriminate between physiological and pathological aging.

However, these preliminary data suggest that FPP®, while confirming its oxidative stress curbing nutrigenomic effect, could also significantly increase telomerase activity and telomerase maintenance genes unlike the antioxidant control formula. This phenomenon seemed to be correlated to a concomitant antioxidant- and DNA-protecting genes upregulation. This study cannot certainly claim to unveil the profound aging mechanisms machinery with its specific alterations in genome architecture and interaction between telomeres and particular loci. However, these results may shed light on the rationale for a foreseeable larger healthspan strategy to pursue effective functional nutraceuticals interventions.

Further studies gathered from far larger samples of healthy subjects and with standardized methods for miRNA detection, will allow the identification of the age-tailored reference range of normality in the quest to face the evil trio of oxidative damage, telomere shortening associated with replicative cell senescence and oncogenesis [38].

Author Contributions

MF and ZN—designed the research study; LA and MF—performed the research; BM, AA and MM—provided help and advice on the ELISA experiments; RS and OM—analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The study design and regulation approved by a joint ethical committee of ReGenera Research Association on Sept. 15, 2019.

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Wang L, Lu Z, Zhao J, Schank M, Cao D, Dang X, *et al*. Selective oxidative stress induces dual damage to telomeres and mitochondria in human T cells. Aging Cell. 2021; 20: e13513.

[2] Azam S, Haque ME, Balakrishnan R, Kim IS, Choi DK. The Ageing Brain: Molecular and Cellular Basis of Neurodegeneration. Frontiers in Cell and Developmental Biology. 2021; 13: 683459.

[3] Saretzki G, Wan T. Telomerase in Brain: The New Kid on the Block and Its Role in Neurodegenerative Diseases. Biomedicines. 2021; 9: 490.

[4] Wissler Gerdes EO, Vanichkachorn G, Verdoorn BP, Hanson GJ, Joshi AY, Murad MH, *et al*. Role of senescence in the chronic health consequences of COVID-19. Translational Research: The Journal of Laboratory and Clinical Medicine. 2022; 241: 96–108.

[5] Mongelli A, Barbi V, Gottardi Zamperla M, Atlante S, Forleo L, Nesta M, *et al*. Evidence for Biological Age Acceleration and Telomere Shortening in COVID-19 Survivors. International Journal of Molecular Sciences. 2021; 22: 6151.

[6] Güneşliol BE, Karaca E, Ağagündüz D, Acar ZA. Association of physical activity and nutrition with telomere length, a marker of cellular aging: a comprehensive review. Critical Reviews in Food Science and Nutrition. 2021; 23: 1–19.

[7] Razgonova MP, Zakharenko AM, Golokhvast KS, Thanasoula M, Sarandi E, Nikolouzakis K, *et al*. Telomerase and telomeres in aging theory and chronographic aging theory (review). Molecular Medicine Reports. 2020; 22: 1679–1694.

[8] Blasiak J, Szczepanska J, Fila M, Pawlowska E, Kaarniranta K. Potential of Telomerase in Age-Related Macular Degeneration-Involvement of Senescence, DNA Damage Response and Autophagy and a Key Role of PGC-1α. International Journal of Molecular Sciences. 2021; 22: 7194.

[9] van der Spek A, Karamujić-Čomić H, Pool R, Bot M, Beekman M, Garmaeva S, *et al*. Fat metabolism is associated with telomere length in six population-based studies. Human Molecular Genetics. 2022: 31: 1159–1170.

[10] Guillén R, Otero F, Mosquera A, Vázquez-Mosquera M, Rego-Pérez I, Blanco FJ, *et al*. Association of accelerated dynamics of telomere sequence loss in peripheral blood leukocytes with incident knee osteoarthritis in osteoarthritis initiative cohort. Scientific Reports. 2021; 11: 15914.

[11] Svenson U, Nordfjall K, Stegmayr B, Manjer J, Nilsson P, Tavelin B, *et al*. Breast cancer survival is associated with telomere length in peripheral blood cells. Cancer Research. 2008; 68: 3618–3623.

[12] Lin J, Epel E. Stress and telomere shortening: insights from cellular mechanisms. Ageing Research Reviews. 2021; 73: 101507.

[13] Denham J, Sellami M. Exercise training increases telomerase reverse transcriptase gene expression and telomerase activity: a systematic review and meta-analysis. Ageing Research Reviews. 2021; 70: 101411.

[14] Patrick M, Weng NP. Expression and regulation of telomerase in human T cell differentiation, activation, aging and diseases. Cellular Immunology. 2019; 345: 103989.

[15] Fernandes SG, Dsouza R, Khattar E. External environmental agents influence telomere length and telomerase activity by modulating internal cellular processes: implications in human aging. Environmental Toxicology and Pharmacology. 2021; 85: 103633.

[16] Wang J, Liu Y, Xia Q, Xia Q, Wang B, Yang C, *et al*. Potential roles of telomeres and telomerase in neurodegenerative diseases. International Journal of Biological Macromolecules. 2020; 163: 1060–1078.

[17] Phu HT, Thuan DTB, Nguyen THD, Posadino AM, Eid AH, Pintus G. Herbal Medicine for Slowing Aging and Aging-Associated Conditions: Efficacy, Mechanisms and Safety. Current Vascular Pharmacology. 2020; 18: 369–393.

[18] Liu Y, Weng W, Gao R, Liu Y. New Insights for Cellular and Molecular Mechanisms of Aging and Aging-Related Diseases: Herbal Medicine as Potential Therapeutic Approach. Oxidative Medicine and Cellular Longevity. 2019; 2019: 4598167.

[19] Pu X, Luo A, Su H, Zhang K, Tian C, Chen B, *et al*. Optimization and mechanism of postponing aging of polysaccharides from Chinese herbal medicine formula. Toxicology Research. 2020; 9: 239–248.

[20] Liu P, Zhao H, Luo Y. Anti-Aging Implications of Astragalus Membranaceus (Huangqi): A Well-Known Chinese Tonic. Aging and Disease. 2017; 8: 868–886.

[21] Salvador L, Singaravelu G, Harley CB, Flom P, Suram A, Raffaele JM. A Natural Product Telomerase Activator Lengthens Telomeres in Humans: A Randomized, Double Blind, and Placebo Controlled Study. Rejuvenation Research. 2016; 19: 478–484.

[22] Vangorder-Braid JT, Sirman AE, Kucera AC, Kittilson JD, Kibble TM, Heidinger BJ. TA-65 does not increase telomere length during post-natal development in house sparrow chicks (passer domesticus). Journal of Experimental Zoology. Part A, Ecological and Integrative Physiology. 2021; 335: 359–366.

[23] Tsoukalas D, Fragkiadaki P, Docea AO, Alegakis AK, Sarandi E, Thanasoula M, *et al*. Discovery of potent telomerase activators: unfolding new therapeutic and anti-aging perspectives. Molecular Medicine Reports. 2019; 20: 3701–3708.

[24] Shibu MA, Lin YJ, Chiang CY, Lu CY, Goswami D, Sundhar N, *et al*. Novel anti-aging herbal formulation Jing Si displays pleiotropic effects against aging associated disorders. Biomedicine & Pharmacotherapy = Biomédecine & Pharmacothérapie. 2022; 146: 112427.

[25] Marotta F, Catanzaro R, Yadav H, Jain S, Tomella C, Polimeni

A, *et al*. Functional foods in genomic medicine: a review of fermented papaya preparation research progress. Acta Bio-Medica: Atenei Parmensis. 2012; 83: 21–29.

[26] Marotta F, Marcellino M, Catanzaro R, Campiotti A, Lorenzetti A, Cervi J, *et al*. Mitochondrial and redox dysfunction in post-menopause as risk factor of neurodegenerative disease: a pilot study testing the role of a validated Japanese functional food. Journal of Biological Regulators and Homeostatic Agents. 2020; 34: 111–121.

[27] Barbagallo M, Marotta F, Dominguez LJ. Oxidative stress in patients with Alzheimer's disease: effect of extracts of fermented papaya powder. Mediators of Inflammation. 2015; 2015: 624801.

[28] Marotta F, Marcellino M, Solimene U, Cuffari B, Yadav H, Khokhlov AN, *et al*. A 2-Year Double-Blind RCT Follow-Up Study with Fermented Papaya Preparation (FPP) Modulating Key Markers in Middle-Age Subjects with Clustered Neurodegenerative Disease-Risk Factors. Clinical Pharmacology & Biopharmaceutics. 2017; 6: 170.

[29] Logozzi M, Di Raimo R, Mizzoni D, Andreotti M, Spada M, Macchia D, *et al*. Beneficial Effects of Fermented Papaya Preparation (FPP®) Supplementation on Redox Balance and Aging in a Mouse Model. Antioxidants (Basel, Switzerland). 2020; 9: 144.

[30] Henriksson S, Rassoolzadeh H, Hedström E, Coucoravas C, Julner A, Goldstein M, *et al*. The scaffold protein WRAP53β orchestrates the ubiquitin response critical for DNA double-strand break repair. Genes & Development. 2014; 28: 2726–2738.

[31] Silwal-Pandit L, Russnes H, Borgen E, Skarpeteig V, Moen Vollan HK, Schlichting E, *et al*. The Sub-Cellular Localization of WRAP53 Has Prognostic Impact in Breast Cancer. PLoS One. 2015; 10: e0139965.

[32] De Rosa M, Johnson SA, Opresko PL. Roles for the 8-Oxoguanine DNA Repair System in Protecting Telomeres from Oxidative Stress. Frontiers in Cell and Developmental Biology. 2021; 9: 758402.

[33] Cosemans C, Van Larebeke N, Janssen BG, Martens DS, Baeyens W, Bruckers L, *et al*. Glyphosate and AMPA exposure in relation to markers of biological aging in an adult population-based study. International Journal of Hygiene and Environmental Health. 2022; 240: 113895.

[34] Kahl VFS, da Silva J. Inorganic elements in occupational settings: a review on the effects on telomere length and biology. Mutation Research. Genetic Toxicology and Environmental Mutagenesis. 2021; 872: 503418.

[35] Chang-Chien J, Huang JL, Tsai HJ, Wang SL, Kuo ML, Yao TC. Particulate matter causes telomere shortening and increase in cellular senescence markers in human lung epithelial cells. Ecotoxicology and Environmental Safety. 2021; 222: 112484.

[36] Turkiewicz S, Ditmer M, Sochal M, Bialasiewicz P, Strzelecki D, Gabryelska A. Obstructive Sleep Apnea as an Acceleration Trigger of Cellular Senescence Processes through Telomere Shortening. International Journal of Molecular Sciences. 2021; 22: 12536.

[37] Al-Daghri NM, Abdi S, Sabico S, Alnaami AM, Wani KA, Ansari MGA, *et al*. Gut-Derived Endotoxin and Telomere Length Attrition in Adults with and without Type 2 Diabetes. Biomolecules. 2021; 11: 1693.

[38] Zhang J, Rane G, Dai X, Shanmugam MK, Arfuso F, Samy RP, *et al*. Ageing and the telomere connection: an intimate relationship with inflammation. Ageing Research Reviews. 2016; 25: 55–69.

[39] Marotta F, Chui DH, Jain S, Polimeni A, Koike K, Zhou L, *et al*. Effect of a fermented nutraceutical on thioredoxin level and TNF-alpha signalling in cirrhotic patients. Journal of Biological Regulators and Homeostatic Agents. 2011; 25: 37–45.

[40] Marotta F, Koike K, Lorenzetti A, Naito Y, Fayet F, Shimizu H, *et al*. Nutraceutical Strategy in Aging: Targeting Heat Shock Protein and Inflammatory Profile through Understanding Interleukin-6 Polymorphism. Annals of the New York Academy of Sciences. 2007; 1119: 196–202.

[41] Fernandez ML, Thomas MS, Lemos BS, DiMarco DM, Missimer A, Melough M, *et al*. TA-65, A Telomerase Activator Improves Cardiovascular Markers in Patients with Metabolic Syndrome. Current Pharmaceutical Design. 2018; 24: 1905–1911.

[42] Freitas-Simoes TM, Cofán M, Blasco MA, Soberón N, Foronda M, Serra-Mir M, *et al*. Walnut Consumption for Two Years and Leukocyte Telomere Attrition in Mediterranean Elders: Results of a Randomized Controlled Trial. Nutrients. 2018; 10: 1907.

[43] Burchat N, Sharma P, Ye H, Komakula SSB, Dobrzyn A, Vartanian V, *et al*. Maternal Transmission of Human OGG1 Protects Mice against Genetically- and Diet-Induced Obesity through Increased Tissue Mitochondrial Content. Frontiers in Cell and Developmental Biology. 2021; 9: 718962.

[44] Roy SG. Regulation of autophagy by miRNAs in human diseases. The Nucleus: An International Journal of Cytology and Allied Topics. 2021: 64: 317–329.

[45] Morais Junior GS, Souza VC, Machado-Silva W, Henriques AD, Melo Alves A, Barbosa Morais D, *et al*. Acute strength training promotes responses in whole blood circulating levels of miR-146a among older adults with type 2 diabetes mellitus. Clinical Interventions in Aging. 2017; 12: 1443–1450.

[46] Ong J, Woldhuis RR, Boudewijn IM, van den Berg A, Kluiver J, Kok K, *et al*. Age-related gene and miRNA expression changes in airways of healthy individuals. Scientific Reports. 2019; 9: 3765.

[47] Olivieri F, Capri M, Bonafè M, Morsiani C, Jung HJ, Spazzafumo L, *et al*. Circulating miRNAs and miRNA shuttles as biomarkers: perspective trajectories of healthy and unhealthy aging. Mechanisms of Ageing and Development. 2017; 165: 162–170.

[48] Lee KH, Kim DY, Kim W. Regulation of Gene Expression by Telomere Position Effect. International Journal of Molecular Sciences. 2021; 22: 12807.