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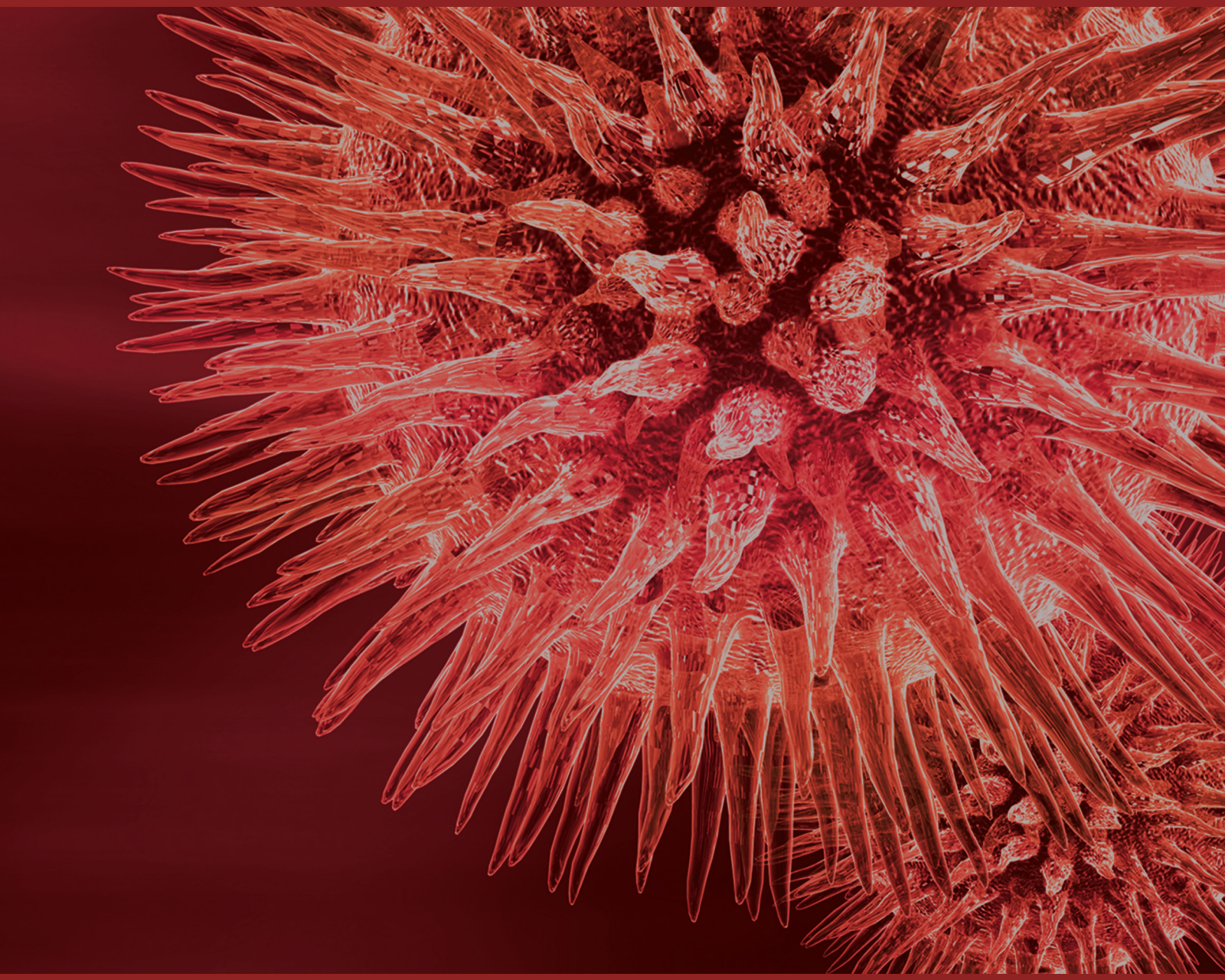
Guest Editors: Giuseppe Passarino, Giuseppina Rose, Dina Bellizzi, Maria De Luca, and Efstathios S. Gonos



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Editorial

Aging and Longevity between Genetic Background and Lifestyle Intervention

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The search for the genetic and molecular basis of aging and longevity has blossomed over the past few decades. Many (correctly, in our opinion) consider that this scientific field started with the experiments of Tom Johnson in the 80s of the last century. Indeed, before then, most gerontologists not only proclaimed the lack of progress in the field, but also suggested that progress in the field was not possible because aging is ineluctable. In their view, aging occurs after reproduction, and then there is no need and also no opportunity for selection to act on genes that are expressed late in life. The analysis of hybrids obtained from different strains of *C. elegans* allowed estimating the heritability of lifespan to be between 20% and 50%. In addition, Johnson found that mutations in a specific gene, named *Age1*, were able to significantly increase lifespan. These experiments triggered a number of genetic studies in both humans and model organisms aimed at identifying the genes and the biochemical pathways that can modulate lifespan. This fruitful quest led to the identification of genes strictly correlated with the maintenance of the cell and of its basic metabolism. Indeed, mutations in genes encoding proteins involved in DNA repair, telomere conservation, heat shock response, and the management of free radicals' levels were found to contribute to longevity or, in case of reduced functionality, to accelerated senescence (cellular aging) and the consequent organism aging. Concurrent efforts also showed that genes implicated in lipoprotein metabolism (especially APOE), immunity, and inflammation play a role in aging, age-related disorders, and organism longevity. Overall, these observations led to the idea that longevity may arise from a particularly efficient process of maintenance of the cellular and organismal activities that could contrast the inevitable time related decline of the organism functionality, which in turn leads to death.

In parallel to the studies mentioned above, a substantial number of findings in model organisms suggested that longevity could "directly" be promoted by some specific pathways. These studies were mostly driven by the finding that calorie restriction (which is a reduction in nutrient intake in the absence of malnutrition) leads to a significant lifespan increase in a variety of organisms. The identification of pathways involved in the molecular mechanisms modulated by calorie restriction has shown that this dietary regime induces an arrest of the start of cell division; consequently, the cell enters in a quiescent status that prolongs its lifespan. In addition, at the tissue level, the dramatic reduction of nutrients correlated with calorie restriction leads to an increase in autophagy, a very efficient way to eliminate old and noxious molecules. It has been largely reported that the pathways associated with nutrient-sensing signaling, such as IGF (insulin-like growth factor)/insulin and TOR (target of rapamycin), have a key role in this process. Since these strategies have recently been studied also in primates

and humans and shown promising results, it has been proposed to search for molecules mimicking the effects of calorie restriction without the side effects that the dramatic reduction of nutrient intake may have on humans (such as depression). To this end, the lack of specific amino acids in the diet and the use of rapamycin are particularly promising interventions to extend health span. Moreover, encouraging studies are currently carried out on the capacity of spermidine to promote autophagy, improve the health of the organism, and, consequently, prolong lifespan.

In the present issue, we have included a number of original articles and updated reviews covering different areas of studies in the field of healthy ageing and longevity. Most of these reports highlight different aspects of the importance of efficient tissue maintenance, especially against oxidative stress (see the papers by M. Ingles et al., S. Heinzel et al., I. Sadowska-Bartosz and G. Bartosz, B. Arosio et al., G. R. Hunter et al., and T. A. Giancaspero et al.). G. Taormina and M. G. Mirisola give an updated review of the effects of calorie restriction on longevity and some possible interventions, taking advantage of the pathways highlighted by studying calorie restriction. The papers by A. Galán-Mercant and A. I. Cuesta-Vargas and S. Garasto et al. show how the close monitoring of health and physical functioning may help in understanding the interventions which may help the quality of human aging. Finally, the paper by P. Garagnani et al. shows the most recent results on the genetic component of longevity and healthy aging suggesting they come from the interactions of three genetic systems.

On the whole, we believe these papers may help the readers have an idea of the different facets of the studies on aging and longevity and of the perspectives they are unveiling.

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Research Article

Alteration of ROS Homeostasis and Decreased Lifespan in *S. cerevisiae* Elicited by Deletion of the Mitochondrial Translocator *FLX1*

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This paper deals with the control exerted by the mitochondrial translocator *FLX1*, which catalyzes the movement of the redox cofactor FAD across the mitochondrial membrane, on the efficiency of ATP production, ROS homeostasis, and lifespan of *S. cerevisiae*. The deletion of the *FLX1* gene resulted in respiration-deficient and small-colony phenotype accompanied by a significant ATP shortage and ROS unbalance in glycerol-grown cells. Moreover, the $flx1\Delta$ strain showed H_2O_2 hypersensitivity and decreased lifespan. The impaired biochemical phenotype found in the $flx1\Delta$ strain might be justified by an altered expression of the flavoprotein subunit of succinate dehydrogenase, a key enzyme in bioenergetics and cell regulation. A search for possible *cis*-acting consensus motifs in the regulatory region upstream SDH1-ORF revealed a dozen of upstream motifs that might respond to induced metabolic changes by altering the expression of Flx1p. Among these motifs, two are present in the regulatory region of genes encoding proteins involved in flavin homeostasis. This is the first evidence that the mitochondrial flavin cofactor status is involved in controlling the lifespan of yeasts, maybe by changing the cellular succinate level. This is not the only case in which the homeostasis of redox cofactors underlies complex phenotypical behaviours, as lifespan in yeasts.

1. Introduction

Riboflavin (Rf or vitamin B_2) is the precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), the redox cofactors of a large number of dehydrogenases, reductases, and oxidases. Most of these flavoenzymes are compartmented in the cellular organelles, where they are involved in energy production and redox homeostasis as well as in different cellular regulatory events including apoptosis, chromatin remodelling, and interestingly, as recently proposed, in epigenetic signalling [1–4]. Consistent with the crucial role of flavoenzymes in cell life, flavin-dependent enzyme deficiency and/or impairment in flavin homeostasis in humans and experimental animals has been linked to several diseases, such as cancer, cardiovascular diseases, anaemia, abnormal fetal development, and different neuromuscular and neurological disorders [5–9]. The relevance of these pathologies merits further research aimed to better describe FAD homeostasis and flavoenzyme biogenesis, especially in those organisms that can be a simple and suitable model for human diseases. The conserved biological processes shared with all eukaryotic cells, together with the possibility of simple and quick genetic manipulation, allowed proposing the budding yeast, *Saccharomyces cerevisiae*, as the premier model to understand the biochemistry and

molecular biology of mammalian cells and to decipher molecular mechanisms underlying human diseases [10–12].

For many years *S. cerevisiae* has been used also as a model to study the complexity of the molecular events involved in the undesired process of aging, in which mitochondria play a major role [13, 14]. The role of mitochondria has been pointed out either because aged respiratory chain is a major source of cellular ROS [14] or because mitochondria actively participate in regulating the homeostasis of the redox cofactor NAD, which regulates yeast lifespan by acting as a substrate of specific deacetylases (EC 3.5.1.-), named sirtuins [15–17]. This might not be the only case in which the homeostasis of redox cofactors underlies complex phenotypical behaviours, as lifespan in yeasts. Here we investigate whether the mitochondrial flavin cofactor status may also be involved in controlling the lifespan of yeasts, presumably by changing the level of mitochondrial flavoenzymes, which are crucial for cell regulation [18, 19].

It should be noted that, even though mitochondria are plenty of flavin and flavoproteins [20, 21], the origin of flavin cofactors starting from Rf in this organelle is still a matter of debate. Yeasts have the ability to either synthesise Rf *de novo* or to take it from outside. The first eukaryotic gene coding for a cellular Rf transporter was identified in *S. cerevisiae* as the *MCH5* gene [22]. Intracellular Rf conversion to FAD is a ubiquitous pathway and occurs via the sequential actions of ATP: riboflavin 5′-phosphotransferase or riboflavin kinase (RFK, EC 2.7.1.26) which phosphorylates the vitamin into FMN and of ATP: FMN adenylyl transferase or FAD synthase (FADS, EC 2.7.7.2) which adenylates FMN to FAD. The first eukaryotic genes encoding for RFK and FADS were identified in *S. cerevisiae* and named *FMN1* [23] and *FAD1* [24], respectively. While there is no doubt about a mitochondrial localization for Fmn1p [23, 25], the existence of a mitochondrial FADS isoform in yeast is still controversial. First a cytosolic localization for Fad1p was reported [24]; thus newly synthesised FAD was expected to be imported into mitochondria via the FAD translocator Flx1p [25]. However, results from our laboratory showed that, besides in the cytosol, FAD-forming activities can be revealed in mitochondria, thus requiring uptake of the FAD precursors into mitochondria [26, 27]. FAD synthesised inside the organelle can be either delivered to a number of nascent client apo-flavoenzymes or be exported via Flx1p into cytosol to take part of an extramitochondrial posttranscriptional control of apo-flavoprotein biogenesis [19, 26].

Besides synthesis and transport, mitochondrial flavin homeostasis strictly depends also on flavin degradation. Recently we have demonstrated that *S. cerevisiae* mitochondria (SCM) are able to catalyze FAD hydrolysis via an enzymatic activity which is different from the already characterized NUDIX hydrolases (i.e., enzymes that catalyze the hydrolysis of nucleoside diphosphates linked to other moieties, X) and it is regulated by the mitochondrial NAD redox status [17].

To prove the relationship between mitochondrial FAD homeostasis and lifespan in yeast we use as a model a *S. cerevisiae* strain lacking the *FLX1* gene which showed a respiratory-deficient phenotype and a derangement in a number of mitochondrial flavoproteins, that is, dihydrolipoamide dehydrogenase (*LPD1*), succinate dehydrogenase (*SDH*), and flavoproteins, involved in ubiquinone biosynthesis (*COQ6*) [18, 25, 26, 28].

We demonstrated here that this deleted strain performed ATP shortage and ROS unbalance, together with H_2O_2 hypersensitivity and altered chronological lifespan. This *flx1*Δ phenotype is correlated to a reduced ability to maintain an appropriate level of the flavoenzyme succinate dehydrogenase (SDH), a member of a complex "flavin network" participating in a nucleus-mitochondrion cross-talk.

2. Materials and Methods

2.1. Materials. All reagents and enzymes were from Sigma-Aldrich (St. Louis, MO, USA). Zymolyase was from ICN (Abingdon, UK) and Bacto Yeast Extract and Bacto Peptone were from Difco (Franklin Lakes, NJ, USA). Mitochondrial substrates were used as TRIS salts at pH 7.0. Solvents and salts used for HPLC were from J. T. Baker (Center Valley, PA, USA). Rat anti-HA monoclonal antibody and peroxidase-conjugated anti-rat IgG secondary antibody were obtained from Roche (Basel, Switzerland) and Jackson Immunoresearch (West Grove, PA, USA), respectively.

2.2. Yeast Strains. The wild-type *S. cerevisiae* strain (EBY157A or *WT* genotype *MATα ura 3–52 MAL2-8^c SUC2 p426MET25*) used in this work derived from the CEN.PK series of yeast strains and was obtained from P. Kotter (Institut für Mikrobiologie, Goethe-Universität Frankfurt, Frankfurt, Germany), as already described in [26]. The *flx1*Δ mutant strain (EBY167A, *flx1*Δ) was constructed as described in [26] and the *WT-HA* (EBY157-SDH1-HA) and *flx1*Δ*-HA* (EBY167-G418S-SDH1-HA) were constructed as described in [19].

2.3. Media and Growth Conditions. Cells were grown aerobically at 30°C with constant shaking in rich liquid medium (YEP, 10 g/L Yeast Extract, 20 g/L Bacto Peptone) or in minimal synthetic liquid medium (SM, 1.7 g/L yeast nitrogen base, 5 g/L ammonium sulphate, and 20 mg/L uracil) supplemented with glucose or glycerol (2% each) as carbon sources. The YEP or SM solid media contained 18 g/L agar.

2.4. Chronological Lifespan Determination. WT and *flx1*Δ strains were grown overnight at 30°C in 5 mL YEP liquid medium supplemented with glucose 0.5% up to the early stationary phase. Each strain was then cultured in SM liquid medium at 30°C for 1, 4, and 7 days. Five serial dilutions from each culture containing 200 cells, calculated from $A_{600\,nm}$, were plated onto SM solid medium and grown at 30°C for two-three days.

2.5. H_2O_2 Sensitivity. *WT* and *flx1*Δ strains were grown overnight at 30°C in 5 mL YEP liquid medium supplemented with glucose 0.5% up to the early stationary phase. Then, each strain was inoculated in SM liquid medium (initial $A_{600\,nm}$ equal to 0.1) containing glucose 2% and H_2O_2 (0.05 or 2 mM).

After 5 or 24 h of growth at 30°C, the H_2O_2 sensitivity was estimated by measuring the $A_{600\,nm}$ of the growth culture.

2.6. Malate and Succinate Sensitivity. *WT* and *flx1*Δ strains were grown overnight at 30°C in 5 mL YEP liquid medium supplemented with glucose 0.5% up to the early stationary phase. Then, each strain was inoculated in SM liquid medium (initial $A_{600\,nm}$ equal to 0.1) containing glucose 2% and succinate or malate (5 mM). After 24 h of growth at 30°C, the H_2O_2 sensitivity was estimated by measuring the $A_{600\,nm}$ of the growth culture.

2.7. Preparation of Spheroplasts, Mitochondria, and Cellular Lysates. Spheroplasts were prepared using Zymolyase. Mitochondria were isolated from spheroplasts as described in [26]. Cellular lysates were obtained by early exponential-phase (5 h) or stationary-phase (24 h) cells harvested by centrifugation (8000 ×g for 5 min), washed with sterile water, resuspended in 250 μL of lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, and 0.2 mM phenylmethanesulfonyl fluoride, supplemented with one tablet of Roche protease inhibitor cocktail every 10 mL of lysis buffer), and vortexed with glass beads for 10 min at 4°C. The liquid was removed and centrifuged at 3000 ×g for 5 min to remove cell debris. The protein concentrations of the spheroplasts, mitochondria, and cellular lysates were assayed according to Bradford [29].

2.8. Quantitation of Flavins, ATP, and Reactive Oxygen Species (ROS). Rf, FMN, and FAD content in spheroplasts and SCM was measured in aliquots (5–80 μL) of neutralized perchloric extracts by means of HPLC (Gilson HPLC system including a model 306 pump and a model 307 pump equipped with a Kontron Instruments SFM 25 fluorometer and Unipoint system software), essentially as previously described [26]. ATP content was measured fluorometrically in cellular lysates by using the ATP Detecting System, essentially as in [30]. NADPH formation, which corresponds to ATP content (with a 1 : 1 stoichiometry), was followed with excitation wavelength at 340 nm and emission wavelength at 456 nm. ROS level was fluorometrically measured on cellular lysates using as substrate 2′-7′-dichlorofluorescin diacetate (DCF-DA) according to [30], with slight modifications. Briefly, the probe DCF-DA (50 μM) was incubated at 37°C for 1 h with 0.03–0.05 mg proteins and converted to fluorescent dichlorofluorescein (DCF) upon reaction with ROS. DCF fluorescence of each sample was measured by means of a LS50S Perkin Elmer spectrofluorometer (excitation and emission wavelengths set at 485 nm and 520 nm, resp.).

2.9. Enzymatic Assays. Succinate dehydrogenase (SDH, EC 1.3.5.1) and fumarase (FUM, EC 4.2.1.2) activities were measured as in [26]. Glutathione reductase (GR, EC 1.6.4.2) activity was spectrophotometrically assayed by monitoring the absorbance at 340 nm due to NADPH oxidation after glutathione addition (1 mM), essentially as in [30]. Superoxide dismutase (SOD, EC 1.15.1.1) activity was spectrophotometrically measured by the xanthine oxidase/xanthine/cytochrome c method, essentially as described in [31].

2.10. Statistical Analysis. All experiments were repeated at least three times with different cell preparations. Results are presented as mean ± standard deviation (SD). Statistical significance was evaluated by Student's t-test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Phenotypical and Biochemical Consequences of FLX1 Deletion. In order to study the relevance of mitochondrial flavin cofactor homeostasis on cellular bioenergetics we introduced a yeast strain lacking the *FLX1* gene, encoding the mitochondrial FAD transporter [26]. This deleted strain showed a small-colony phenotype, on both fermentable and nonfermentable carbon sources, due to an impairment in the aerobic respiratory chain pathway [32]. The deleted strain, *flx1*Δ, grew normally on glucose medium but failed to grow on nonfermentable carbon sources (i.e., glycerol), thus indicating a respiration-deficient phenotype (Figure 1(a)). The growth defect on nonfermentable carbon source, which was restored by complementing the deleted strain with the YEpFLX1 plasmid [26], was not rescued by the addition of tricarboxylic acid (TCA) cycle intermediates such as succinate or malate (Figure 1(a)).

Among the mitochondrial flavoenzymes which were demonstrated to be altered in *flx1*Δ strain [25, 26, 28], we showed before [19, 32] and confirmed in Figure 1(b) a significant reduced level of the apo-flavoprotein Sdh1p, resulting in an altered functionality of SDH or complex II of the respiratory chain. This reduction was revealed by creating a strain in which three consecutive copies of the human influenza hemagglutinin epitope (HA epitope, YPYDVPDYA) were fused in frame to the 3′ end of the *SDH1 ORF* in the genome of both the WT and *flx1*Δ strains. The chimera protein, namely, Sdh1-HAp, carrying the HA-tag at the C-terminal end of Sdh1p, lost the ability to covalently bind the flavin cofactor FAD [19, 33], but not its regulatory behaviour, that is, its inducible expression in galactose or in nonfermentable carbon sources. In all the growth conditions tested, the FAD-independent fumarase (FUM) activity, used as a control, was not affected by *FLX1* deletion (see histogram in Figure 1(b)).

A significant decrease of Sdh1-HAp level was accompanied in galactose, but not in glycerol, by a profound derangement of flavin cofactors, particularly evident in cell grown at the early exponential phase (Table 1), in agreement with [25, 26], respectively. The reason for these carbon source-dependent flavin level changes, which is not easily explainable, is addressed in Section 4.

Consistent with an altered functionality of SDH, the *flx1*Δ strain also showed impaired isolated mitochondria oxygen consumption activity, specifically detectable when succinate was used as a respiratory substrate [19]. Similar phenotype was also observed in yeast strains carrying either a deletion

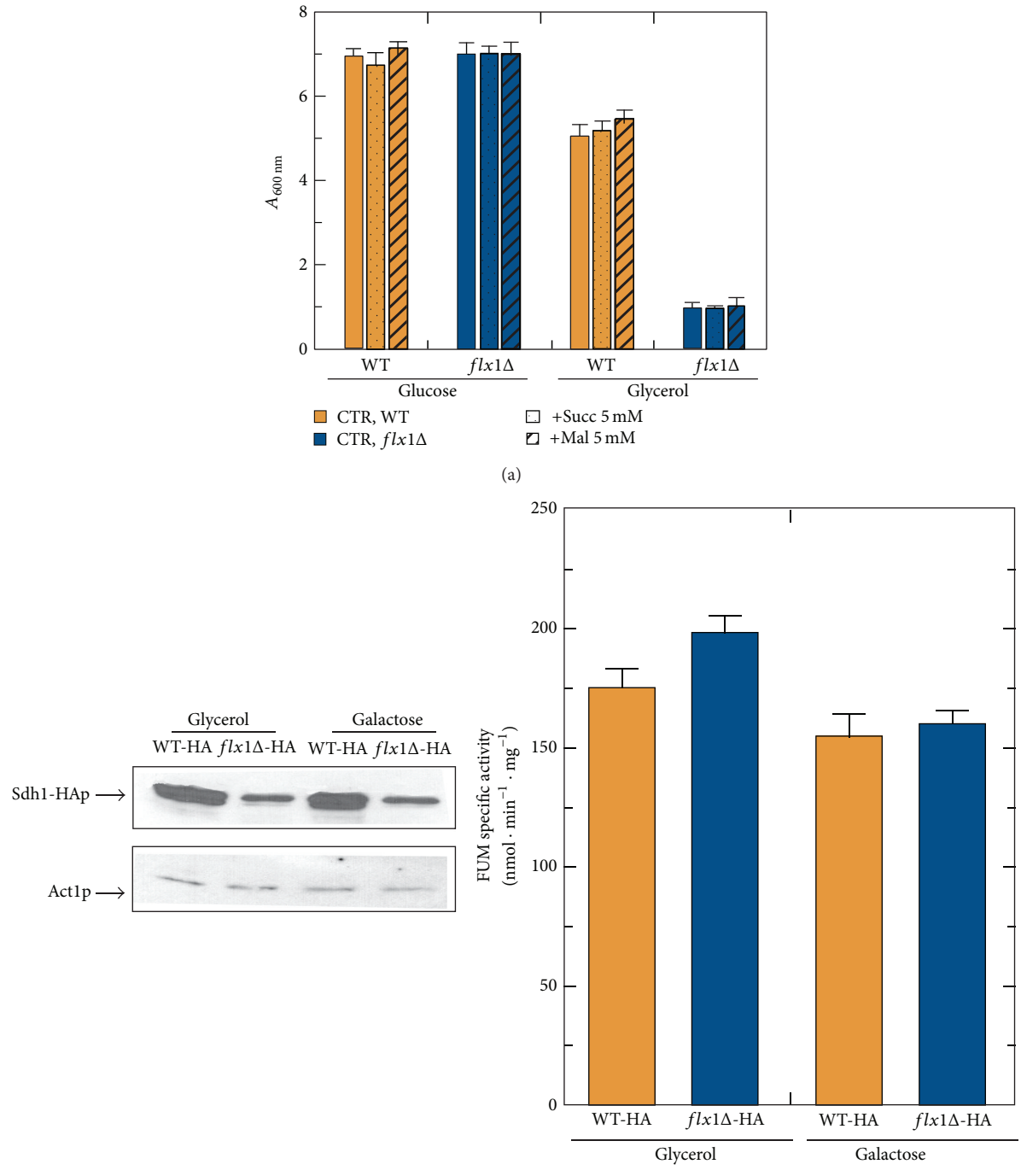

Figure 1: (a) Respiratory-deficient phenotype of *flx1*Δ strain: effect of succinate and malate addition. WT and *flX1*Δ cells were cultured at 30°C in YEP liquid medium supplemented with either glucose or glycerol (2% each) as carbon source. Where indicated either 5 mM succinate (Succ) or 5 mM malate (Mal) was added. Cell growth was estimated at the stationary phase (24 h) by measuring the absorbance at 600 nm ($A_{600\,nm}$) of a ten-fold dilution of each growth culture, consistently, corrected for the dilution factor. The values reported in the histogram are the means (±SD) of three experiments. (b) Changes in the recombinant Sdh1-HAp level in *flx1*Δ strain. Cellular lysates were prepared from *WT-HA* and *flX1*Δ*-HA* cells grown at 30°C up to the exponential growth phase (5 h) in YEP liquid medium supplemented with either glycerol or galactose (2% each) as carbon source. Proteins from cellular lysates (0.05 mg) were separated by SDS/PAGE and transferred onto a PVDF membrane. In each extract, Sdh1-HA protein was detected by using an α-HA and its amount was densitometrically evaluated. The values reported in the histogram are the means (±SD) of three experiments performed with different cellular lysates preparations. Statistical evaluation was carried out according to Student's t-test ($^{*}P < 0.05$). As a control, the specific activity of the enzyme fumarase (FUM) was determined in each cellular lysate preparation.

Table 1: Endogenous flavin content in spheroplasts and mitochondria.

Carbon source	Strain	Spheroplasts			SCM		
		FAD pmoli mg^{-1}	FMN pmoli·mg^{-1}	FAD/FMN	FAD pmoli mg^{-1}	FMN pmoli·mg^{-1}	FAD/FMN
Glycerol	WT	157 ± 7	153 ± 7	*1.1*	160 ± 10°	30 ± 10°	*4.8*
	*flx*1Δ	126 ± 11	110 ± 10	*1.1*	140 ± 30°	40 ± 10°	*4.5*
Galactose	WT	263 ± 10	189 ± 8	1.4	538 ± 32	103 ± 7	5.2
	*flx*1Δ	207 ± 8*	195 ± 8	1.1	306 ± 15*	67 ± 11*	4.8

Spheroplasts and mitochondria (SCM) were prepared from WT and *flx*1Δ cells grown in glycerol or galactose (2%) up to the exponential growth phase (5 h). FAD and FMN content was determined in neutralized perchloric acid extracts, as described in *Materials and Methods*. Riboflavin amount was not relevant, and thus its value has not been reported. The means (±SD) of the flavin endogenous content determined in three experiments performed with different preparations are reported. °Data published in (Bafunno et al., 2004) [26]; statistical evaluation was carried out according to Student's *t*-test (* $P < 0.05$).

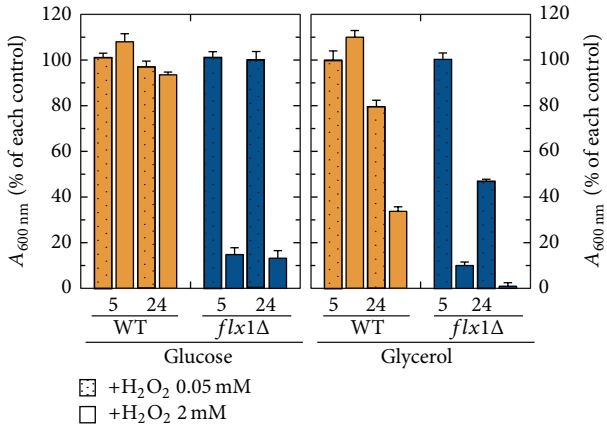

Figure 2: Sensitivity to H_2O_2. WT and *flX1*Δ cells were cultured at 30°C in YEP liquid medium supplemented with either glucose or glycerol (2% each) as carbon source. Where indicated, H_2O_2 at the indicated concentration was added. Cell growth was estimated at the exponential (5 h) and stationary phase (24 h) by measuring the absorbance at 600 nm ($A_{600\,nm}$). In the histogram, the $A_{600\,nm}$ of the cell cultures grown in the presence of H_2O_2 is reported as a percentage of the control (i.e., the $A_{600\,nm}$ of cell cultures grown in the absence of H_2O_2, set arbitrary equal to 100%). The values reported in the histogram are the means (±SD) of three experiments.

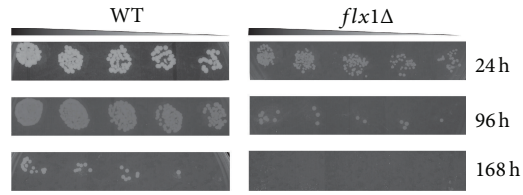

Figure 3: Chronological lifespan determination. WT and *flX1*Δ strains were cultured in SM liquid medium at 30°C. Dilutions from each culture containing about 200 cells (as calculated from $A_{600\,nm}$ by taking into account that one $A_{600\,nm}$ is equivalent to 3×10^7 cell/mL) were harvested after 24, 96, and 168 h and plated onto SM solid medium and grown at 30°C for two-three days.

of *SDH1* [34] or a deletion of *SDH5*, which encodes a mitochondrial protein involved in Sdh1p flavinylation [35]. Another respiration-related phenotype of *flx1*Δ strain was investigated in Figure 2, by testing H_2O_2 hypersensitivity of cells grown on both fermentable and nonfermentable carbon sources. In glucose, the WT cells grew up to the stationary phase (24 h) in the presence of H_2O_2 (0.05 or 2 mM) essentially as the control cells grown in the absence of H_2O_2. In glycerol, their ability to grow up to 24 h was reduced of about 20% at 0.05 mM H_2O_2 and of 60% at 2 mM, with respect to the control cells in which no H_2O_2 was added.

In glucose, *flx1*Δ cells did not show H_2O_2 hypersensitivity at 0.05 mM. At 2 mM H_2O_2, their ability to grow was significantly reduced (of about 85%) with respect to *flx1*Δ cells grown in the absence of H_2O_2. The ability of the *flx1*Δ cells to grow in glycerol, which was *per se* drastically reduced by deletion, was reduced at 24 h by the addition of 0.05 mM H_2O_2 (about 50% with respect to the control cells grown in the absence of H_2O_2). An even higher sensitivity to H_2O_2 was observed in the presence of 2 mM H_2O_2, having their growth ability reduced of about 85% with respect to control cells in which no addition was made. The impairment in the ability to grow under H_2O_2 stress conditions clearly demonstrates an impairment in defence capability of the *flx1*Δ strain. Interestingly, the same phenotype was observed also in the yeast *sdh5*Δ [35], *sdh1*Δ, and *sdh2*Δ [36] strains.

To understand whether mitochondrial flavoprotein impairment, due to *FLX1* deletion, influenced aging in yeast, we carried out measurements of chronological lifespan on both WT and *flx1*Δ cells cultured at 30°C in SM liquid medium supplemented with glucose 2% as carbon source (Figure 3). Following 24 h (1 day), 96 h (4 days), and 168 h (7 days) of growth, the number of colonies was determined by spotting five serial dilutions of the liquid culture and incubating the plates for two-three days at 30°C. The results of a typical experiment are reported in Figure 3. A reduced number of small colonies were counted for the *flx1*Δ strain, with respect to the number of colonies counted for the WT strain. This phenotype, particularly evident after 96 h and 168 h of growth time, clearly indicated a decrease in chronological lifespan of the *flx1*Δ strain. Essentially the same phenotype was observed in *sdh1*Δ and *sdh5*Δ strains [35]. Thus, it seems quite clear that a correct biogenesis of mitochondrial flavoproteome, and in particular assembly of SDH, ensures a correct aging rate in yeast. When *flx1*Δ *cells* were grown on glycerol, they lost the ability to form colonies following 24 h growth time (data not shown).

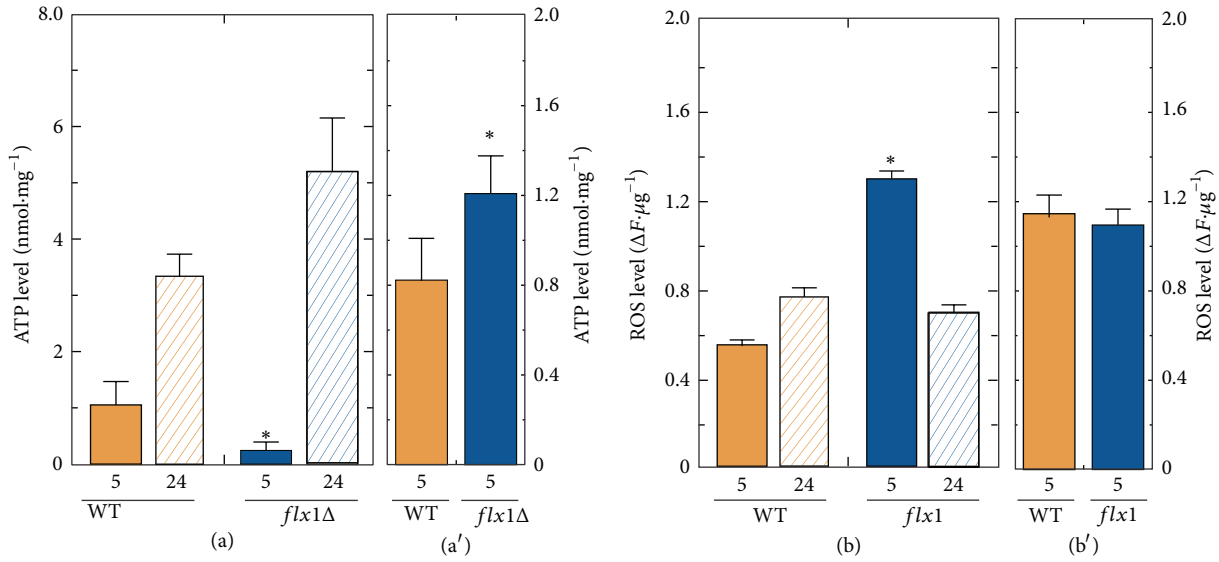

FIGURE 4: Bioenergetic and redox impairment in *flx1*Δ strain: ATP and ROS content. Cellular lysates were prepared from WT and *flx1*Δ mutant strains grown in glycerol ((a), (b)) up to either the exponential (5 h) or the stationary phase (24 h) or in glucose ((a′), (b′)) up to the exponential phase (5 h). ATP content ((a), (a′)) was enzymatically determined following perchloric acid extraction and neutralization. ROS content ((b), (b′)) was fluorometrically measured as described in Section 2. The values reported in the histograms are the means (±SD) of three experiments performed with different cellular lysate preparations. Statistical evaluation was carried out according to Student's t-test ($^{*}P < 0.05$).

In order to correlate the observed phenotype with an impairment of cellular bioenergetics, we compared the ATP content and the ROS amount of the *flx1*Δ strain with that of the WT. In Figure 4, panel (a), the ATP cellular content was enzymatically measured in neutralized perchloric extracts prepared from WT and *flx1*Δ cells grown on glycerol. At the exponential growth phase (5 h), a significant reduction was detected in the *flx1*Δ cells in comparison with the WT (0.21 versus 1.05 nmol·mg^{-1} protein). At the stationary growth phase (24 h), the ATP content increased significantly in WT cells (3.4 nmol·mg^{-1} protein) and even more in the deleted strain (5.2 nmol·mg^{-1} protein). The temporary severe decrease in ATP content induced by the absence of Flx1p was not observed in glucose-grown cells (Figure 4, panel (a′)), as expected when fermentation is the main way to produce ATP.

FLX1 deletion induced also a significant increase in the amount of ROS (135% with respect to the WT cells), as estimated with the fluorescent dye DCFH-DA on the cellular lysates prepared from cells grown in glycerol up to the exponential growth phase (Figure 4, panel (b)). At the stationary phase the *flx1*Δ cells presented almost the same ROS amount measured in the WT cells (Figure 4, panel (b)). In glucose-grown cells, the amount of cellular ROS in the *flx1*Δ strain was not significantly changed with respect to the WT (Figure 4, Panel (b′)), as expected when a mitochondrial damage is the major cause of ROS unbalance.

In line with the unique role of flavin cofactor in oxygen metabolism and ROS defence systems [20, 30, 37, 38], we further investigated whether the impairment of the ROS level in glycerol-grown *flx1*Δ strain was due to a derangement in enzymes involved in ROS detoxification, such as the flavoprotein glutathione reductase (GR) or the FAD-independent superoxide dismutase (SOD); their specific enzymatic activities were measured in cellular lysates from WT and *flx1*Δ cells grown on glycerol and glucose, while assaying the FAD-independent enzyme FUM as control (Figure 5). Figure 5, panel (a), shows a significant increase in GR specific activity in *flx1*Δ strain (65%) at the exponential growth phase with respect to that measured in WT. The GR specific activity in the *flx1*Δ reached the same value measured in the WT cells (about 35 nmol·mg^{-1} protein) at the stationary phase. In cells grown in glucose up to the exponential growth phase (Figure 5, panel (a′)) a slight, but not significant, reduction in GR specific activity was detected in the *flx1*Δ strain with respect to the WT (25 versus 31 nmol·mg^{-1} protein).

As regards SOD, in the glycerol-grown *flx1*Δ cells after 5 h growth time (Figure 5, panel (b)), the SOD specific activity was significantly higher than the value measured in the WT cells (16 versus 9 standard U·mg^{-1}). At the stationary phase, the SOD specific activity in the *flx1*Δ significantly decreased, reaching a value of 6.6 standard U·mg^{-1}, that is, about two-fold lower than the SOD specific activity measured in WT cells. In glucose-grown cells after 5 h growth time (Figure 5, panel (b′)), a slight, but significant, reduction in SOD specific activity can be detected in the *flx1*Δ strain with respect to the WT (9.2 versus 12.2 nmol·mg^{-1} protein). This reduction might be explained by a defect in FAD dependent protein folding, as previously observed in [30, 39].

In all the growth conditions tested, the FUM activity, used as a control, was not affected by *FLX1* deletion (Figure 5, panels (c) and (c′)).

3.2. The Role of Flx1p in a Retrograde Cross-Talk Response Regulating Cell Defence and Lifespan. Results described in

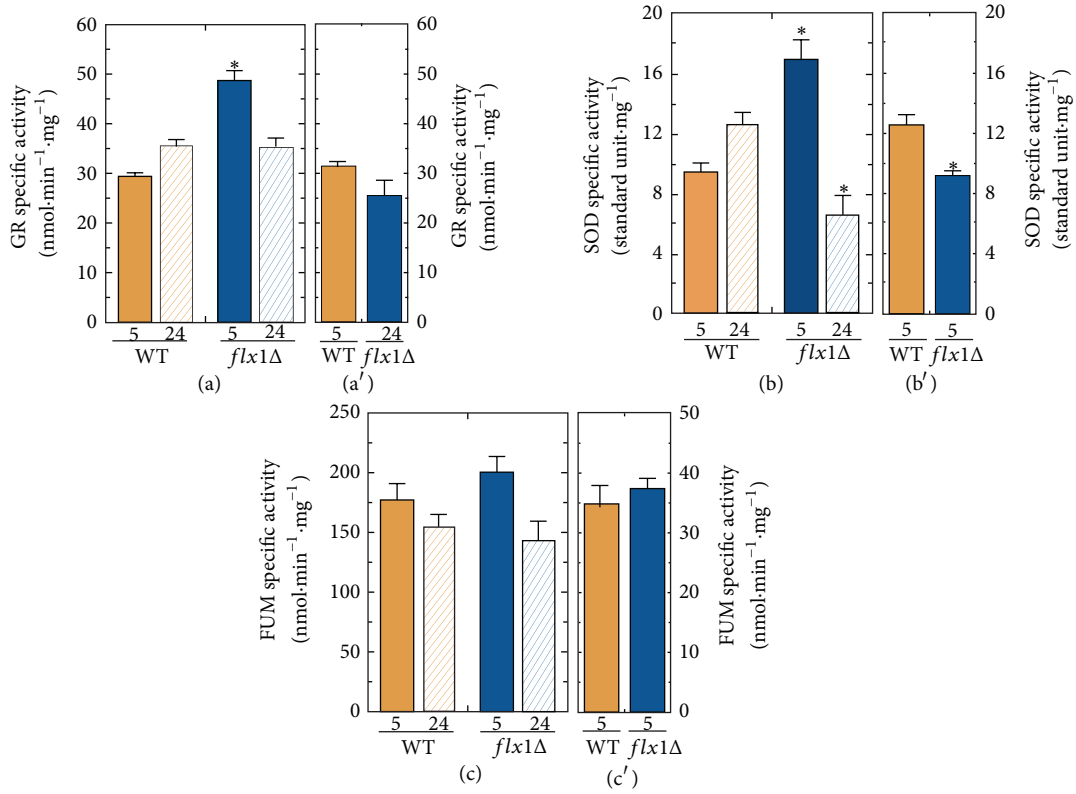

Figure 5: GR and SOD activities in *flx1*Δ strain. Cellular lysates were prepared from WT and *flx1*Δ mutant strains grown in glycerol ((a), (b), and (c)) up to either the exponential (5 h) or the stationary phase (24 h) or in glucose ((a′), (b′), and (c′)) up to the exponential phase (5 h). GR ((a), (a′)) and SOD ((b), (b′)) specific activities were spectrophotometrically determined as described in Section 2. As control FUM specific activity ((c), (c′)) was measured as described in Section 2. The values reported in the histograms are the means (±SD) of three experiments performed with different cellular lysate preparations. Statistical evaluation was carried out according to Student's *t*-test ($^*P < 0.05$).

the previous paragraph strengthen the relevance of Flx1p in ensuring cell defence and correct aging by maintaining the homeostasis of mitochondrial flavoproteome. As concerns SDH, in [19] we gained some insight into the mechanism by which Flx1p could regulate Sdh1p apo-protein expression, as due to a control that involves regulatory sequences located upstream of the SDH1 coding sequence (as reviewed in [40]).

To gain further insight into this mechanism, we searched here for elements that could be relevant in modulating Sdh1p expression, in response to alteration in flavin cofactor homeostasis. Therefore, first we searched for *cis*-acting elements in the regulatory regions located upstream of the *SDH1 ORF*, first of all in the 5′UTR region, as defined by [41], which corresponds to the first 71 nucleotides before the start codon of *SDH1 ORF*. No consensus motifs were found in this region by using the bioinformatic tool "Yeast Comparative Genomics—Broad Institute" [42]. Indeed, it should be noted that no further information is at the moment available on the actual length of the 5′UTR of *SDH1*.

Thus, we extended our analysis along the 1 kbp upstream region of *SDH1 ORF* and we found twelve consensus motifs that could bind regulatory proteins, six of which are of unknown function. Among these motifs, summarised in Table 2, the most relevant, at least in the scenario described by our experiments, seemed to be a motif which is located at −80 nucleotides upstream the start codon of *SDH1 ORF* and, namely, motif 29 (consensus sequence shRCCCYTWDt), that perfectly overlaps with motif 38 (consensus sequence CTCCCCTTAT). This motif is also present in the upstream region of the mitochondrial flavoprotein *ARH1*, involved in ubiquinone biosynthesis [28], but not in that of flavoprotein *LPD1* and *COQ6* [25, 26, 28]. Interestingly, this motif 29 is also present in the upstream regions of the members of the machinery that maintained Rf homeostasis, that is, the mitochondrial FAD transporter *FLX1* [25], the FAD forming enzyme *FAD1* [25], and the Rf translocator *MCH5* [22]. Moreover, this motif is also present in the upstream regulatory region of the mitochondrial isoenzyme *SOD2*, but not in the cytosolic one, *SOD1*, and in one of the five nuclear succinate sensitive JmjC-domain-containing demethylases, that is, *RPH1* [43]. According to [42], this motif is bound by transcription factor Msn2p and its close homologue Msn4p (referred to as Msn2/4p), which under nonstress conditions are located in the cytoplasm. Upon different stress conditions, among which oxidative stress, Msn2/4p are hyperphosphorylated and shuttled from the cytosol to the nucleus [44]. The pivotal role played by Msn2/4p in chronological lifespan in yeast was first discovered by [45] and recently exhaustively reviewed by [46].

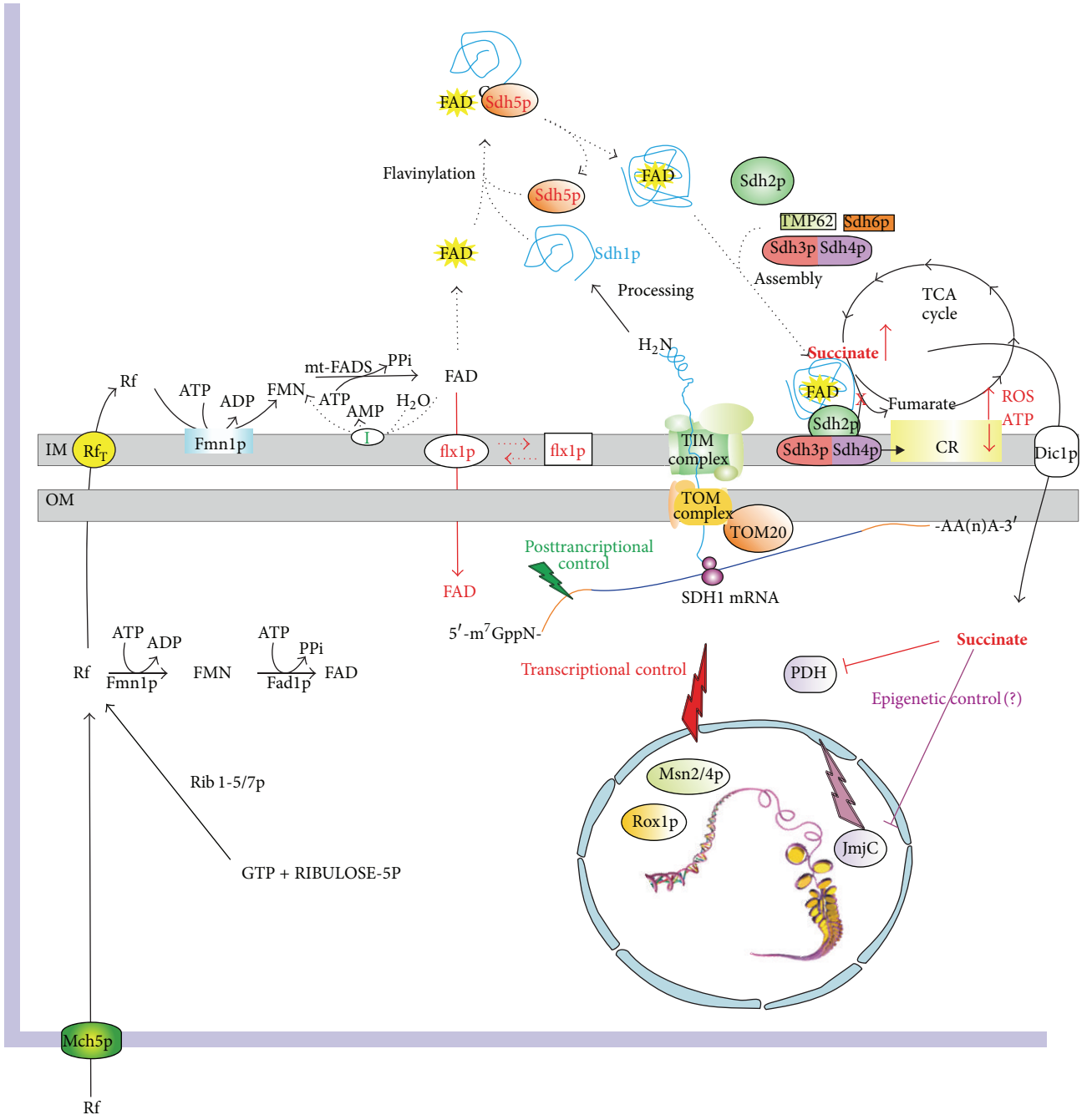

Figure 6: A possible correlation between mitochondrial FAD homeostasis and chronological lifespan. The scheme summarizes results from studies described in this and other papers [17, 19, 22, 26, 35, 36, 40, 50, 53]. Mch5p, plasma membrane Rf transporter; Rib1-5/7p, enzymes involved in Rf *de novo* biosynthesis; Rf_T, mitochondrial riboflavin transporter; Fmn1p, riboflavin kinase; mtFADS, mitochondrial FAD synthase; Flx1p, mitochondrial FAD exporter; I, FAD pyrophosphatase; Sdh1p, succinate dehydrogenase flavoprotein subunit; Sdh5p, protein required for Sdh1p flavinylation; Sdh2/3/4p, other subunits of succinate dehydrogenase complex; Tmp62p/Sdh6p, factors required for SDH complex assembly; TCA cycle, tricarboxylic acid cycle; TOM complex/TIM complex, proteins involved in mitochondrial protein import; Dic1p, mitochondrial dicarboxylic acid carrier; PDH, prolyl hydroxylase; JmjC, JmjC-domain-containing demethylases, Rox1p, heme-dependent repressor of hypoxic genes; Msn2/4p, transcriptional factors activated in stress conditions.

A further comparison between the 5′UTRs of *SDH1* and of proteins involved in FAD homeostasis revealed another common motif of unknown function located at −257 nucleotides upstream the start codon of *SDH1 ORF*, namely, the motif 14 (consensus sequence YCTATTGTT) [42]. Besides *SDH1*, this motif is also present in the upstream region of *MCH5* and its homologue *MCH4*, in *FAD1*, and also in a number of mitochondrial flavoproteins, including

Table 2: List of motifs localized in the 1000 nucleotides upstream region of SDH1 ORF and identified by enriched conservation among all Saccharomyces species genome using the "Yeast Comparative Genomics—Broad Institute" database.

Number	Motif	Number of ORFs	Binding factor	Function
2	RTTACCCGRM	865	Reb1	RNA polymerase I enhancer binding protein
14	**YCTATTGTT**	**561**	**Unknown**	**/**
26	DCGCGGGGH	285	Mig1	Involved in glucose repression
29	**hRCCCYTWDt**	**442**	**Msn2/4**	**Involved in stress conditions**
38	**CTCCCCTTAT**	**218**	**Msn2/4**	**Involved in stress conditions**
39	GCCCGG	152	Unknown	Filamentation
41	CTCSGCS	77	Unknown	/
47	TTTTnnnnnnnnnnngGGGT	359	Unknown	/
57	CGGCnnMGnnnnnnnCGC	84	Gal4	Involved in galactose induction
61	GKBAGGGT	363	TBF1	Telobox-containing general regulatory factor
63	GGCSnnnnnGnnnCGCG	80	mbp1-like	Involved in regulation of cell cycle progression from G1 to S
70	CGCGnnnnnGGGS	156	Unknown	/

HEM14, *NDI1*, and *NCP1*. The binding factor and the functional role of the motif 14 have not yet annotated in "Yeast Comparative Genomics—Broad Institute" (Table 2). Searching in the biological database "Biobase-Gene-regulation-Transfac" we found that this motif is reported as bound by Rox1p (YPR065W, a heme-dependent repressor of hypoxic genes—SGD information). Rox1p is involved in the regulation of the expression of proteins involved in oxygen-dependent pathways, such as respiration, heme, and sterols biosynthesis [47]. Thus, *SDH1* expression is downregulated in *rox1*Δ strain under aerobiosis [47]. This finding strengthens the well-described relationship between oxygen/heme metabolism and flavoproteins [18, 37]. A possible involvement of this transcriptional pathway in the scenario depicted by deletion of *FLX1* remains at the moment only speculative.

4. Discussion

This paper deals with the role exerted by the mitochondrial translocator Flx1p in the efficiency of ATP production, ROS homeostasis, H_2O_2 sensitivity, and chronological lifespan in *S. cerevisiae*, starting from the previous demonstrations of the derangements in specific mitochondrial flavoproteins which are crucial for mitochondrial bioenergetics, including Coq6p [28], Lpd1p, and Sdh1p [19, 25, 26]. The alteration in Sdh1p expression level in different carbon source is confirmed here (Figure 1) and it is accompanied by an alteration in flavin cofactor amount in galactose, but not in glycerol-grown cells (Table 1), in agreement with [19, 25], respectively. In the attempt to rationalize the reason for the carbon source dependence of the flavin level changes, we hypothesized different subcellular localization for Fad1p in response to carbon sources. Experiments are going on in our laboratory to evaluate this possibility.

The *flx1*Δ strain showed impaired succinate-dependent oxygen consumption [19]. Since no reduction in the oxygen consumption rate was found by using alternative substrates, such as NADH or glycerol 3-phosphate, possible defects in the ubiquinone or heme biosynthesis [28] could not be relevant for mitochondrial respiration, at least under this nonstress condition.

To evaluate the consequences of *FLX1* deletion on bioenergetics and cellular redox balance, the ATP content and ROS level (Figure 4) were compared in WT and *flx1*Δ strains, accompanied by measurements of the enzymatic activities of GR and SOD, enzymes involved in ROS detoxification (Figure 5). ATP shortage and ROS unbalance were observed in *flx1*Δ cells grown in glycerol up to the exponential growth phase, but not in cells grown in glycerol up to the stationary phase or in glucose. The findings are in agreement with the mitochondrial origin of these biochemical parameters. More importantly, the observation that lifespan was changed in glucose (not accompanied by a detectable ROS unbalance) allows us to propose that the lifespan shortage induced by the mitochondrial alteration due to absence of *FLX1* gene (correlated to flavoprotein impairment) may act also independently of ROS level increase.

The *flx1*Δ strain showed also H_2O_2 hypersensitivity (Figure 2). Since the same respiratory-deficient phenotype was previously observed in the yeast strain *sdh1*Δ and *sdh5*Δ strains [35], these results could be explained by the incapability of the *flx1*Δ strain to increase the amount of Sdh1p in response to oxidative stress.

In this paper, for the first time, a correlation between deletion of *FLX1* and altered chronological lifespan was reported (Figure 3). A similar phenotype was also previously demonstrated for *sdh5*Δ strains [35]. Thus, it seems quite clear that a correct biogenesis of mitochondrial flavoproteome, and in particular assembly of SDH, ensures a correct aging rate in yeast. This conclusion is also consistent with the recent observations made in another model organism, that is, *C. elegans*, in which the FAD forming enzyme FADS coded by *flad-1* gene was silenced [30, 48].

To understand the molecular mechanism by which FAD homeostasis derangement and flavoproteome level maintenance are correlated, a bioinformatic analysis was performed which revealed at least two *cis*-acting motifs which are located in the upstream region of genes encoding SDH1, other mitochondrial flavoproteins, and some members of

the machinery that maintain cellular FAD homeostasis. Therefore, the analysis describes the ability of yeast cells to implement under H_2O_2 stress condition and aging a strategy of gene expression coordinating flavin cofactor homeostasis with the biogenesis of a number of mitochondrial flavoenzymes involved in various aspects of metabolism ranging from oxidative phosphorylation to heme and ubiquinone biosynthesis. Even though no experimental evidence still exists to test the direct involvement of these *cis*-acting motifs in flavin-dependent cell defence and chronological lifespan, their involvement in the scenario depicted by deletion of *FLX1* appeared to be a fascinating purpose to be pursued. Experiments in this direction are at the moment going on in our laboratory.

In [19] we demonstrated that the early-onset change in apo-Sdh1p content observed in the *flx1*Δ strain appeared consistent with a posttranscriptional control exerted by Flx1p, as depicted in Figure 6. Thus, an inefficient translation of SDH1-mRNA is expected in *flx1*Δ strain due to the posttranscriptional control [19], even when putative mRNA levels may change in response to cell stress and/or aging. In this pathway the transcription factors Msn2/4p and Rox1p could play a crucial role.

Moreover, scheme in Figure 6 outlines how *FLX1* deletion, causing a change in expression level of Sdh1p, could activate a sort of retrograde cross-talk directed to nucleus. In our hypothesis besides ROS increase, a key molecule mediating nucleus-mitochondrion cross-talk should be the TCA cycle intermediate succinate, whose amount is expected to increase when altering the activity of SDH. The increased amount of succinate in turn may alter the activity of the α-ketoglutarate- and Fe(II)-depending dioxygenases among which there are (i) the JmjC-domain-containing demethylases [36], which may be causative of epigenetic events at the basis of precocious aging (for an exhaustive review on this point see [49]), and (ii) the prolyl hydroxylase (PDH), which may mimic a hypoxia condition in the cell [50].

5. Conclusions

Here we prove that in *S. cerevisiae* deletion of the mitochondrial translocator *FLX1* results in H_2O_2 hypersensitivity and altered chronological lifespan, which is associated with ATP shortage and ROS unbalance in nonfermentable carbon source. We propose that this yeast phenotype is correlated to a reduced ability to maintain an appropriate level of succinate dehydrogenase flavoprotein subunit [19], which in turn can either derange epigenetic regulation or mimic a hypoxic condition. Thus, *flx1*Δ strain provides a useful model system for studying human aging and degenerative pathologic condition associated with alteration in flavin homeostasis, which can be restored by Rf treatment [51, 52].

Abbreviations

Rf: Riboflavin
RFK: Riboflavin kinase
FADS: FAD synthase
SCM: *Saccharomyces cerevisiae* mitochondria
WT: Wild-type
FUM: Fumarase
SDH: Succinate dehydrogenase
GR: Glutathione reductase
SOD: Superoxide dismutase
DCF-DA: 2′-7′-Dichlorofluorescin diacetate
TCA cycle: Tricarboxylic acid cycle.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Calorie Restriction in Mammals and Simple Model Organisms

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Calorie restriction (CR), which usually refers to a 20–40% reduction in calorie intake, can effectively prolong lifespan preventing most age-associated diseases in several species. However, recent data from both human and nonhumans point to the ratio of macronutrients rather than the caloric intake as a major regulator of both lifespan and health-span. In addition, specific components of the diet have recently been identified as regulators of some age-associated intracellular signaling pathways in simple model systems. The comprehension of the mechanisms underpinning these findings is crucial since it may increase the beneficial effects of calorie restriction making it accessible to a broader population as well.

1. Introduction

The amount and quality of nutrient intake during lifetime are commonly regarded as main health-span regulators. Diet is in fact one of the lifestyle components capable of affecting the quality and the duration of life in a wide range of living organisms. The list of human pathologies, directly or indirectly affected by nutrients, is growing at a fast pace and includes major causes of mortality and morbidity such as cardiovascular diseases, diabetes, cancer, inflammation and neurodegeneration. Considering that population aging and disabilities are major concerns industrialized countries are going to face in next years, the possibility to increase the health-span with a consequent reduction of related healthcare costs is of general interest. It is therefore surprising that the most straightforward nutritional intervention to prolong lifespan is almost 80 years old but has had only limited application so far.

McCay published, in 1935, the first paper demonstrating that reduced intake of nutrients without malnutrition (Calorie Restriction, CR) could increase the mean as well as the maximum lifespan of rats [1]. The amount of calorie deprivation and the age at which the reduction in calorie intake starts influence the magnitude of the modification observed. Many other investigators, throughout the world, have confirmed this observation in all the other model systems tested. Yeasts, fruit flies, nematodes, fishes, hamsters, and several strains of mice as well as rats consistently increase their lifespan when the nutrient availability drops between 30% and 75% of the normal calorie supplementation, according to the species considered. Not only calorie restricted rodents lived longer than the ad libitum fed counterparts, but a significant part of them (about 30%) died without any apparent pathology, raising the striking possibility that aging is not necessarily tightly linked with costly pathologies.

However, accumulating data in both human and non-humans suggest that not only calorie restriction but also the balance of nutrients such as protein, amino acid, fat, mineral and phytochemicals may have an important role in regulating both lifespan and health-span. Protein restriction, methionine restriction, and alternate day fasting, without overall reduction in calorie intake, are some examples of interventions with outcome similar to that observed following a calorie restricted diet regimen. There is a growing interest in this field also because, while calorie restriction may encounter limited compliance on the population scale, dietary restriction promises to have broader application. Here we review the effects of calorie restriction in different model organisms and the molecular mechanisms by which dietary interventions may modulate lifespan in simple model organisms and mammals.

2. CR in Yeast

The simple genetic techniques, the low cost, the possibility to do multiple tests and the short lifespan have tempted the research community to use yeast to precisely dissect the molecular mechanisms involved in nutrient responses. Glucose depletion, the most common practice to mimic calorie restriction in yeast cultures, progressively increases the mean and maximum life span when glucose concentration drops from 2% up to 0.01% [2]. On the contrary, addition of glucose to starved yeasts modifies one-third of the yeast transcriptome modulating both PKA and Sch9p activities [3]. However, Ras/PKA pathway seems to have a predominant role in this response; in fact incubation of yeast cultures with limited glucose availability (0.5%) do not further extend the lifespan of long-lived *cdc25-10* mutants (the Ras2p exchange factor) [2] or the stress resistance of *ras2* deleted mutants [4]. Phosphorylation of Bcy1p, the PKA regulatory subunit, which results in increased Bcy1p inhibitory function, seems to be involved in glucose regulation of PKA activity as well [5].

Many studies have shown that also the availability of amino acids and nitrogen bases affect the lifespan of yeasts [6]. This is consistent with the observation that mutations in genes involved in amino acid biosynthesis or nitrogen uptake influence the life span [7]. Nitrogen limitation has been linked to ROS increase and promotes autophagy induction [8, 9] by the sphingolipids biosynthetic pathway [10, 11]. The relative concentration of each available amino acid also affects yeast longevity [7, 12–15] as well as the ratio of essential to nonessential amino acids [16]. It is not surprising that single amino acid addition or depletion is sufficient to affect yeast longevity. As observed in rodents, methionine restriction extends the lifespan even of glucose-depleted cultures (0.5% glucose), while a 6-fold excess of glutamic acid has a pro-longevity effect [16, 17]. It is interesting to note that the effect of these amino acids is not influenced by *SIR2* or *SOD2* deletions whereas Gcn2p, which is a modulator of amino acid deprivation response, was shown to impair lifespan extension induced by the depletion of these amino acids [16]. Finally, since methionine restriction does not extend the lifespan of strains lacking Sch9p, the latter protein must be involved in methionine response [16]. A role for methionine in growth promotion and autophagy inhibition has been identified; this process involves the methionine product S-adenosyl-methionine that acts as a methyl donor during these processes [18]. Very recently, the role of single amino acid in the regulation of longevity pathways and stress resistance has been clarified at the molecular level [4]. The study demonstrates the existence of at least two different amino acid response pathways: the first one transduces threonine and valine through TORC1 activation; the second one transduces serine activating PDK1 orthologs Pkhsp [4, 19]. Both pathways modify Sch9p, promoting its phosphorylation at specific amino acidic residues [4]. It is interesting to note that the restriction of each of these amino acids is capable to significantly increase both the lifespan and stress resistance of yeast cultures even in the presence of high glucose concentration, thus confirming that the effect of specific amino acids is not simply due to their role as energy source.

However, the observation that extreme starvation can double maximum life span when stationary phase cells are switched into water, not only in wild type, but also in *ras2sch9* double deleted mutants cells [20], supports the hypothesis that nutrients can trigger pathways alternative to the two identified so far. Consistent with this hypothesis *RIM15* deletion, which reverses life span extension associated with the deletion of *TOR1*, *RAS2*, or *SCH9*, has only a partial effect on the life span extension under extreme CR, an observation that suggests the existence of at least another yet to be discovered prolongevity mechanism [19].

Many metabolic changes are associated with CR and some of them must be responsible for the effect on life span observed. CR accelerates ethanol and neutral lipids catabolism as well as gluconeogenesis [21, 22]. It promotes trehalose and glycogen storage, while glycogen catabolism takes place at later stages. Neutral lipids, the storage molecules free fatty acids that diacylglycerol and ergosterol are derived from, regulate energy homeostasis as well as membrane stability. In addition, they can activate apoptosis and phospholipids biosynthesis, which in turn trigger multiple transduction networks. Therefore CR, promoting lipids consumption, may have synergistic effects with many processes [16]. ATP levels are high in calorie-restricted cells; in fact, CR enhances mitochondrial activity. ROS levels are higher in cells grown on 0.2% glucose media compared to those grown on 0.5%. It has been observed that shifting the metabolism toward respiration has the same effects on lifespan and transcriptome than CR [23, 24], and that this increased respiration fuels ROS production. Therefore, probably, the amount of ROS produced with lower glucose concentration is not sufficient to damage cellular components but at the same time activates stress-protecting processes like the increase in cytosolic and mitochondrial ROS scavenging proteins (mitohormesis) [22]. It has recently been reported that such ROS production may involve epigenetic silencing of subtelomeric chromatin [25, 26].

These and other findings support the hypothesis that nutrient composition and not simply calorie restriction might be the key regulator of lifespan [12]. In particular Sch9p the appears to be the major nutrient, especially amino acids, sensing factor [4].

3. CR in *Caenorhabditis elegans*

The nutrition of laboratory-based nematodes relies on bacteria, mainly *E. coli*, and calorie restriction metabolic state is usually obtained either diluting these bacteria or reducing worm eating capability as well as nutrient transportation pathways. In fact, a reduction of the bacterial density by 10-fold results in 60% increased lifespan [27], whereas higher bacterial dilution can extend the lifespan of worms up to 150% [28]. Mutations in genes that affect feeding mechanics (e.g., *eat-2* which causes a pharyngeal pumping defect) increase life span by about 30–60% [29]. Decreased activity of the gut

sodium dicarboxylate transporter NAC-3 or NAC-2 (high-affinity sodium-dicarboxylate cotransporters that accept a range of tricarboxylic acid-cycle intermediates with 4-5 carbon atoms), obtained using RNAi, produces an increase in life span varying from 15% to 19% [30, 31]. Like in other model organisms, inactivating the Ins/IGF-1 pathway significantly prolongs life span; but many experiments have shown that life span extension caused by dilution or absence of *E. coli* or eat-mutation does not completely overlap with this pathway [32, 33]. Indeed, *eat-2/daf-2* double mutant lived 20% longer than *daf-2* alone [33], and, while *daf-2* mutant lived 69% more than the wild type, the lifespan of the same mutants, grown in the absence of bacteria, increased by 274% compared to the wild type. Furthermore, *daf-2*, *daf-2/daf-12*, and *daf-16* mutants are still sensitive to nutrients as judged by SOD and catalase activities measurement [33].

Other evidences link CR to a better oxidative stress response in an insulin/IGF-1 independent way. CR response is mediated by thioredoxin 1 (trx-1) a protein that has oxidoreductase activity and is conserved in many animals. Trx-1 regulates aging and stress resistance; its deletion shortens adult lifespan and increases the sensitivity to paraquat-induced oxidative stress. It has also been discovered that *trx-1* deletion completely suppresses the lifespan increase of both the *eat-2* mutant and the dietary deprived regimen but only partially affects the lifespan of the *daf-2* mutant. At the same time *trx-1* overexpression failed to further extend the long lifespan of *eat-2* mutant. Finally, *trx-1* overexpression in the ciliated sensory neurons (ASJ) of wild-type animals extends adult lifespan but only under dietary deprivation [34].

Hansen and coworkers identified four genes extending the life span in *daf-16* (the FOXO ortholog) but not in *eat-2* mutants: *sams-1* (encoding S-adenosyl methionine synthetase), *rab-10* (encoding a Rab-like GTPase), *drr-1* (dietary restriction response, of unknown function), and *drr-2* (encoding a putative RNA-binding protein). Expression of all four genes is reduced in *eat-2* mutant suggesting these genes may be involved in longevity responses to CR [35].

More recently, Greer and Brunet proposed that specific pathways might respond to different dietary restriction regimens [36]. Low-energy sensing AMP-activated protein kinase AMPK/Aak-2 and the Forkhead transcription factor Foxo/Daf-16 are necessary for longevity induced by a CR regimen, while AMPK and Foxo are necessary for longevity induced by some but not all CR regimens.

However, the role of specific nutrients as regulators of longevity is consistent with other literature data. For example O'Rourke and coworkers have recently attributed a role to polyunsaturated fatty acids (PUFA) as regulators of longevity. The underpinning mechanism involves autophagy activation in response to PUFA supplementation [37], whereas malate and fumarate supplementation increase worm lifespan likely increasing respiration [38]. The role of amino acid supplementation has also been confirmed into this organism. Pep-2 deletion, which reduces the uptake of peptides, determines an increase in life span and stress tolerance and synergizes with reduced insulin signaling [39]. In addition, metformin, a common drug used to treat type II diabetes, increases the worm lifespan through alteration of folate and methionine metabolism [40] suggesting that amino acid metabolism may have a role different from simply being energy source also in this organism.

4. CR in *Drosophila melanogaster*

The idea that the effect of dietary restriction regimen on lifespan relies on the reduced intake of calories [41] was strengthened by whole-genome transcripts profile experiments in *Drosophila*. It has been observed that calorie restriction reverts the transcriptional changes normally observed during the aging process of flies and downregulates the expression of genes involved in cell growth, metabolism, and reproduction [42]. Recent experiments, however, challenged the idea that calorie restriction owes its beneficial effects on the reduced intake of calories suggesting that the depletion of specific nutrients, rather than the reduction of the overall energy intake, is responsible for the increased longevity observed in calorie restricted animals [43, 44].

A growing body of evidence points to the ratio between protein and carbohydrate (P : C), two major macronutrients, as the most important regulator of lifespan and reproduction in the fruit fly diet [45]. Higher ratio shortens lifespan whereas lower ones do the opposite [46]. A P : C ratio = 1/16 prolongs *Drosophila* lifespan, while higher protein content (P : C ratio 1/2) maximizes egg production and shortens the lifespan [47]. But it is hard to distinguish between life span extension due to protein restriction or to carbohydrate excess. Probably, both carbohydrate excess and protein depletion have crucial effects, since the longest lived flies are those which are subjected to a quite high C : P ratio and have an absolute high carbohydrate content. Because hydrolyzed yeast, the common protein source of the fruit fly, consists not only of proteins but also of vitamins, minerals, and carbohydrates, casein was used as an alternative protein source aiming at clarifying if other nutrient components could have a role in the regulation of longevity. Using this pure source of proteins overlapping results were obtained, thus confirming the major role of proteins in the aging process of this organism [48]. However, while low protein and high carbohydrate consumption maximizes lifespan a further increase of carbohydrate content does the opposite [49], probably because increasing the carbohydrate amount over a certain threshold could have other additional effects. Indeed, high carbohydrates consumption promotes obesity whereas increased protein intake suppresses adiposity, (Skorupa and coworkers). In addition, higher sucrose level enhanced the influence of proteins on lifespan, suggesting that both proteins and carbohydrates promote aging in a synergistic way [50].

One by one nutrient replenishment to otherwise severely calorie-restricted fruit fly demonstrates that only amino acids addition is effective in decreasing the lifespan and increasing fecundity, indicating that the amount of calories *per se* does not affect the lifespan [47]. In addition, essential and non-essential amino acids appear to have different roles in regulating longevity, the former being capable to negatively affect longevity, while the latter does not. The previously

demonstrated tight link between longevity and fecundity is weakening since methionine addition, one of the essential amino acids, has been demonstrated to be sufficient to increase fecundity at the same extent of full feeding but had no influence on lifespan, raising the possibility that the trade-off between fecundity and longevity is not a mandatory scenario [47]. The latter observation has been confirmed by *chico* mutants which have increased lifespan without impairment in oogenesis [51].

Other experiments suggest methionine restriction, rather than glucose depletion, as a prolongevity intervention supporting the hypothesis that the amount of macronutrients rather than the total amount of energy is the key to extend the lifespan [52]. A very recent report limits the efficacy of methionine restriction on longevity only when the overall amino acid supplementation is low thus suggesting the existence of cross talk mechanisms between the various amino acid response pathways [53].

At the molecular level, in spite of the many observations relating single diet components to life span, the underpinning molecular mechanisms have been poorly understood.

Insulin/IGF-like signaling pathway is central to control longevity in all living organisms and *Drosophila* makes no exception to this general rule. Mutations in Chico protein, the substrate of IGF1-receptor, extend fruit fly median lifespan by up to 48% in homozygotes and 36% in heterozygotes. Nevertheless some evidences suggest the existence of pathways alternative to insulin/IGF1 pathway by which nutrients can exert their action. Indeed, *chico* mutants continue to respond to CR suggesting that IIS and CR have only partially overlapping mechanisms [54]. In addition, *dFoxo* overexpression in thoracic and abdominal fat body increased longevity of 42% when flies were maintained on restricted diet, but had only a limited effect when flies were maintained on a high-yeast diet (high protein content) [55]. In addition, because null *dFoxo* mutants still respond to CR, *dFoxo*, even though its activity can modulate this response, is not the central mediator of diet response [56]. Regarding the role of ROS on lifespan, protein restricted diet, which increases lifespan in a Tor dependent manner, reduces oxidative stress resistance probably through the downregulation of antioxidant genes, while low sugar-high protein diet does the opposite; on the other hand life span increasing due to protein restriction with high sugar level is suppressed by Sod1 reduction suggesting high sugar level increases ROS production, while low protein level leads to reduced Tor signaling and promotes longevity [57].

Finally, inhibition of fatty acid synthesis or oxidation genes, in particular in the muscle tissue, inhibits lifespan extension upon DR [58].

5. CR in Mammals

Calorie restriction extends the lifespan of rodents [1]. This extension is accompanied with a lower incidence of most chronic diseases and results in a more youthful metabolic state [41, 59–61]. In addition, a significant proportion of the calorie-restricted rodents reaches very old age without any sign of disease [62]. CR protects from cancer [63, 64] although the underlying mechanism is not fully understood [65, 66].

One hypothesis is that energy restriction alters cell cycle regulation, inhibiting cell proliferation and increasing apoptosis [67]. On the contrary increased levels of IGF-1 reverse cancer prevention due to CR in mice probably stimulating cell proliferation and inhibiting apoptosis [68]. Notably, ames dwarf mice, which are deficient in IGF-1 production [69], postpone the incidence of neoplastic disease [70]. Other authors have reported that CR enhances the efficiency of DNA repair mechanisms therefore reducing the oxidative damage on DNA molecules [71, 72]; this is consistent with the overall upregulation of cellular and molecular defense systems during calorie restriction [73, 74].

CR attenuates aging-associated shrinkage of telomeres in many mouse tissues and reduces the incidence of tumors in mice that overexpress telomerase [75].

In male mice some of the effects of calorie restriction, such as improved physical performance, increased insulin sensitivity and reduced low-density lipoprotein as well as cholesterol levels are similar to those induced by metformin, a drug commonly used to treat type 2 diabetes. In fact, the reduction of both oxidative damage and chronic inflammation is associated with increased cellular protection [76] during metformin treatment.

The first clues that protein intake and amino acid composition could regulate mammalian longevity are derived from studies in mice and rodents. In these model systems CR causes a 40% increase in lifespan whereas protein restriction (PR) is capable of 20%, suggesting that about 50% of the CR effect on lifespan relies on PR. In addition, mtROS decreases during PR resulting in less DNA and protein oxidative damage [77].

It has been suggested that methionine restriction (MetR) could be responsible for the beneficial effects observed in protein-restricted animals [76, 78] since MetR mice have lower levels of serum IGF-1, insulin, glucose, and thyroid hormone and reduced visceral fat deposition. Levels of hormones such as leptin and adiponectin are increased in methionine-restricted animals with respect to controls and independently of overall energy restriction [79]. Furthermore, they show a delay in developing cataract and age-related changes in T-cell subclasses [80]. Conversely, methionine supplementation produces different damages on cardiovascular system [81]. Mouse has been useful also as Alzheimer's disease model. A study conducted at the Los Angeles Longevity Institute shows that periodic protein restriction cycles, without CR, in mice already displaying significant cognitive impairment and Alzheimer's disease (AD)-like pathology can promote changes in circulating growth factors (reduction of IGF-1 and increase of IGFBP-1) as well as decrease of tau phosphorylation in the hippocampus with a consequent reduction of the age-dependent impairment in cognitive performance [82].

Rats consuming no cysteine/cystine and low amount of methionine (which are the limiting amino acids for GSH synthesis) show an improvement in survival parameters and no decrease in GSH levels [83], suggesting the existence of a compensatory mechanism [84]. Likewise rats fed with 80% methionine reduction show an increase of free GSH in blood

according to a drop in oxidative stress biomarkers such as plasma 8-hydoxydeoxyguanosine and 8-isoprostane, even if the activities of GSH reductase and superoxide dismutase in liver do not change [85].

Some possible mechanisms have been proposed: MetR, like PR and CR, decreases the amount of mitochondrial complex I, III, and IV in different rat tissues; excess of methionine could impair gene expression because methionine is a methyl groups donor during DNA methylation [86, 87]; furthermore, proteins rich in methionine are less resistant to oxidative modification [88, 89]; MetR avoids the production of methionine cycle metabolites like S-adenosyl-methionine, S-adenosyl-homocysteine, and homocysteine that increase the risk for degenerative diseases associated with aging and free radicals [90, 91] and represents a thiol agent that enhance mtROS production [92].

Methionine is not the only proaging amino acid in mice; in fact tryptophan has been identified as another amino acid capable of influencing the lifespan of mice and one-third restriction of this amino acid extends maximum life span by 23% [87, 93, 94].

Also lipid metabolism seems to have an important role in aging and it could be influenced by diet. Long lived mammals have tiny amount of unsaturated fatty acids in their cellular membranes, since these macromolecules are the most susceptible to oxidative stress, their depletion result in increased cellular protection against lipid peroxidation [95–99]. Sphingolipids are a class of lipids important in cellular processes for their bioactive role. Two classes of them: ceramides and glycosphingolipids are implicated in many kidney pathologies [100, 101], and sphingolipid levels change during aging in brain and liver [102]. CR prevents the accumulation of the long chain glycosphingolipids hexosylceramide and lactosylceramide (which are elevated also in fibroblasts derived from elderly humans) in the kidneys of mice during aging [103, 104]; this could be one of the mechanisms that allow CR to maintain kidney function during aging [105–111].

However, the effect of calorie restriction on primates appears to be more controversial than it has been observed in other model organisms. Two different studies on the effect of calorie restriction on rhesus monkey are presently ongoing, one at the Wisconsin National Primate Research Center (WNPRC) and another one at the National Institute of Aging (NIA). Regarding the safety of long-term calorie restriction practice both studies agree that a 30% calorie restriction, even for long term, is both feasible and safe for primates. Regarding the effectiveness of this energy-based nutritional intervention on longevity, the two studies differ since WNBRC indicates a 50% decrease in the incidence of cancer, cardiovascular diseases, type 2 diabetes, and glucose intolerance [112, 113] for the calorie restricted versus the ad libitum fed rhesus monkeys.

On the contrary, the NIA study did not find a significant improvement in survival in the calorie-restricted group. The different method used in the two studies to calculate the nutritional demands has been claimed as a possible explanation of such a discrepancy [114]. This may have led to a minor calorie reduction in the NIA study or, as very recently suggested, even the control group may have been maintained under calorie restriction diet regimen thus masking the beneficial effects of calorie restriction [115]. In addition, diet composition is quite different in the two studies; WNBRC is similar to a typical western diet whereas NIA looks more like a Mediterranean/Japanese diet thus suggesting that diet composition could underpin the different conclusions of the two studies.

However, even if there are no definitive results about the effect on human lifespan upon CR, it has been reported that this intervention protects against many age-associated pathologies in particular cardiovascular diseases like atherosclerosis and hypertension and lowers risk factors for obesity, insulin resistance, and inflammation [116, 117]. Short-term studies indicate that CR in humans lowers fasting insulin, core body temperature, and DNA damage and possibly decreases cancers [62, 118].

It has also been demonstrated that humans with growth hormone receptor deficiency also exhibit a high reduction of IGF-1 and insulin level resulting in a highly reduced incidence of cancer and diabetes mortality [119]. Another study has reported a similar protection from cancer development in GHRD [120]. On the other hand, protein restriction or the depletion of specific amino acid, namely, methionine and tryptophan, from the diet has the potential to reduce the level of the circulating IGF-1 and to increase the level of the IGF-1 binding proteins [121–123]. Consequently, similar dietary regimen inhibits tumor growth in human xenograft models [124]. It is interesting to note that the observed association between protein restriction and lower free IGF-1 is independent from calorie intake and relies only on diet composition. Recent epidemiological and cellular studies have confirmed the association between protein consumption and IGF-1 level in humans [125]. In addition, the group consuming a high protein diet has a fourfold risk developing a cancer and a 75% enhanced risk of all causes of death. It must also be noted that the detrimental effect of the high protein diet on 65 and younger is counterbalanced by a milder positive effect on older people raising the possibility that aging should be considered as a dynamic process and that each phase of this process has different nutritional demands.

6. Conclusions

The usefulness of calorie restriction diet regimen has been demonstrated in all the species tested from the simplest unicellular eukaryotes to mammals. Even the discrepancies between the two primate studies have recently been solved confirming the efficacy of calorie restriction also in these long-lived species. However, recent research articles suggest that the effect of calorie restriction relies on the reduced uptake of single component of the diet and not on the overall energy uptake. Protein restriction and variations in the ratio between macronutrients demonstrated their efficacy in several model systems including humans. Methionine restriction efficacy has been confirmed in most species although the molecular mechanism is not yet fully understood.

In addition, the molecular mechanism underlying the effect of selected amino acids has recently been clarified in simple model organisms suggesting their role as longevity regulators. Human studies have also revealed that nutritional intervention may have different outcomes at different ages suggesting caution transferring the results obtained in model systems to human.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Peripheral Blood Mononuclear Cells as a Laboratory to Study Dementia in the Elderly

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The steady and dramatic increase in the incidence of Alzheimer's disease (AD) and the lack of effective treatments have stimulated the search for strategies to prevent or delay its onset and/or progression. Since the diagnosis of dementia requires a number of established features that are present when the disease is fully developed, but not always in the early stages, the need for a biological marker has proven to be urgent, in terms of both diagnosis and monitoring of AD. AD has been shown to affect peripheral blood mononuclear cells (PBMCs) that are a critical component of the immune system which provide defence against infection. Although studies are continuously supplying additional data that emphasize the central role of inflammation in AD, PBMCs have not been sufficiently investigated in this context. Delineating biochemical alterations in AD blood constituents may prove valuable in identifying accessible footprints that reflect degenerative processes within the Central Nervous System (CNS). In this review, we address the role of biomarkers in AD with a focus on the notion that PBMCs may serve as a peripheral laboratory to find molecular signatures that could aid in differential diagnosis with other forms of dementia and in monitoring of disease progression.

1. Introduction

The prevalence of dementia has increased globally, most noticeably in the ageing populations of the developed world. Alzheimer's disease (AD) is the most common type of dementia (60% of cases). Individuals affected by AD are 5.4 million in the United States and more than 33.9 million worldwide [1]. Moreover, AD prevalence is estimated to triple over the next 40 years and this will place a heavy burden on society and its health-care systems in terms of both economic costs and human impacts. The steady and dramatic increase in the incidence of AD and the lack of effective treatments have stimulated the search for strategies to prevent or delay its onset and/or progression.

There is general agreement that the epidemiological impact of dementia can be reduced by detecting and treating classical vascular risk factors since different studies provide evidence in favour of a coexistence of vascular and degenerative components in its pathogenesis [2].

In western countries vascular dementia (VD) is the second most common cause of dementia after AD among the elderly. A meta-analysis of the European studies on the incidence of dementia showed that VD constitutes 17.6% of all dementias [3]. In Europe and North America, AD is more common than VD in a 2 : 1 ratio; in contrast, in Japan and China VD accounts for almost 50% of all dementias. Also, the possibility of concomitant AD often confounds the relationship between cerebrovascular disease and VD.

AD is characterized by neurofibrillary tangles (NFT) and extracellular amyloid deposits. The former are composed of intraneuronal aggregates of hyperphosphorylated tau proteins and the latter are made of amyloid-beta (Aβ) peptides stemming from the sequential cleavage of a transmembrane precursor named amyloid precursor protein (APP).

Vascular pathology, namely arteriosclerosis, endothelial proliferation, and neovascularization, have been often found to be associated with NTF and amyloid plaques [4].

A number of autopsy studies have confirmed that among cases of dementia, AD-related pathology was associated with vascular lesions in nearly one-third of cases [5]. In addition, many epidemiological reports have demonstrated that the presence of vascular factors increases the risk of developing AD.

However, it is still a matter of controversy whether neurodegenerative AD-like disease and cerebrovascular lesions are coexisting but unrelated pathologies or whether they represent different results of synergistic pathogenic mechanisms.

It is hypothesized that an alteration of the neurovascular unit, which is the functional unit encompassing vascular cells, astrocytes, and perivascular neurons, is an early event in the pathogenesis of AD [6]. Dysfunction of the neurovascular unit results in impaired blood brain barrier (BBB) functions, dysregulation of cerebral blood flow, and impairment of Aβ clearance leading to an increase of oligomers and soluble Aβ forms [7]. Vascular oxidative stress and inflammation underlie many of these deleterious effects and are potential therapeutic targets even if, at present, there is no cure for AD and only a few medications aimed at slowing down memory deficits and clinical symptoms are available, with limited benefits.

Consequently, there is a pressing need for the identification of biomarkers that will aid in the differential diagnosis between AD and other forms of dementia and that will allow the detection of AD at early stages. Within the scenario of dementia, biomarker research may thus play an important role in paving the way towards novel diagnostic or therapeutic strategies.

2. Biomarkers

A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention [8, 9]. Many tests commonly used in clinical practice are biomarkers; biochemical tests provide soluble biomarkers, whereas physiological assessment and imaging measures provide anatomical and functional biomarkers. The majority have been identified on the basis of biological insight or underlying physiology. With increasing knowledge and practical experience, many of these tests have evolved into measurable end points in clinical research, applied as indicators of change, be it for the better or for the worse [10].

Biomarkers have also gained an important role in the field of clinical management and have established a close link with bedside medicine, by providing metrics of quality in medical care alongside meaningful costing. With effective translation into many clinical guidelines, biomarkers can facilitate the delivery of evidence-based medical care [11].

The evaluation of biomarkers may aid in the identification of diseases and may also allow correlations to be made with the progression or the susceptibility to a disease or a given treatment.

Yet, single biomarkers are unlikely to capture the complex process of human pathophysiology. Thus research may need to be geared towards sets of biomarkers, reflecting different, but intercalated, processes, which may enable a better assessment of disease states.

Biomarkers can be measured, for instance, in saliva, sweat, breath, blood/serum, urine, and cerebrospinal fluid (CSF). The fact that the collection of these biological fluids is significantly less invasive than biopsies is an important practical issue when studying neurodegenerative disorders like AD [12].

It has been reported that the sensitivity (definitely positive/(definitely positive + false negative)) of an "ideal" biomarker to detect AD should be at least 85%. Similarly, the specificity (definitely negative/(definitely negative + false positive)) in differentiating AD patients from controls and from patients with other forms of dementia should be at least 75% [10].

2.1. Biomarkers and Alzheimer. Despite the enormous advances in modern medicine, the diagnosis of AD remains largely clinical, based on patient history/examination, neuropsychological testing, and imaging techniques. Unfortunately the clinical diagnosis of AD suffers from limitations in that it only allows us to speak of probable or possible AD [13] with a 93% sensitivity and a 55% specificity. Furthermore, the diagnosis becomes far more difficult in the case of early or unusual presentations of the disease.

With the expansion of current knowledge on AD and the increasing availability of technical tools, there is an emerging need for the development of accurate biochemical and imaging tests that support the diagnosis [14, 15]. In this context the diagnostic criteria for AD proposed in 2007 [14] highlight the usefulness of genetic studies since they would enable a definite diagnosis to be made based on the demonstration of mutations in any of the three genes responsible for autosomal dominant disease: the gene for APP on chromosome 21, for presenilin 1 (on chromosome 14), and for presenilin 2 (on chromosome 1).

As to the more prevalent sporadic cases of AD, the need for a biological marker has proven to be urgent, for both the diagnosis and monitoring of the disease [16, 17].

Indeed, an ideal biomarker for AD would assist in the identification of preclinical disease, early disease diagnosis, staging of disease progression, and response to treatment [18]. Early diagnosis and identification of preclinical AD are particularly important issues considering the development of underlying neuropathology in those yet to display clinical symptoms. In particular, Mild Cognitive Impairment (MCI) is a well-described prodromal state of cognitive decline preceding dementia, with an accelerated conversion to AD estimated at 10–12% per year [19].

Over the past decade, biomarker discovery has become a rapidly advancing area of AD research.

With the development of structural, functional, and molecular techniques, neuroimaging is increasingly being employed as a diagnostic and prognostic tool in AD. Quantitative magnetic resonance imaging (MRI) is used to assess neurodegenerative changes in AD, which include reducing whole brain volume and cortical thickness associated with ventricular enlargement [20]. Early degeneration is also apparent in the hippocampus, entorhinal cortex, and medial temporal lobe of AD patients relative to controls [21]. In fact, MRI-determined hippocampal atrophy is currently the most established structural biomarker for AD and has been shown to predict the conversion from MCI to AD in about 80% of cases [22]. Additional neuroimaging techniques include functional MRI (fMRI), positron emission tomography (PET), and single-photon emission computed tomography (SPECT) which reveal abnormalities in brain synaptic activity, metabolism, and perfusion, respectively. Recent advances include the development of a number of amyloid-binding compounds, the most extensively studied being 11C-PIB (Pittsburgh Compound B, PIB). Several PET studies have detected an increased uptake of PIB in AD patients, which was found to correlate with the extent of cerebral atrophy and memory impairment [23]. Notably, longitudinal studies suggest that PIB imaging is able to predict the progression from normal cognition and MCI to symptomatic AD [24].

In view of the close relationship of the CSF with the brain and spinal cord, it is believed that the composition of this fluid may reflect biochemical changes in the CNS and thus provide information on the pathological changes occurring in neurodegenerative disorders [18]. Multiple studies have examined CSF for potential AD biomarkers. It is generally recognized that AD subjects compared to age-matched controls exhibit decreased CSF levels of soluble Aβ42 and increased CSF levels of total tau and phosphotau [25]. Importantly, diagnostic accuracy is improved by using the tau/Aβ42 ratio instead of either single biomarker, and this is reflected in an increase in sensitivity and specificity to 86% and 97%, respectively [26]. Moreover, this combination also appears to predict the subsequent development of AD in both cognitively normal and MCI patients [27, 28]. These findings have thus established CSF Aβ42 and tau as the most sensitive and specific diagnostic and predictive biomarkers for AD.

It should however be remarked that although neuroimaging and CSF biomarkers seem to be the most promising, they also carry some limitations. They are generally expensive to perform routinely and lumbar puncture is invasive and often unpleasant. Moreover a large variability exists in the literature as to CSF biomarker diagnostic accuracies and cut-offs, hampering or delaying their everyday application in the clinical setting [29, 30] and their potential use as indicators of prodromal AD.

Also, it is worth noting that the process of biomarker discovery involves many critical steps including study design, sample preparation, protein and peptide separation and identification, and bioinformatics and data integration issues that must be carefully controlled before achieving independent confirmation and validation.

Lastly, patient age is an important confounding factor in these biomarker studies and could explain some of the variability in published diagnostic accuracies and cut-offs [30]. Indeed a consistent number of subjects affected by Lewy Body Dementia (LBD), Frontotemporal Lobar Degeneration (FTLD), VD, and Corticobasal Degeneration (CBD) display an AD-like CSF biomarker profile [31].

3. Inflammation and Alzheimer

In the human brain several cell types are responsible for initiating and amplifying a specific inflammatory response. In AD signs of an inflammatory activation of microglia and astroglia are present both inside and outside amyloid deposits. Cell cultures and animal models suggest an interactive relationship between inflammatory response activation, reduced neuronal functioning, and amyloid deposition. Furthermore cells associated with extracellular plaques within AD brains can produce a variety of cytokines, chemokines, and other related proteins that influence plaque and tangle formation [32].

There is strong evidence that inflammation exacerbates neuronal loss [33, 34]. In fact, local inflammatory processes can exert a direct neurotoxicity, interfere with Aβ expression and metabolism, and maintain a chronic intracerebral acute phase protein secretion, which in turn favours formation of Aβ oligomers [35].

On the other hand, microglial activation leads to an increased brain expression of major histocompatibility complex type II and an increased secretion of proinflammatory cytokines and chemokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), and interleukin-8 (IL-8), as well as complement components and acute phase proteins [36].

A "cytokine cycle" has been proposed where [37] the anti-inflammatory cytokines (IL-4, IL-10, and IL-13) regulate Aβ-induced microglial/macrophage inflammatory responses and modify the microglial activity surrounding amyloid neuritic plaques [38]. Such cytokines can inhibit the induction of IL-1, TNF-α, and MCP-1 in differentiated human monocytes and, above all, IL-10 causes dose-dependent inhibition of the IL-6 secretion induced by Aβ in these cells and in murine microglia [37].

Accordingly, several reports make it appears that the risk of AD is substantially influenced by polymorphisms in the promoter region and other untranslated regions, of genes encoding inflammatory mediators. Alleles that favour an increased or decreased expression of inflammatory mediators are more frequent in patients with AD than in controls [39].

Aβ has also been shown to induce a phagocytic response in microglia, suggesting a neuroprotective defense mechanism [40]. This is, however, coupled to an increased release of signalling molecules and reactive oxygen and nitrogen species, which may further promote neuronal damage [41].

Despite these findings, clinical trials of nonsteroidal anti-inflammatory drugs (NSAIDs) in AD patients have been disappointing [42].

3.1. Peripheral Blood Mononuclear Cells. Nowadays it remains the need for a reliable, minimally invasive, and inexpensive biomarker for dementia, leading many to investigate peripheral blood. Blood collection is simple, inexpensive, and less invasive than lumbar puncture, allowing for repeated sampling. Approximately 500 mL of CSF is absorbed into the blood daily [43] and there is also evidence for blood-brain barrier (BBB) dysfunction in AD and other neurodegenerative disorders, which may enhance protein exchange between both fluids [44]. Consequently, the leakage of CNS metabolites into the peripheral system may reflect neurodegenerative disease status and could offer a suitable source of disease biomarkers.

AD also affects PBMCs that are defined as any blood cell with a round nucleus (i.e., lymphocytes, monocytes, or macrophages). These blood cells are a critical component of the immune system which provide defence against infection and respond to intruders. The lymphocyte population consists of CD4+ and CD8+ T cells, B cells and natural killer cells, CD14+ monocytes, basophils, neutrophils, eosinophils, and dendritic cells.

Although studies are continuously providing additional data that emphasize the central role of inflammation in AD, PBMCs have not been sufficiently investigated in this context. Indeed, only scant studies have used PBMCs to measure cytokine release, showing a significantly different production of these inflammatory components in AD and MCI subjects compared to controls [39, 45] (Figure 1) as well as a greater IL-1 and TNF-α production, associated with an increased risk of AD, in elderly controls [46].

Delineating biochemical alterations in AD blood constituents may enable the identification of accessible footprints that mirror degenerative processes within the CNS.

Moreover PBMCs could reflect inflammatory mechanisms in a more specific way compared with the serum/plasma, and PBMC-associated biomarkers could thus provide novel insight into the pathogenesis of AD.

In the following paragraphs we discuss the potential of PBMCs to serve as a peripheral laboratory to find molecular signatures in AD that could aid both in the differential diagnosis with other forms of dementia and in the monitoring of disease progression.

4. Peptidyl-prolyl *cis-/trans*-Isomerase Pin1 in PBMCs

The peptidyl-prolyl *cis-*/*trans*-isomerase Pin1 is a cytosolic protein that isomerizes the peptide bond of a phosphorylated serine or a phosphorylated threonine followed by a proline (pSer-/pThr-Pro). Pin1 catalyzes the *cis-*/*trans*-isomerization of its substrates, consequently potentiating the accessibility of the phosphate residue for further dephosphorylation by protein phosphatases such as the protein phosphatase PP2A. Alternatively, the binding of Pin1 to other highly phosphorylated substrates can repress their dephosphorylation by calcineurin. Therefore, through isomerization of pSer-/pThr-Pro, Pin1 regulates the function or degradation of a growing number of proteins including transcription factors and cytoskeletal, mitotic, or proapoptotic proteins [47].

Pin1 consists of 2 functional domains. The binding domain corresponds to the amino-terminal region consisting of a group IV WW domain (Trp-Trp domain) that specifically binds to pSer-/pThr-Pro motifs. The carboxyl-terminal region is the catalytic domain [48]. Pin1 substrate-binding and isomerase activity are regulated by phosphorylation. Indeed, 3 phosphorylation sites of Pin1 have been characterized. In particular, serine 16 is located in the WW domain and is phosphorylated by protein kinase A. Phosphorylation of Pin1 at serine 16 represses substrate recognition [49]. Pin1 has several additional putative phosphorylation sites (e.g., human Pin1 has 29 residues of serine or threonine and 3 tyrosines).

Phosphorylation of proteins is a key signalling mechanism in diverse of physiological and pathological processes. Pin1-catalysed conformational changes can have profound effects on phosphorylation signalling by regulating a spectrum of target activities. Interestingly, Pin1 deregulation is implicated in a number of conditions, notably ageing and age-related diseases, including cancer and AD. Pin1 is overexpressed in most human cancers; it activates numerous oncogenes or growth enhancers and also inactivates a large number of tumour suppressors or growth inhibitors. By contrast, ablation of Pin1 prevents cancer but eventually leads to premature ageing and neurodegeneration. Recent studies have demonstrated the reemergence within the brain of cell cycle proteins as patients progress from MCI into AD. Pin1 plays an important role in regulating the activity of key proteins, such as CDK5, GSK3-β, and PP2A, that are involved not only in the cell cycle but also in the phosphorylation state of Tau [50].

Indeed, Pin1 facilitates tau dephosphorylation [51] and regulates APP metabolism, thus providing additional support to the hypothesis that it has a neuroprotective function against AD [52–56].

It has been reported that Pin1 activity is repressed by oxidation in AD [52–58] and that Pin1 is localized to granular vesicles in AD and FTD but not to tau aggregates [55, 59–63].

It should be remarked that the expression and activity of Pin1 are tightly regulated at a transcriptional level and that a Pin1 gene polymorphism (−842G/C) has been found to be associated with reduced levels of Pin1 in blood cells and with an increased risk for AD in an Italian cohort [64].

Interestingly, a depletion of the soluble form of Pin1 has been described in neurons from AD subjects [57, 65] and differences in Pin 1 molecular and biochemical parameters have been reported in PBMCs from late-onset AD (LOAD) compared with control subjects [66].

In particular, in PBMCs from LOAD we observed a significant increase in Pin1 gene expression together with a significant decrease in gene promoter methylation [66].

This latter finding holds particular relevance, since so far little is known about epigenetic patterns in AD. Moreover, epigenetic mechanisms have already been proposed as markers of AD in PBMC-derived DNA [67] and it has been claimed that DNA methylation in peripheral cells could be taken as a model of epigenetic gene regulation in the brain [68].

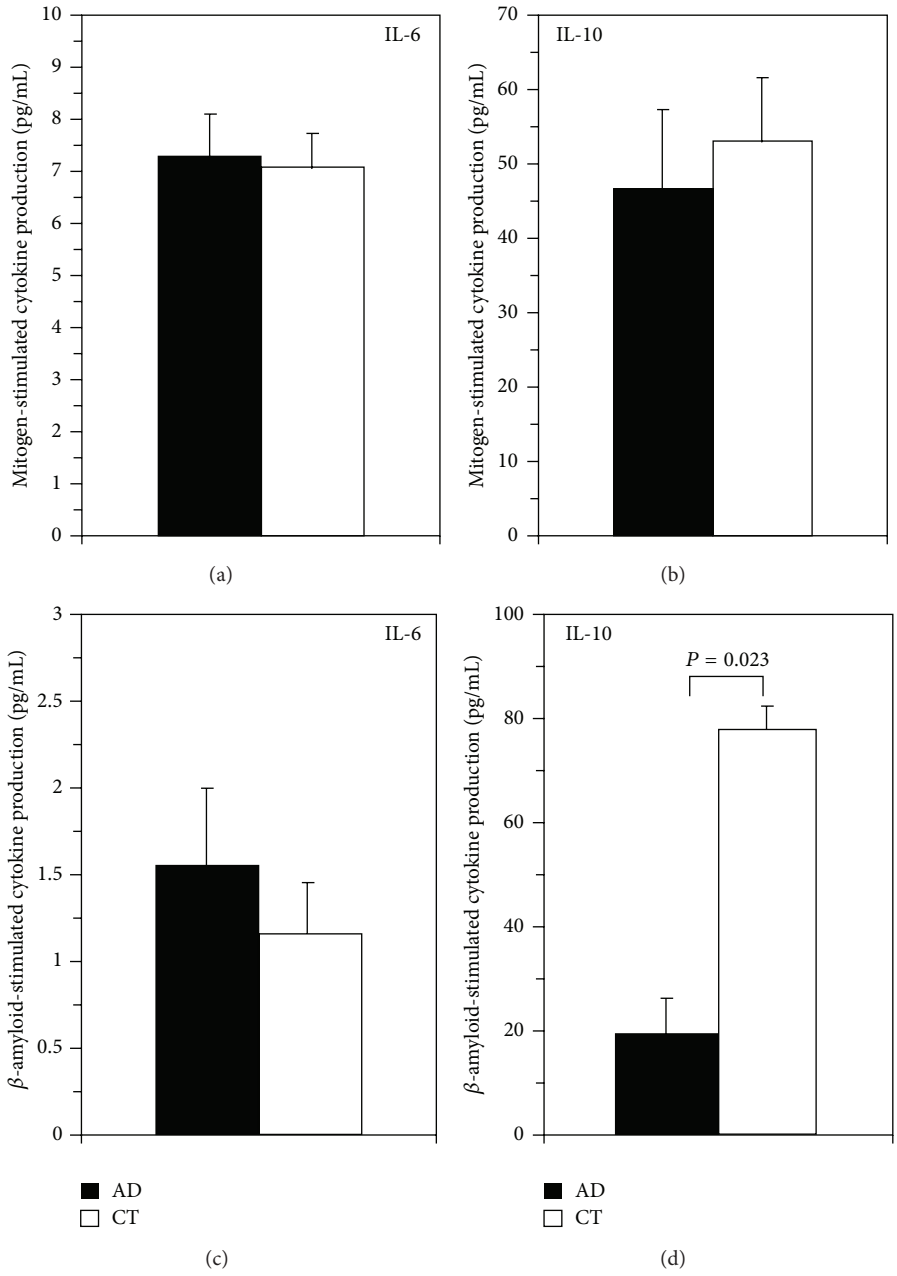

Figure 1: PBMCs of AD patients and age- and sex-matched controls (CT) were stimulated with a mitogen (LPS) and with a pool of three Aβ peptides (Aβ fragment 25–35; Aβ fragment 1–40; Aβ fragment 1–16). The production of IL-10 and IL-6 was measured by means of ELISA. There were no differences in mitogen-stimulated IL-6 and IL-10 production in AD and controls. In contrast, when Aβ-stimulated production of IL-6 and IL-10 was analysed, a marginally increased IL-6 production and a significantly decreased IL-10 generation were observed in AD patients compared to controls, suggesting an antigen-specific impairment in the production of these cytokines.

We have also shown that in LOAD subjects Ser^{16} phosphorylation levels of Pin1 were lower than in controls (Figure 2).

Phosphorylation of Pin1 must therefore be a key factor in regulating its localization, function, and metabolism and tau seems to be involved in controlling the balance between the phosphorylation/dephosphorylation of Pin1 in brain cellular lysate [69].

Moreover, Wang et al. [70] suggested that reduced Pin1 activity in the frontal cortex of patients with MCI contributes to the initial accumulation of hyperphosphorylated tau and is then followed, in a more advanced stage of the disease, by a compensatory upregulation of the Pin1 gene that counteracts Aβ plaque formation.

In particular, with regard to our finding of lower Ser^{16} phosphorylation levels of Pin1 in LOAD subjects relative to controls, different interpretations can be put forward: the presence in LOAD patients of rare gene variants of Pin1 that could influence its phosphorylation state [71] and the effects on Pin1 of a higher blood concentration of Aβ42 [72]. In

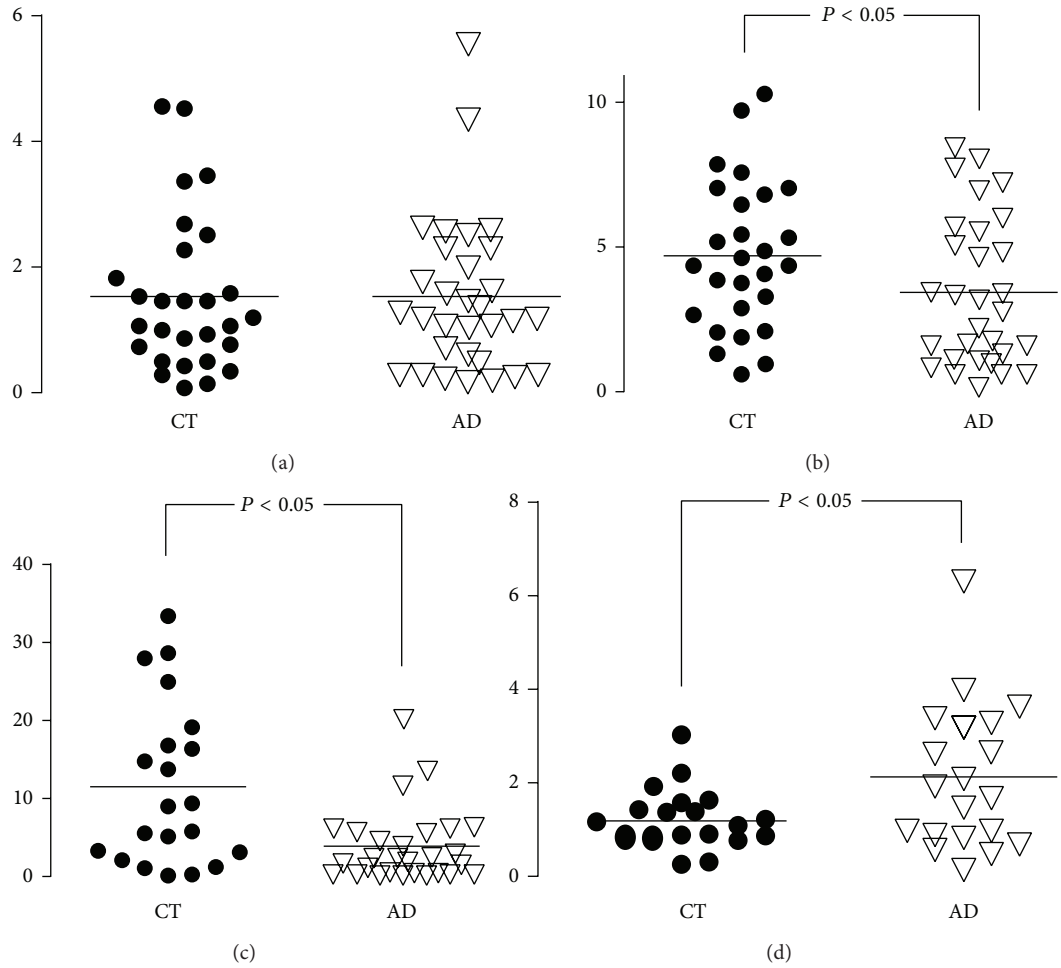

Figure 2: Scatter dot plots showing the distributions of molecular and biochemical parameters of PBMCs from controls (CT) and LOAD: activity (a), Ser^{16} phosphorylation (b), methylation (c), and gene expression (d). The lines across the boxes indicate median values.

keeping with the latter hypothesis, in rat hippocampal cells, treatment with Aβ42 oligomers has been shown to promote a transient Pin1 dephosphorylation on Ser^{16} associated with a decrease in phosphorylated $TauThr^{231}$ [73]. Whatever the specific explanation is, the modifications of Pin1 observed in LOAD subjects make it reasonable to suppose that Pin1 is involved in AD [74] and that epigenetic mechanisms (i.e., Pin1 promoter methylation) play a role in the disease. Therefore, alterations in easily accessible peripheral cells may prove to be valuable biomarkers in the diagnosis and follow-up of AD and, potentially, also of some tauopathies.

5. Epigenetics

Literally meaning "above the genome" the epigenome comprises the heritable changes in gene expression that occur in the absence of changes to the DNA sequence itself. Epigenetic mechanisms include chromatin folding and attachment to the nuclear matrix, packaging of DNA around nucleosomes, covalent modifications of histone tails, and DNA methylation in the whole genome and/or in specific gene promoters [75].

DNA methylation, in particular, consists of the transfer of a methyl group to position 5 of the cytosine pyrimidine ring of a cytosine guanine dinucleotide (CpG), which ultimately blocks the binding of transcription factors causing chromatin compaction and gene silencing [76].

The influence of regulatory small RNAs and microRNAs on gene transcription is also increasingly recognized as a key mechanism of epigenetic gene regulation [77].

Indeed, microRNAs (miRNAs), small regulatory RNAs in cells, probably constitute one of the most investigated extracellular RNAs in body fluidsand the levels of certain miRNAs in the circulation correlate well with different pathological conditions (i.e., miR-499 and miR-1 are associated with cardiovascular conditions) [78–81].

Epigenetic mechanisms are important in cell growth and differentiation [82]. Epigenetic change can be stochastic [83] or internally orchestrated as part of ageing [84]. Longitudinal changes in global and gene-specific DNA methylation clusters within families suggest there is a genetic control to methylation status [85].

Epigenetics is destined to change across the lifespan. In fact a loss of global DNA methylation and promoter

hypermethylation of several specific genes occurs during ageing.

In particular ageing-associated DNA hypermethylation occurs predominantly in genes involved in the development of anatomical structures, organs, and multicellular organisms and in the regulation of transcription.

This phenomenon may be considered a new aspect of the age remodeling process, a continuous adaptation of the body to the deteriorative changes occurring over time. However, it is not clear how relevant these epigenetic changes are in the context of functional changes in gene expression [86].

Inappropriate epigenetic changes are associated with many diseases including cancer [87], Rett syndrome [88], Beckwith-Wiedemann syndrome [89], and other imprinting disorders.

Environmental signals can trigger epigenetic responses and may be an important mechanism by which environmental exposures are associated with disease [90]. Furthermore, epigenetic mechanisms may play an important role in the developmental origins of adult health and disease by providing a mechanism underlying the latent effects of adverse fetal, infant, and childhood environments on late-life chronic disease [91–93].

5.1. Epigenetic Epidemiology and Alzheimer's Disease. Epigenetic epidemiology is the study of the effects of heritable epigenetic changes on the occurrence and distribution of diseases in populations [94]. This research includes both transgenerational and intraindividual cellular epigenetic inheritance systems. Epigenetic changes are associated not only with ageing [95, 96], but also with psychiatric outcomes [97, 98] and neurodegeneration [99].

Evidence for the role of epigenetics in AD pathogenesis can be found in human studies of various tissues, in animal models, and in cell cultures [100–102]. Global changes associated with AD have been observed in DNA methylation, miRNAs, and histone modifications.

Discordant data have been reported on specific epigenetic modifications of tau- and amyloid-processing genes. On the one hand an altered regulation was reported across multiple brain regions [103–105], and on the other hand no differences were seen in DNA methylation in regions associated with MAPT, PSEN1, and APP [103].

Human postmortem case-control studies have demonstrated global hypomethylation in the entorhinal cortex of AD subjects [106] and in the temporal neocortex of an AD monozygotic twin relative to the cognitively normal twin [107].

An AD case-control study in the postmortem human parietal lobe cortex has revealed a differential regulation of several miRNAs, including miR-204, miR-211, and miR-44691 [108].

Age-matched AD cases have been found to exhibit an increased neuronal global phosphorylation of histone 3 relative to controls, as determined by immunolabeling in the hippocampus, and such histone modification suggests mitotic activation [109].

In experiments where neuroblastoma cells were cultured under low folate and vitamin B12 conditions, PSEN1 and BACE1 were hypomethylated, mRNA expression of BACE1 and PSEN1 was significantly induced, and Aβ production was increased [110].

An additional study using human neuroblastoma cells and male rat brain tissue reports that APP mRNA expression is repressed by thyroid hormone (T3) sensitive histone modifications [111].

5.2. Epigenetics in PBMCs. The study of gene regulation in blood cells from living patients offers the possibility to go through the whole history of the disorder (including the response to pharmacological, metabolic, and environmental events) in a more comprehensive perspective, compared to postmortem studies which allow only pinpoint assessment.

It is important to note that PBMCs may also be a useful model of epigenetic gene regulation in the brain [68]. In fact, it has been shown that PBMCs share much of the nonsynaptic biochemical environment of neurons and contain the full complement of epigenetic enzymes found in most tissues, including neurons and peripheral nucleated cells [112, 113].

For instance, our group has investigated the role of DNA methylation in the PBMCS from LOAD subjects compared to controls and has demonstrated an altered Pin1 gene expression and promoter methylation [66], as detailed above, along with changes in fatty acid amide hydrolase (FAAH) and 5-lipoxygenases (5-LOX) genes (Faah EC 3.5.1.99 and Alox5 EC 1.13.11.34), proteins, and activity [114].

Also, by comparing DNA methylation of Faah and Alox5 promoters we found a direct correlation between these two genes [114, 115].

It has been shown that oxygenation of the FAAH substrates by lipoxygenase activity modulates recognition of these molecules by their protein targets [116], with potential implications for their biological activity [117].

These results might suggest that a parallel increase of FAAH and 5-LOX expression in AD patients could evoke a sustained inflammatory condition, thus reinforcing neurodegeneration [114, 115].

This finding in peripheral cells is in agreement with previous results in postmortem AD brains [118], where FAAH protein upregulation within plaques was suggested to lead to an increase in metabolites from endocannabinoid anandamide (AEA) degradation (such as arachidonic acid). Such metabolites could contribute to the inflammatory process occurring in AD.

Recently, there has been considerable interest in exploring the therapeutic potential of anti-inflammatory agents to prevent, treat, or slow down the progression of AD [119]. However, nonsteroidal anti-inflammatory drugs were found to be ineffective in AD patients with mild to moderate cognitive impairment [120], emphasizing the importance of an early diagnosis and therapy. Furthermore, pharmacological interventions based on chronic treatment with COX inhibitors, or treatment with anticytokine therapies, are not ideal for a long-term use, due to their gastrointestinal (COX1-selective inhibitors), cardiovascular (COX2-selective inhibitors), and immunosuppressive (anticytokine therapies) side effects [121].

Taken together, these lines of research converge towards the notion that novel anti-inflammatory targets may provide a safer strategy for the prevention and the treatment of AD. In such scenario PBMCs stand out as potential peripheral markers of disease within the CNS.

6. Adenosine A_{2A} Receptors in PBMCs

Nutritional alterations have been linked to the epigenetic modulation of some AD-related genes and seem to play a role in AD pathology. There is also evidence in favour of the epigenetic modulation of genes involved in the pathways activated by some dietary factors, both in ageing and disease, further supporting the involvement of epigenetic mechanisms in AD. A number of dietary elements have been reported to be either risk or protective factors for the development of AD. These include fat, fatty acids, antioxidants, fish, vitamins, alcohol, and, more recently, caffeine [122].

The neuroprotective effect of caffeine consumption on AD pathology is currently emerging from both basic and epidemiological studies [123]. *In vitro* and animal studies have provided convincing data on caffeine's neuroprotective effects against and in the presence of AD pathology [124–126]. Human studies have begun to demonstrate the presence of a similar neuroprotective role in the ageing and demented population.

However, due to the conflicting results from some longitudinal studies, there is no consensus about the role of caffeine in the onset of AD [124–128].

Caffeine is one of the most consumed psychoactive drugs and acts mostly by blocking adenosine receptors [129]. The purine ribonucleoside adenosine (Ado) is a naturally occurring metabolite that is ubiquitously distributed throughout the body as a metabolic intermediary. Intra- and extracellular Ado levels rise in response to physiological stimuli and with metabolic/energetic perturbations, inflammatory challenges, and tissue injury [130, 131].

The physiological responses to Ado take place as a result of the binding and activation of different transmembrane receptors: the high-affinity A_1 and A_{2A} (A_{2A}) receptors, the low-affinity A_{2B} receptor, or the low-abundance A_3 receptor [132].

These receptors are G-protein coupled receptors that regulate, in opposite directions, the second messenger cAMP; while A_1 is inhibitory Gi-coupled, A_{2A} is excitatory Gs-coupled, thereby decreasing and increasing cAMP levels, respectively [133]. The activation of these receptors is also able to modulate Ca^{2+} channels and the phospholipase C pathway. Through these actions and by modulating both the release and the uptake of different neurotransmitters, the balance between the activation of adenosine A_1 and A_{2A} receptors allows the fine tuning of synaptic transmission and plasticity in the hippocampus [134].

In particular A_{2A} is present in a wide variety of tissues, including the nervous system and the peripheral immune system, where they display different levels of expression: significant levels in neurons and peripheral cells (lymphocytes and neutrophils) and lower levels in glial cells [132]. The different levels of expression of A_{2A} in different tissues are consistent with the sophisticated, multifaceted neurochemical, and molecular effects of the Ado system. On the basis of *in vitro* [135, 136] and *in vivo* [137] studies, it has become clear that A_{2A}, through complex mechanisms which are still poorly understood [138–141], plays a critical role in the modulation of inflammatory reactions, influencing functional outcome in a broad spectrum of pathologies including neurodegeneration [142, 143].

Moreover it has been demonstrated that A_{2A} is able to prevent Aβ-induced synaptotoxicity in animal models and cell cultures [144] and it has been shown to control NMDA currents and glutamate outflow in the hippocampus [145, 146].

Contrasting data have been reported so far on the beneficial/detrimental effects of A_{2A} on brain cells [147]. The blockade of A_{2A} alleviates the long-term burden of brain disorders such as ischaemia, epilepsy, Parkinson's disease, or AD [138, 145, 148, 149]. On the other hand, agonists of A_{2A} can protect the CNS against several insults, including ischemia and excitotoxins [143, 150].

In the periphery A_{2A} contributes to coronary endothelial dilatation in mice [151], can inhibit endothelial apoptosis [152], and preserves vascular reactivity following hemorrhagic shock in rats [153].

We recently investigated A_{2A} gene expression and density in the PBMCs of patients with amnestic MCI (a-MCI), multiple cognitive domain MCI (mcd-MCI), outright AD, VD, and controls. We found that A_{2A} expression is upregulated in the peripheral cells of a-MCI but not AD subjects, supporting an involvement of the Ado system in the early stages of AD [154]. We also showed that A_{2A} expression is lower in the PBMCs of subjects with VD than AD, highlighting its possible relevance as a biomarker that may help differentiate two forms of dementia that are often closely associated (Figure 3).

Indeed, ROC analysis data showed that A_{2A} possesses a moderate degree of sensitivity and specificity for identifying VD patients from a heterogeneous group composed of VD and AD patients. The lower A_{2A} expression in VD compared to AD subjects seems to suggest a differential role of the Ado system in these dementias [155].

The methylation of the ADORA2A promoter gene, which codes for A_{2A}, may explain its different expression in these pathological conditions as well as in the ageing process, as already mentioned [156].

Moreover, A_{2A} represents the main Ado receptor involved in inflammation and it is interesting to note that also other inflammatory biomarkers are differently expressed in VD and AD subjects, such as alpha1-globulin and alpha2-globulin in the serum [157] and C3a and C4a in the CSF [158].

On the other hand the decreased A_{2A} levels in VD could be a defence mechanism since it has been demonstrated that pharmacologic inactivation or genetic deletion of $A_{2A}R$ reduces neuronal injury after global and focal cerebral ischemia in many animal models [149, 159, 160]. From our results it can be concluded that A_{2A} may play an important but differential role in both types of dementia: its upregulation in the preclinical stages of AD could counterbalance the existing inflammatory state and its downregulation in

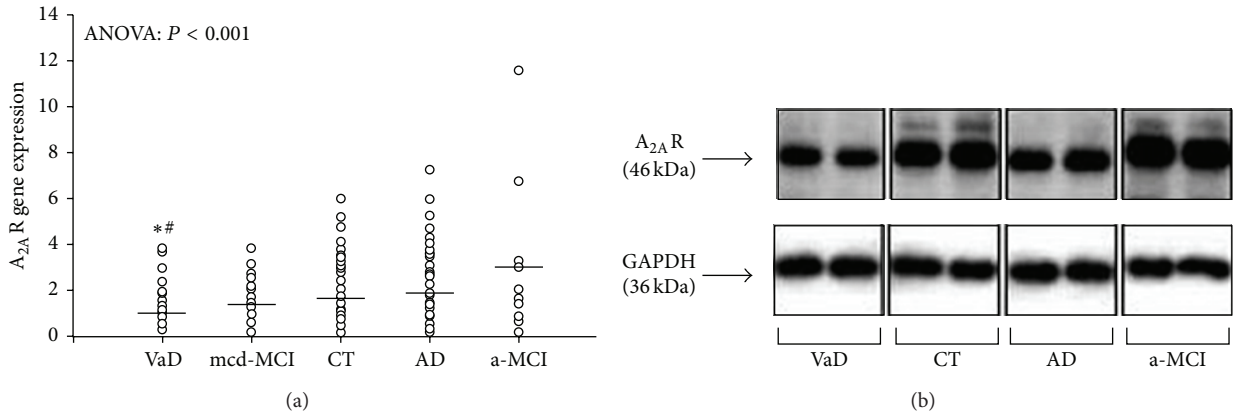

Figure 3: (a) Scatter plot of A_{2A} gene expression in PBMCs from VD, mcd-MCI, controls (CT), AD, and a-MCI subjects (the lines represent the mean value for each group). $^{*}P < 0.001$ versus AD; $^{\#}P < 0.05$ versus a-MCI. (b) Representative picture of the western blot analysis of the A_{2A} densities in PBMCs extracts, running in duplicate, from one subject from the VD, CT, AD, and a-MCI groups, respectively.

VD could reflect the effects of A_{2A} on the brain vasculature [161]. It can therefore be suggested that A_{2A} could serve as a biomarker in the differential diagnosis between VD and AD.

7. Conclusions

Peripheral cells and in particular PBMCs seem to directly participate to neurodegenerative processes. They play critical roles in immune response, metabolism, and communication with other cells as already pointed out many years ago [162]. Moreover, PBMCs have been shown to share much of the nonsynaptic biochemical environment of neurons and contain the full complement of epigenetic enzymes and machinery, which are found in both neurons and peripheral nucleated cells, as in most other tissues.

The substantial evidence in favour of the notion that PBMCs provide a window into the CNS holds particular relevance in neurodegenerative disorders in which, unlike most other diseases, the affected tissue is not directly accessible to evaluation. On a final note, it should be mentioned that the value of biochemical dysfunctions in PBMCs as mirrors of CNS defects appears to extend well beyond dementia.For instance, FAAH and other elements of the endocannabinoid system show alterations in the blood that resemble those within the CNS in a broad spectrum of clinical conditions including Parkinson's and Huntington's disease, multiple sclerosis, schizophrenia, minor depression, and headache [163].

Nowadays we do not know if PBMCs biomarkers are better or worse than the CSF biomarkers. Our study is only a preliminary study, instead multiple studies have examined CSF to establish sensitivity and specificity of CSF biomarkers. Moreover, despite these many studies, a large variability exists in the literature as to CSF biomarker diagnostic accuracies and cut-offs. As biomarker discovery in PBMCs is an ongoing process and PBMCs biomarkers are still immature, we need further analysis to enlarge design population.

It will be also of relevance the possibility to utilize intracellular biomarkers in specific blood cell subpopulations. In fact the differences in between subjects could also be due to different composition of their PBMCs pools, even if separating PBMCs into subpopulations would not permit the cell-cell interactions required for activation of lymphocytes.

Finally, we assume that the combination of peripheral and CSF markers may be utilized to categorize patients since early stages of dementia and to understand mechanisms underlying dementia.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Three Genetics (Nuclear DNA, Mitochondrial DNA, and Gut Microbiome) of Longevity in Humans Considered as Metaorganisms

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Usually the genetics of human longevity is restricted to the nuclear genome (nDNA). However it is well known that the nDNA interacts with a physically and functionally separated genome, the mitochondrial DNA (mtDNA) that, even if limited in length and number of genes encoded, plays a major role in the ageing process. The complex interplay between nDNA/mtDNA and the environment is most likely involved in phenomena such as ageing and longevity. To this scenario we have to add another level of complexity represented by the microbiota, that is, the whole set of bacteria present in the different part of our body with their whole set of genes. In particular, several studies investigated the role of gut microbiota (GM) modifications in ageing and longevity and an age-related GM signature was found. In this view, human being must be considered as "metaorganism" and a more holistic approach is necessary to grasp the complex dynamics of the interaction between the environment and nDNA-mtDNA-GM of the host during ageing. In this review, the relationship between the three genetics and human longevity is addressed to point out that a comprehensive view will allow the researchers to properly address the complex interactions that occur during human lifespan.

1. Introduction

Longevity is a complex trait whose genetics has been extensively studied since many years. Understanding the genetic makeup that predisposes to longevity is an urgent challenge owing to the explosion of the elder population in western as well as in emerging countries.

Usually the studies on the genetics of human longevity are restricted to the analysis of nuclear genome (nDNA). However, another essential genome, that is, the mitochondrial genome (mtDNA), is part of the genetic machinery of each cell. Despite its limited length, the mtDNA encodes for few genes that constitute a quantitatively relevant group because of the high copy number of mtDNA in each cell.

These two genomes do not work in the void and life/survival, as well as ageing and longevity, depends on their complex interaction with environment/lifestyle. To this scenario we have to add another level of genetic complexity represented by the microbiota, that is, the whole set of bacteria that live in different anatomical districts of our body with their whole set of genes (microbiome). Indeed, the most comprehensive view is to consider human being as a "metaorganism" resulting from the close relationship with symbiont microbial ecosystems. A particular attention has been recently devoted to the gut microbiome (GM). The GM probably represents the most adaptable genetic counterpart of the human metaorganisms, being extremely plastic in response to age-related physiological changes in diet and modification in lifestyle.

Thus, the result of the ageing process is defined by the sum of a number of factors both biological and nonbiological (environmental and stochastic). Therefore while the ageing research based on the study of animal models starts assuming the existence of major genes that determine longevity, in humans this assumption represents an oversimplification. The study of human model imposes a more holistic view of the genetics to grasp the complex dynamics of the interaction between the environment, stochasticity, and the three genetics of the host (nDNA, mtDNA, and GM).

The main aim of this review is to sum up the state-of-the-art of the knowledge of the three genetic components in human longevity to demonstrate that within this comprehensive view the genetics moves from a crystallized concept (genes are forever) to a much more flexible and dynamic perspective, in which the complex interaction between genetic makeup and environment across the long-lasting human lifespan is properly addressed.

2. The Nuclear Genome

The study of genetics of ageing in human being is tangled given the high complexity of the interaction between heterogeneous environmental factors and genetic variability across a long period of time. A strategy for disentangling this complexity is to focus on robust human models of longevity such as centenarians.

Centenarians are a model of successful ageing as in most cases they display medical histories free from most of the major age-related diseases, including cancer, dementia, diabetes, and cardiovascular diseases. Their ability to reach the extreme limit of human life—escaping, or largely postponing, age-associated pathologies—is the result of the combination of a well-preserved and efficient immune system, a good capability to cope with different stressors, an appropriate lifestyle, and a robust genetic background [1–5].

At the beginning, studies on longevity were conducted on lower organisms and animal models, providing evidences that longevity could be influenced by many conserved genetic variants with small effects [6]. Complying with this idea, several association studies have been conducted, comparing centenarians' genetic profile to that of younger cohorts. Several gene variants have been found to be associated with longevity, including *IL6* -174 C/G [7], *IL10* -1082 A/G [8, 9], *PON1* gene [10], *SOD2* 401nt T/C [11], the arginine to proline amino acid substitution in *TP53* gene at codon 72 [12–15], and insulin/IGF1 signal response pathway [16–21], but replication studies have provided contradictory results [22].

This can be due to different reasons that include the effect of population structure [23] and the lack of an appropriate control group [24]. The best control group for centenarians should include subjects born in the same birth cohort since younger subjects could be grown up in very different environmental and socioeconomic conditions. Furthermore, the recruitment of an elderly cohort until the age of 100 is demanding also from the "experimental" point of view. Considering that in Italy 1/4000 individuals is centenarian, to perform a study with 100 centenarians researchers should consider a pool of about 400000 people and a much larger cohort to perform a longitudinal study with a final cohort of a hundred of survivors over 100 years of age [25]. This calculation clearly explains the difficulties in carrying out longitudinal studies on human longevity that include the extreme decades of life.

Technological advances in the last 10 years have fostered the study of the genetics of complex traits by means of genome-wide approaches that allow the simultaneous analysis of thousands of genetic variants on large cohorts.

Many genome-wide association studies (GWASs) have been conducted assuming that long-lived individuals could share several common genetic variants that influence human lifespan.

Nebel et al. genotyped 1848 Germans, 763 individuals aged 94–110 years and 1085 controls aged 45–77 years, and replicated the results on an independent cohort of 1614 subjects. They reported a statistical significant association only for one SNP, rs4420638, that is located near *APOC1* and is in linkage with *APOE* [26].

Deelen et al. [27] compared 403 unrelated nonagenarians and 1670 younger controls from the Leiden Longevity Study cohort and tested the emerged SNPs on 4149 nonagenarians and 7582 controls from the Rotterdam Study, Leiden 85-plus study, and Danish 1905 cohort. Only rs2075650 is associated with survival to old age also in the replication stage (OR = 0.71, 95%CI = 0.65–0.77, and $P = 3.39 * 10^{-17}$). This SNP is located in *TOMM40* gene, close to *APOE* gene and, even if it displays only a moderate linkage with *APOE ε4*

determining variant, authors report an *APOE* dependent effect of rs2075650 on longevity.

Another GWAS was performed by Sebastiani et al. [28] considering initially 801 long-lived individuals (95–119 yrs) and 914 matched controls from the New England Centenarian Study. They identified 281 SNPs (about 130 genes) that they used to build a genetic risk model to distinguish cases (long-lived) from controls with 89% sensitivity/specificity. It is interesting to note that about 50% of the SNPs included in the model are located in intergenic regions, underlining that the regulatory machine plays a major role in longevity. This model has been tested in two independent cohorts (253 centenarians + 341 controls and 60 centenarians + 2863 controls) providing results that are less exciting than those obtained from the training datasets (60–58% and 78–61% sensitivity/specificity, resp.). Overfitting problems are common when high-dimensional data are managed and may play a role also in the relatively poor accuracy reported for the proposed model on the test datasets. Furthermore, authors reported that sensitivity of the predictor increases with increasing age, supporting the hypothesis that the influence of genetics on longevity gets stronger with increasing age. Moreover, this approach showed that different genetic signatures can be used to group centenarians into different "longevity classes" according to factors such as the prevalence or the age of onset of age-related diseases [29]. Nevertheless, some centenarians did not show a genetic signature of exceptional longevity, suggesting that exceptional longevity might be better explained by rare or private genetic variants. The 281 SNPs include rs2075650 in *TOMM40* gene but the contribution of this variant to the predictive power of the genetic risk model is poor. This is probably because of the low frequency of GG genotype that is almost absent in centenarians (frequency: 0.1%) and that makes the prediction of lifespan of AG and AA carriers uncertain without further genetic information [28]. It is relevant to note that this particular SNP (rs2075650) shows a cline in minor allele frequency from south to north Europe and this stresses the importance of stratifying models by ancestry.

Sebastiani and colleagues tested the 281 identified SNPs on 5 studies of centenarians from USA, Europe, and Japan and they found that 128 SNPs reached statistical significance, bringing out biological pathways deeply involved in exceptional longevity [30].

APOE is the only gene accounted as a "longevity determinant" by several independent GWASs. *APOEε* variants have been extensively analyzed and the frequency of *ε4* allele has been found decreased in long-lived subjects [31–33] but this result varies among different populations [34, 35]. Recently, Tan et al. proposed a method to identify the signature of mortality deceleration at late age. They estimated the effect of *APOEε4* variant in the Danish 1905 Birth Cohort and they found that relative risk of *ε4* allele does not increase linearly with age, supporting the idea that this allele exerts its deleterious effect also in the last decades of life [36].

Overall, GWASs have proved to be rather disappointing for identifying genetic determinants of complex traits, that is, longevity or many age-associated diseases, and established loci account only for a small proportion of trait heritability. The lack of robust results can be attributed to different reasons that include, among others, the need to conduct these studies on hardly available large cohorts, the phenotypic heterogeneity of longevity, the gene-environment interactions, and the failure to identify both low frequency variants with large effects and rare variants. One point to take into account is that each cohort in GWASs could be characterized by population-specific genetic makeup. This phenomenon has been suggested by De Benedictis and Franceschi [37] from the observation that the demographic males/females ratio among centenarians is consistently different depending on the geographic origin of individuals. The authors sustain that this observation provides evidence of the existence of a genetics characteristic of each population and it makes less and less impressive the results of GWASs, which include different populations, even more difficult to interpret. The advent of next-generation sequencing (NGS) has renewed interest and hopes of the researchers in the study of the genetics of longevity, as this technology allows a previously unattainable systematic discovery of low frequency variants in thousands of samples. In the context of longevity, NGS has been applied to assess whether ageing is accompanied by an accelerated accumulation of somatic DNA mutations that affects the primary structure of proteins, ultimately compromising organismal functions [38]. Ye et al. performed whole genome sequencing of two pairs of monozygotic twins aged 40 and 100 years old, by using two independent NGS platforms and validating potentially discordant single-base substitutions by Sanger, Roche 454, and Ion Torrent sequencing. Authors found that NGS can detect somatic single nucleotide substitutions and that getting centenarian is not accompanied by an increase in the number of detectable somatic mutations. Furthermore, the authors highlighted that the low background somatic variation reported within twin pairs is advantageous if discordant twins are considered for the identification of disease-related mutations.

Studies carried out so far were unable to discriminate between two basic assumptions, that is, whether the ability to reach the extreme limits of human life is due to the presence of polymorphisms in "longevity genes" that promotes the achievement of old age or to the absence of harmful variants [39]. The study of extreme phenotypes has proved particularly useful to clarify these clinical issues [40]. In the field of longevity, this approach should include centenarians and individuals severely affected by age-associated diseases. Recently, interesting results were reported using this method to study type 2 diabetes (T2D). 1349 Italians, including 562 T2D patients, 558 unrelated controls, and 229 centenarians, were genotyped for 31 SNPs mapping within or nearby genes involved in T2D. rs7903146 in *TCF7L2* gene showed a progressive increase in the frequency of risk genotype (TT) from centenarians to diabetic patients who developed one or more complications and the strongest genotypic association was detected when diabetic patients were compared to centenarians ($P = 9.066 * 10^{-7}$). The use of centenarians in this kind of studies has proved to be useful to evaluate the biological relevance of genetic variants emerging from GWASs. Authors speculate that if a SNP is considered a statistically significant but "weak" risk factor for the disease

and it is present at similar frequency in centenarians and in patients, its biological relevance can be assumed as negligible. On the contrary, if the frequency of a genetic risk variant is much higher in patients than in centenarians, it is likely that this SNP plays a consistent biological role in the pathogenesis of the disease under study [41]. It is also interesting to note that the T risk allele and the TT risk genotype were present in few centenarians, suggesting that they were not able to foster the T2D phenotype, likely because of being counteracted by healthy lifestyle.

In addition to centenarians, a widely used model to investigate longevity consists of long-lived families. Indeed, it was observed that siblings of centenarians have an increased relative risk of reaching 100 years, sustaining the existence of a heritable component of human longevity [42–44]. This heritable component has been estimated to account for about 25–32% of the observed variation in human population, with an increasing influence after 60 years of age [42, 45, 46]. To understand whether this heritable component results mostly from the genetics or from the familial environment, Schoenmaker et al. [47] proposed to consider spouses of long-lived subjects as an additional control group. They included in their analysis 100 families with at least two long-living siblings and they evaluated standardised mortality ratios, finding a survival benefit for all siblings of the long-living participants, for their parents, and for their offspring but not for their spouses. This result allowed authors to sustain that the families considered are genetically enriched for extreme survival and that it is unlikely that environmental factors play a major causative role in familial longevity. The environment effect on longevity was studied also by Montesanto et al. [48] on 202 long-lived families from Calabria. Authors compared the survival functions of nonagenarians' siblings to those of their spouses to estimate the genetic component of longevity, minimizing the effects of environmental factors. Authors confirmed that both parents and siblings of the nonagenarians had a significant survival benefit. They also reported for the first time a gender-effect restricted to males. Indeed, only male siblings showed a substantial survival advantage and the presence of a male nonagenarian in a family significantly decreased the mortality rate throughout lifetime for all the siblings, suggesting that genetic factors in males strongly affect the possibility of becoming long-lived. Family studies of exceptional longevity were performed also to identify rare genetic variants that cannot be discovered with population-based studies. One of the largest European projects aimed at identifying genes involved in healthy ageing, the Genetics of Healthy Ageing (GEHA) project, was focused on a sophisticated familial model of longevity, that is, nonagenarian sib pairs, that is, two or more siblings aged 90 years or older [49, 50]. During GEHA project, 2535 families comprising 5319 nonagenarian siblings were recruited from 15 regions in 11 European countries. In addition, younger persons aged 50–75 years were included as unrelated controls but coming from the same geographical area as the sib pairs. The comprehensive phenotype description and an estimation of the survival rate of a subset of GEHA subjects were performed on this exceptional cohort to identify survival predictors [51]. In particular, some predictors of longevity, including sociodemographic, physiological, clinical, and haematochemical parameters, were examined at baseline and 6 years after the recruitment in 1160 Italian GEHA 90+ siblings. It was observed that better specific cognitive and functional parameters (SMMSE, ADL scale, and hand grip strength), self-reported health, and clinical parameters (haemoglobin, creatinine, and total cholesterol) in 90+ sib-ships were also effective survival predictors. Cevenini et al. also suggest that this combination of the parameters identified in the GEHA model of healthy ageing is influenced by familiarity/genetics [51].

A genome-wide linkage analysis on 2118 European nonagenarian full sibships of the GEHA project was performed to identify chromosomal regions involved in longevity [52]. By using Illumina HumanLinkage12 Genotyping BeadChip, four regions (14q11.2, 17q12-q22, 19p13.3-p13.11, and 19q13.11-q13.32) were identified, together with three loci that were linked to longevity in a sex-specific manner (8p11.21-q13.1 (men), 15q12-q14 (women), and 19q13.33-q13.41 (women)). A GWAS performed in the same GEHA 90+ sibships and controls showed that only rs4420638 was significantly associated with longevity. As expected by results obtained in other genome-wide studies, this SNP tags the linkage disequilibrium block harboring the *TOMM40*, *APOE*, and *APOC1* genes. The analysis of *APOEε* variants in GEHA nonagenarians siblings showed that *ε4* allele frequency was significantly lower than that reported for the geographically matched younger controls (6.8% versus 12.7%). In agreement with this finding, *ε4* allele carriers have about 50% lower chance to become nonagenarians than the non-*ε4* carriers (OR = 0.48, 95%CI = 0.42–0.55).

3. The Mitochondrial Genome

Several theories on ageing process and longevity posed mitochondria in a central position. Mitochondria produce the cellular energy through oxidative phosphorylation (OXPHOS) and many metabolic pathways are located in these organelles as well as the pathway controlling apoptosis. The two main mechanisms that link mitochondria to ageing are the mutagenesis of mitochondrial DNA (mtDNA) and the production of reactive oxygen species (ROS). The relative contribution of these two mechanisms and their interplay in the ageing process are still matter of debate, but a detailed analysis of the history of these theories is out of the scope of this review and it is well described elsewhere [53]. However, the more recent hypothesis states that ROS generation is not *per se* a cause of ageing, but rather a consequence of the age-dependent accumulation of mtDNA damage. Indeed, it is well known that mtDNA mutations increase with age, and recent findings show that this increment is likely due to errors in replication machinery or to unrepaired damage, placing ROS mutagenic effect in the background. Two studies support this hypothesis: (i) a study by Kennedy et al. [54] showed that in mice the mtDNA damage during ageing is not characterized by an accumulation of transvertions (i.e., the type of mutations

caused by ROS) but by a higher prevalence of transition; (ii) the study of Trifunovic et al. [55] showed that defects in the proofreading function of gamma polymerase (polγ) lead to a high accumulation of mtDNA mutations and to a consequent premature ageing of mice that carry these defects.

3.1. Longevity and mtDNA Somatic and Inherited Variability. Recent GWASs on age-related diseases [56–59] have identified some nuclear loci implicated in mitochondrial bioenergetics (*PPARγ, PGC-1α,* and *UCPs*) providing further support to the hypothesis that mitochondria play a central role in the ageing process [60–62]. In humans, the processes influenced by mitochondrial activity and their effects on degenerative diseases and ageing appear to be modulated by mtDNA common variants as addressed by many studies [60, 63–73].

Recently, the association between recurrent or sporadic mtDNA mutations and longevity has been highlighted by the results of the GEHA project. In this project the control region of 3000 samples and the complete mtDNA of 1292 samples were sequenced including 650 ultranonagenarians (90+) and a comparable number of controls, enrolled in Denmark, Finland, Southern Italy, and Greece [74]. A haplogroup classification and a specific analysis for evaluating the burden of nonsynonymous mutations in different mtDNA regions were performed [75]. The results showed that the number of nonsynonymous mutations in mtDNA genes coding for subunits of OXPHOS Complexes I, III, and V is different between 90+ subjects and controls. In particular, the presence of mutations on complex I may be beneficial for longevity, while the cooccurrence of mutations on both complexes I and III or on both I and V might be detrimental to attain longevity. As haplogroup *J* is characterized by mutations in complex I genes, this result might explain previous contrasting findings emerging from association studies on *J* haplogroup and longevity [65, 67, 70, 76–78]. This result points out the need of complete sequencing of mtDNA in this type of genetic studies and the inadequacy of studies based on haplogroup classification. Intriguingly, the analysis of mtDNA sequences in 90+ and controls has also shown that many mtDNA mutations associated with a variety of mitochondrial and degenerative diseases are as frequent in 90+ as in younger controls (e.g., 4336T > C mutation in the tRNAGln in Alzheimer's disease), supporting the idea that the effect of mtDNA mutations is highly influenced by the individual-specific genetic background (the combination of nuclear and mitochondrial genome variants), as well as by stochastic events [68, 79, 80]. In conclusion, a major result of the GEHA study is that the interaction between mutations concomitantly occurring on different mtDNA genes can affect human longevity [74]. Moreover, such an effect of mtDNA variability on longevity seems to be mainly due to the cooccurrence of rare, private mutations, which are not detected by haplogroup analysis.

The complex relationship between mitochondrial genetics and longevity has been further puzzled when somatic mtDNA variability is considered. Indeed in one cell many mitochondrial genomes exist and the cooccurrence of mutated and wild type copies of mtDNAs is named heteroplasmy. Many studies [81–83] showed an accumulation of heteroplasmy during ageing in different tissues (such as muscle, brain, etc.). Recent findings demonstrated that even low-frequency heteroplasmic mutations can be inherited from the mother [84] suggesting a potential role of these variants as primer to potentiate the effect of somatic mutations that accumulate during ageing [85]. This component of mtDNA variability is difficult to study, as heteroplasmy pattern observed in adulthood is a mixture of both inherited and somatic (acquired) mtDNA mutations. Moreover, the proportion of mutated mtDNA can vary according to the tissues and cells considered. Only few studies were able to establish a link between heteroplasmy and healthy ageing and longevity [86, 87] and even less studies addressed the role that these accumulations may have in promoting longevity. A high incidence of the C150T transition in centenarians' leukocytes was observed and a remodeling event of mtDNA replication origin associated with this mutation was hypothesized. These studies also indicate that the level of heteroplasmy at position 150 is similar between relatives and correlates in parent-offspring pairs, thus suggesting a genetic influence. However previous technologies (such as DHPLC, pyrosequencing) have not allowed a high-resolution analysis of mtDNA variants occurring at a very low frequency. The advent of NGS technologies, with their capability for very high coverage, allows the analysis of mtDNA mutations at very low frequency with high accuracy [88]. This new technology applied to powerful models of longevity, such as centenarians and their offsprings, is expected to answer some of the open questions in this hot topic.

4. Gut Microbiome

In humans most of the microorganisms reside in the intestinal tract and their role is so central that all these microbes are considered as an additional organ, characterized by its own genome [89]. The gut microbial community is predominantly bacterial and the most characterized part inhabits the distal colon where two bacterial phyla—the Firmicutes and the Bacteroidetes—constitute more than 90% of the total community [90]. The relationship between human GM and the host is highly plastic, with the potential to readily adapt to changes in diet, life style, and geography, as well as to the different host ages, defining a process which is fundamental to maintain host health and homeostasis [91]. This plasticity has been recently highlighted by David and colleagues [92] that reported the effects on GM composition of different diets, that is, one based on animal products and another one based on plant products. Authors observed that the short-term consumption of these two kinds of diet alters microbial community structure and bypasses the interindividual differences in the microbial gene expression.

The composition of the microbiota strongly impacts on the host health. Indeed, several studies report that the dysbiosis of the microbiome occurs in different chronic conditions, including obesity, inflammatory bowel diseases, and diabetes [93–96].

Regardless of whether it is a cause or a consequence of diseases, the GM can actively contribute to diseases consolidation. Indeed, several studies on disorders show that a pathologic phenotype can be transmitted from a diseased animal to a healthy recipient through the graft of the microbiota and this also applies to complex diseases where host genetics and environmental factors play a role [97, 98]. The first study that linked microbiota to human ageing dates back to 1908 when Metchnikoff postulated that ageing can be caused by gut microbiota dysbiosis [99].

The age-related changes in gut microbiota are very controversial and results from recent studies are not always concordant. In fact the study by Biagi et al. [100], performed on Italian subjects, showed that *Bacteroidetes* proportion remains unchanged in elderly, whereas in a study on Irish elderly [101] *Bacteroidetes* strikingly increase and overcome the *Firmicutes*. The influence of different cultural habits and lifestyle in the two populations considered is likely the major force underlying the observed differences [102], even if a "study effect" cannot be excluded [103]. This observation is obviously complicated by a high level of interindividual variability in the composition of gut microbiota. However, studies agree that old individuals are characterized by a lower GM diversity [100] and by an enrichment in "pathobionts," that is, proinflammatory bacteria that usually are present at low concentration in healthy individuals [104, 105]. Interestingly, in centenarians this GM profile has been associated with inflammaging, a condition that is characterized by a high level of blood inflammatory markers. A shift in microbial composition towards an age-related pattern was observed in inflammatory disorders [106], supporting the proinflammatory nature of an aged-type microbiota. However, the causes and the effects of a direct association between microbiota modifications and immunosenescence and inflammaging are still unclear [107]. Recently, an alteration of GM functional profile was also observed in extreme ageing. The age-related trajectory of the human gut microbiome was shown to be characterized by loss of genes for short chain fatty acid production—well-known anti-inflammatory GM metabolites [108]—and an overall decrease in the saccharolytic potential, while proteolytic functions were more abundant in centenarians'GM than in the intestinal metagenome of younger adults. Treatments that include prebiotics/probiotics seem to produce beneficial effects on healthy ageing but an extensive review of these concepts is outside the purpose of this review and they are extensively described elsewhere [107].

5. The Remodeling Theory of Ageing

Human physiology undergoes profound changes from birth to old age. In the elderly, these changes are mainly the result of adaptive strategies at the molecular and cellular levels to compensate the damage accumulation that occurs over time. A major contribution to these changes is played by the cell microenvironment. A growing body of evidence supports the idea that the systemic environment is the repository of danger signal products and of the whole garbage that the senescent cells and the impaired tissues produce. Several studies provide evidence of rejuvenation of aged cells by exposure to a young systemic environment, suggesting that it is the microenvironment to which the cells are exposed to that causes/maintains the old phenotype [109–112]. Therefore, the assumption that a given allele has the same biological effect(s) in the body (systemic body environment) of young, adult, old, and very old people is simplistic. Within this perspective, the remodeling theory was postulated. This theory poses that the same allele has different effects on the probability of survival according to age-related physiological conditions (Figure 1), by modifying gene expression and, as a consequence, the composition of cell microenvironment which in turn modifies again gene expression in an amplifying vicious circle, which eventually is responsible for the systemic age-related decline. Indeed, as described above, the individual gene-environment interaction changes with age and a significant contribution to the remodeling could be provided by the three genomes interactions.

6. Mitochondrial-Nuclear Crosstalk

More than 90% of the factors required for mitochondrial function are encoded by the nuclear genome. Coevolution of mtDNA and nDNA is a crucial process that preserves biological functionality and cell activities [113]. This is demonstrated by Kenyon and Moraes who mark the interaction between nDNA and mtDNA, focusing on species-specific compatibility between these genomes [114]. In particular, using xenomitochondrial cybrids they showed that only our closest relatives, that is, chimpanzee and gorilla, were able to restore oxidative phosphorylation when placed in a human nuclear background, whereas distant relatives with a mtDNA that is more different from an evolutionary point of view, that is, orangutan and lemur, were not. It is also relevant to note that the time that leads to incompatibility between nuclear and mitochondrial genomes could be shorter than the divergence of species. Indeed, alterations in this equilibrium could alter cell functionality, leading to an increased disease susceptibility or pathological processes [115]. mtDNA is fundamental for energy production in the cell, and both nuclear and mitochondrial genes are needed to assemble the mitochondrial translation machinery and to carry out major processes, such as OXPHOS.

Similar evidences come from studies on *Drosophila* showing that epistatic interactions can cause incompatibilities that decrease fitness [116]. In particular, in *Drosophila,* the cooccurrence of naturally occurring mutations in a mitochondrial tRNA and in a nuclear-encoded tRNA synthetase that show little effect on their own severely compromises development and reproduction. The effect of this interaction affects mitochondrial functionality, supporting the hypothesis that the variable penetrance of mitochondrial DNA mutation is likely affected by mitochondrial-nuclear interactions [116].

6.1. From the Nucleus to the Mitochondria and Back. Interactions between nDNA and mtDNA products are possible at many levels such as protein-protein interactions in

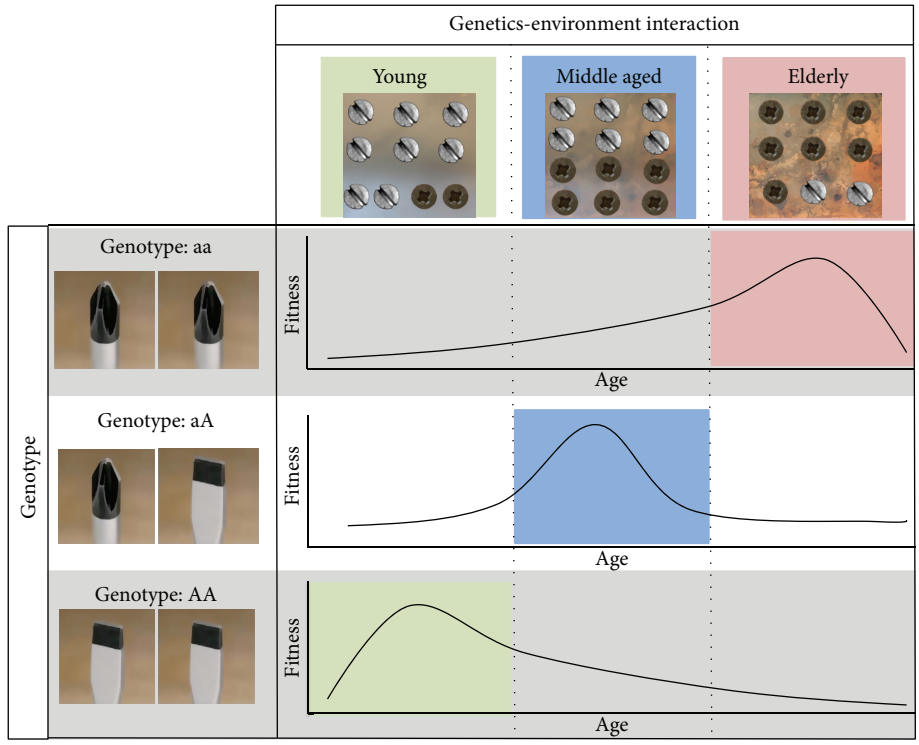

FIGURE 1: Schematic representation of the remodeling theory. The genetics-environment interactions as a function of age are represented as the fitness between the screwdrivers (genotype) and screw head shape (cross or cut). In particular, the different gene-environment interaction is represented by different ratio between the screw head shape. According to the remodeling theory, the interaction between the same genotype and time-related physiological conditions could result in different fitness, as shown in the three plots (fitness versus age). In particular, the highest fitness values for each genotype are coloured (red, blue, and green).

the OXPHOS, protein-RNA interactions in the mitochondrial ribosome, or nuclear factors-mtDNA recognition sites interactions in transcription and replication processes [115, 117, 118]. The interaction between these two genomes is bidirectional, meaning that there are both a flow of information from the nucleus toward the mitochondria and a mitochondrial retrograde signaling pathway.

Regarding the first type of communication (from the nucleus to the mitochondria), the nDNA encodes for all potential factors that regulate mtDNA replication, transcription, and processing, including mtDNA polymerase (polγ). Nevertheless, nDNA encodes not only regulatory factors, but also structural proteins that constitute the respiratory multimeric protein complexes, as well as some noncoding RNAs that are imported subsequently into the mitochondria. Complex I contains 7 mtDNA gene products and at least 25 nDNA gene products, complex II contains no mtDNA gene products and 4 nDNA gene products, complex III is made of 1 mtDNA gene product and 10 nDNA gene products, complex IV contains 3 mtDNA gene products and 10 nDNA gene products, and complex V contains 2 mtDNA gene products and 11 nDNA gene products [119]. Regarding the nucleus-encoded RNAs, many import mechanisms exist but are still not fully understood [120].

Communication from the mitochondria to the nucleus involves metabolic signals (including ROS) but this field of research suffers from the lack of human studies. However, recent studies in *C. elegans* demonstrated that neuronal cells that experienced mitochondrial stress (i.e., electron transport chain (ETC) impairment) produce a signal that is transmitted from the mitochondria to the nucleus. Then, these cells produce other extracellular signals (called "mitokines") that are able to induce a stress response (mitochondrial unfolded protein response) in the intestine, without altering the ETC functionality in intestinal cells. As it was observed that this mechanism extends lifespan, the authors speculate that a diffusible molecule released from one tissue might spread a sort of "longevity signal" to other tissues [121, 122].

The mechanisms that regulate the communication between these two genomes are not completely elucidated. To date, it is well known that the balance of the crosstalk between the nDNA and the mtDNA is essential for cellular homeostasis and events that perturb this delicate equilibrium increase the vulnerability of the cell and, thereby, the rate of ageing. Regarding the ageing process, a particular attention should be devoted to SNPs located in nDNA or in mtDNA that may affect the communication between these genomes. An interesting papers by Bertolin et al. [123] focuses on the role of TOMM machinery (a multiprotein complex responsible for importing most of mitochondrial proteins) in the mitochondrial clearance. Under normal conditions, damaged mitochondria are removed via autophagy but during ageing autophagy declines [124, 125], leading to accumulation of dysfunctional mitochondria. Bertolin and

colleagues demonstrated that the degradation of the core structure of the TOMM complex is crucial in mitochondrial clearance and that mutations in *PARK2*, a cytosolic E3 ubiquitin-protein ligase recruited for proteasomal-mediated degradation of outer mitochondrial membrane, significantly affect the interaction with TOMM70A and TOMM40. These results let us hypothesize that also SNPs located in genes of the TOMM machinery could be involved in the effectiveness of autophagic mitochondrial clearance. From this point of view, rs2075650 in *TOMM40* that is associated to longevity, as previously described, could affect the relative configuration of the partners of TOMM complex, resulting in a more efficient clearance of damaged mitochondria. In this context, further studies are needed to elucidate the interactions between mtDNA and nuclear gene variants assessing their association with longevity in human.

The study of epistatic interactions represents a challenge [126] that is gradually becoming more achievable with the availability of NGS techniques. Indeed, the significant drop in costs and time commitment required to obtain a complete mtDNA sequence caused a burst in the number of available samples in both private and public databases [127]. It is noteworthy that about 22% of the whole database of the complete human mtDNA sequences was deposited in the last 12 months. The nDNA and mtDNA interactions could represent a critical issue when considered in the context of age-related heteroplasmy accumulations. Indeed, locally heteroplasmic mutations that alter this complex interaction can spread with age and impair cellular and tissues homeostasis.

7. Gut Microbiota-Host Genes Crosstalk and Role of the Diet

The three-way interaction between human genetics, environment, and microbiota fundamentally shaped the biological history of modern human populations and continues to affect healthy globally. The disruption of this interaction and stability by modifying one or more of these three components may be a trigger for the development of diseases.

A recent paper tried to determine to what extent the gut microbiota is determined by the host or by environmental factors, such as diet. This study demonstrated that the composition of gut microbiota among great ape species is phylogenetically conserved and pattern of relationship inferred from the analysis of the microbial communities was very similar to that inferred from host mitochondrial DNA, suggesting that host genome is a fundamental factor in determining microbial composition [128]. Ley and colleagues [129] suggested that both diet and phylogeny have driven the coevolution of mammalians and their gut microbiome. While, at large taxonomic scale, "diet" appears to be the major driving factor, at lower taxonomic scale, "phylogeny" well reflects microbiome composition. However, in certain circumstances, a highly specialized diet can override the phylogenetic inertia, resulting in an adaptive microbiome convergence of phylogenetically distant hosts that share a well-defined dietary specialization [130].

The role of human genetics in the assembly of the human intestinal microbiota is still controversial. Turnbaugh et al. [93] reported that monozygotic and dizygotic twins showed a comparable degree of similarity between their intestinal microbial communities. Since family members had a more similar intestinal microbiota profile than unrelated individuals, the authors concluded that the host genetics is secondary to environmental exposures in shaping the gut microbial ecology. However, another fingerprinting study of monozygotic and dizygotic twins showed a slightly reduced microbiota similarity profile in dizygotic twins [131].

A recent study in mice investigated how environmental stimuli and host genetic factors combine together to shape microbiota composition. In particular, this is the first study that tries to estimate the effects of maternal environment and host genetics. The results showed 18 host quantitative trait loci (QTL) in linkage with specific microbial taxa. In particular some of these loci affect species composition, others affect taxa, and others exert a pleiotropic effect. The authors suggest that the core measurable microbiota can be used in GWASs in humans [132].

Moreover, there is a clear evidence that host-microbe crosstalk involves the immune system, and in particular the gut-associated lymphoid tissue (GALT) likely developed to oversee the interaction between bacteria that live close to the intestinal mucosal surfaces [133]. In this process, enterocytes exert a very crucial role steering GALT toward tolerance or responsiveness, depending on the perceived degree of treats [134].

From an evolutionary perspective, the GALT-GM immunological crosstalk and the related tolerance of the immune system to the gut microbes have been a basal trait in mammalian evolution. Thus, the coevolutionary trajectories between mammals and their GM have been shaped by adaptive changes in the host genes that play a primary role in the crosstalk with the harbored microbial communities.

GWASs identified genes of both innate and adaptive immunity as relevant for inflammatory diseases, and interestingly several of these genes have been shown to have a role in shaping the gut microbial community. Genetic variants of several nuclear genes for components of immune system showed to have an impact on the composition of the gut microbiome. Mediterranean Fever (*MEFV*) gene encodes a protein called pyrin/marenostrin that controls inflammation by interacting with the cytoskeleton in white blood cells and that, when mutated, leads to an autoinflammatory disorder called "familial Mediterranean fever" [135]. Khachatryan et al. [136] demonstrated that mutations in *MEFV* gene are associated with a shift in the *Bacteroidetes*, *Firmicutes,* and Proteobacteria phyla and that patients with familial Mediterranean fever have a lower microbial diversity. *MYD88* gene is involved in communications between sensors of microbial products during inflammatory responses and loss of *MYD88* compromises the innate immune response to pathogens [137]. The microbiota from *MYD88*-deficient and wild-type mice was compared and it was found that three bacterial families (Lactobacillaceae, Rikenellaceae, and Porphyromonadaceae) differ in their relative abundance [138]. Studies on IgA locus were performed on both mice and

humans. It was reported that mice deficient in IgA harbor an increase abundance of *Candidatus Savagella* [139]; furthermore, a decrease in proportion of IgA-coated bacteria in humans was associated with weight loss [140]. Other genes having roles in metabolism have been identified. APOA1 is the major component of high density lipoprotein in plasma and SNPs in *APOA1* gene have been associated with the risk of cardiovascular diseases [141] and with T2D [142]. Zhang et al. observed that the microbiota of *APOA1*-deficient mice has a different community structure in comparison with that of wild-type mice [143].

However, despite this clear coevolution between gut microbiota and host genomes, external stimuli are likely to play a crucial role in determining interindividual variability [144]. Diet is one of the external stimulus able to influence microbial compositions, and its effect on the microbiota is already reviewed elsewhere [145]. It has been demonstrated that a change from low in fat and rich in polysaccharide diet to a western diet, high in saturated and unsaturated fats, alters the microbial profile [146] and a high fat diet changes drastically the ratio between Bacteroidetes and Firmicutes. Furthermore, a recent study explores the intestinal microbiota from European children (characterized by western diet) and from children born in an African rural village in Burkina Faso (characterized by a fiber rich diet). The results suggest that consumption of sugar, animal fat, and calorie-dense foods in industrialized countries is rapidly limiting the adaptive potential of the microbiota by reducing functionality itself [147]. However, little is known regarding the relationship between environment and nuclear genetics in shaping this biodiversity. Studies by Franceschi and by other research groups clearly show that the ecology of the gut microbiota changes with age [100, 101, 104–108]. However, it is yet unclear how the drivers of this change combine with inflammaging, diet/environment, and genetic background. In any case it can be predicted that the age-related changes in the GM can stress and amplify the different genetic makeup of the host.

8. Conclusions: The Omic Challenge

This overview clearly shows that the knowledge of the genetics of human ageing relies on three pillars, that is, nuclear genetics, mitochondria genetics, and microbiome genetics. Until now this research field has suffered the fact that these three genetic domains have been studied separately, and it is urgent to set up investigations capable of analyzing these three genomics altogether. If these considerations are correct, the phenotypic characterization of the people/models used in genetic studies of ageing and longevity becomes critical, particularly regarding the lack of information of GM in most of the studies. The poor knowledge of GM genetics is critical because it is closely related to anthropological variables such as nutrition and diet. Similar considerations must be applied to the fact that mitochondrial genetics is not considered in GWASs, as the mitochondrial genome is closely related to human population evolution that is in turn molded by environmental adaptation to climate and diet. Finally, all the three genetic components of longevity change with age, and this is particularly evident in GM and mtDNA but it also applies to more subtle, but quantitatively more important, mutations in nDNA. Overall, the emerging scenario is that of a continuous and dynamic interaction between the different components of the genetics of human longevity that was unpredictable until recently and is currently largely unexplored.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Paolo Garagnani and Chiara Pirazzini contributed equally to this work.

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Research Article

PTEN Mediates the Antioxidant Effect of Resveratrol at Nutritionally Relevant Concentrations

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Introduction. Antioxidant properties of resveratrol have been intensively studied for the last years, both *in vivo* and *in vitro*. Its bioavailability after an oral dose is very low and therefore it is very important to make sure that plasma concentrations of free resveratrol are sufficient enough to be active as antioxidant. *Aims.* In the present study, using nutritionally relevant concentrations of resveratrol, we aim to confirm its antioxidant capacity on reducing peroxide levels and look for the molecular pathway involved in this antioxidant effect. *Methods.* We used mammary gland tumor cells (MCF-7), which were pretreated with different concentrations of resveratrol for 48 h, and/or a PTEN inhibitor (bpV: bipy). Hydrogen peroxide levels were determined by fluorimetry, PTEN levels and Akt phosphorylation by Western Blotting, and mRNA expression of antioxidant genes by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). *Results.* Resveratrol treatment for 48 h lowered peroxide levels in MCF-7, even at low nutritional concentrations (1 nM). This effect was mediated by the activation of PTEN/Akt pathway, which resulted in an upregulation of catalase and MnSOD mRNA levels. *Conclusion.* Resveratrol acts as an antioxidant at nutritionally relevant concentrations by inducing the expression of antioxidant enzymes, through a mechanism involving PTEN/Akt signaling pathway.

1. Introduction

In the last two decades, life expectancy at birth has increased by 5–10 years [1]. As a consequence, the population is growing older and therefore there is increasing interest in how to face age-related problems. Oxidative damage to biomolecules caused by reactive oxygen species (ROS) plays an important role in the aging process, as stated in the free radical theory of aging [2]. This occurs when an imbalance between the production of free radicals and the ability of the natural antioxidant defenses to scavenge them exists. We have previously suggested the possibility that the intake of nonvitamin antioxidants such as nutrients or natural compounds may be effective in increasing antioxidant defenses, by upregulating the activity of antioxidant enzymes, which are normally present in cells [3]. One of these plausible antioxidants is resveratrol (*trans*-3,5,4′-trihydroxystilbene), a natural polyphenol found in many plants and fruits, such as blueberries, blackberries, peanuts, and grapes, and specially in red wine, the main source in the human diet [4]. Resveratrol properties as an antioxidant have been intensively studied for the last years [5–10], both *in vivo* and *in vitro*, but the mechanism involved remains unclear. Moreover, its bioavailability after an oral dose is very low. In fact, plasma concentrations of free *trans*-resveratrol after ingestion of 600 mL of red wine [11] or an oral dose of 25 mg [12, 13] are extremely low, of the order of nanomolar or low micromolar. Therefore, it is very important to make sure that those nutritionally relevant low plasma concentrations of free resveratrol are sufficient enough to be active as an antioxidant [13, 14].

One of the antioxidant effects attributed to resveratrol is the ability to protect cells against H_2O_2-induced oxidative

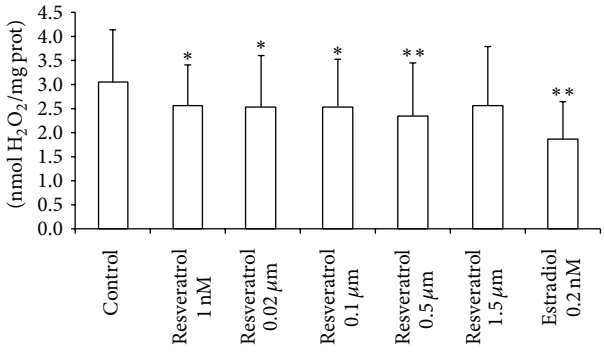

Figure 1: Resveratrol diminishes hydrogen peroxide levels in MCF-7 cells. Peroxide levels were determined by fluorimetry using homovanillic acid (see Section 3). Cells were treated with resveratrol or with estradiol for 48 h. Data are expressed as means + SD for 15 different experiments; $^{*}P < 0.05$; $^{**}P < 0.01$ *versus* control.

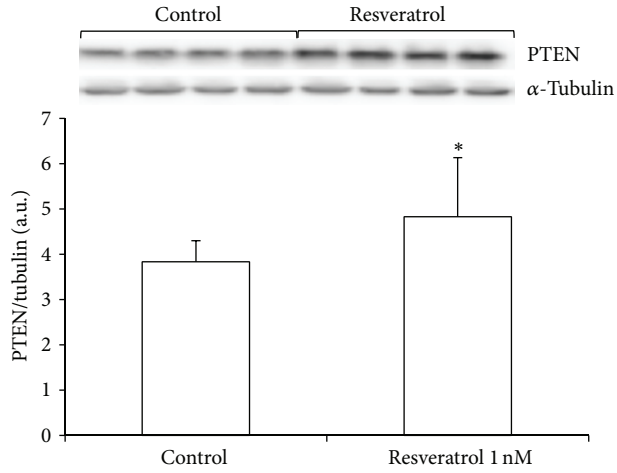

Figure 2: Resveratrol activates the PTEN signaling pathway. Levels of PTEN were measured in cells treated for 48 h with resveratrol (1 nM). Data are expressed as means + SD for 4 different experiments; $^{*}P < 0.05$ *versus* control.

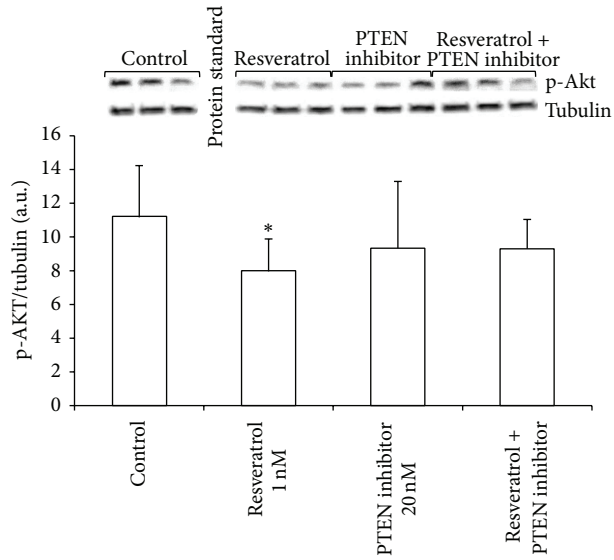

Figure 3: Resveratrol inactivates the Akt signaling pathway through PTEN activation in MCF-7 cells. Phospho-Akt levels were measured by Western blotting, after 48 h incubation with resveratrol (1 nM) alone, potassium bisperoxo (bipyridine) oxovanadate (V) as a PTEN inhibitor alone (20 nM), or both together. Histograms represent densitometric measurement of specific bands of phospho-Akt content using tubulin levels as housekeeping control. Data are expressed as means + SD for 4 independent experiments; $^{*}P < 0.05$ *versus* control.

stress [15, 16]. Furthermore, resveratrol is able to increase the phosphatase and tensin homolog PTEN [17, 18], a well-known tumor suppressor that antagonizes the activity of phosphatidylinositol 3-kinase type I (PI3K), thus leading to decreased phosphorylated-Akt (P-Akt) levels [19]. Interestingly, it has been recently reported that PTEN is able to increase energy expenditure and improve organismal survival independently of its effect on cancer, thus suggesting that PTEN might have multiple protective functions [20].

We have previously reported that oestradiol and genistein are able to decrease hydrogen peroxide levels in MCF-7 cells [21, 22]. Using the same cell line, we report that nutritionally relevant concentrations of resveratrol are able to decrease hydrogen peroxide levels not because of its phenolic structure, but because of the fact that it induces the expression of antioxidant genes, such as catalase (Cat) and manganese superoxide dismutase (MnSOD), through a mechanism that involves phosphatase and tensin homolog (PTEN) and protein kinase-B (PKB or Akt) signaling pathway. This finding may be useful to support the idea that, despite having low bioavailability, it is possible to consider resveratrol as an important nonvitamin antioxidant and to provide new insights into the mechanism involved in it.

2. Results

2.1. Nutritional Concentrations of Resveratrol Decrease Hydrogen Peroxide Levels in MCF-7 Cells. Figure 1 shows that resveratrol treatment for 48 h lowers hydrogen peroxide levels, except at the highest dose (1.5 μM). These concentrations are similar to those of free *trans*-resveratrol found in plasma after ingestion of 600 mL of red wine [11] or an oral dose of 25 mg [12, 13]. Therefore, we find antioxidant effects of resveratrol at nutritionally relevant concentrations.

2.2. Resveratrol Increases PTEN Protein Levels in MCF-7 Cells. In order to find out the mechanism by which resveratrol acts as an antioxidant, we tested if PTEN signaling pathway could be involved in its antioxidant effect. We found that resveratrol treatment for 48 h increased PTEN protein levels in MCF-7 (Figure 2).

2.3. Resveratrol Decreases Akt Phosphorylation via PTEN Activation in MCF-7 Cells. Figure 3 shows that incubation of MCF-7 cells with physiological concentrations of resveratrol (1 nM) for 48 h reduces the phosphorylation of Akt. This effect can be seen within 5 minutes of incubation and

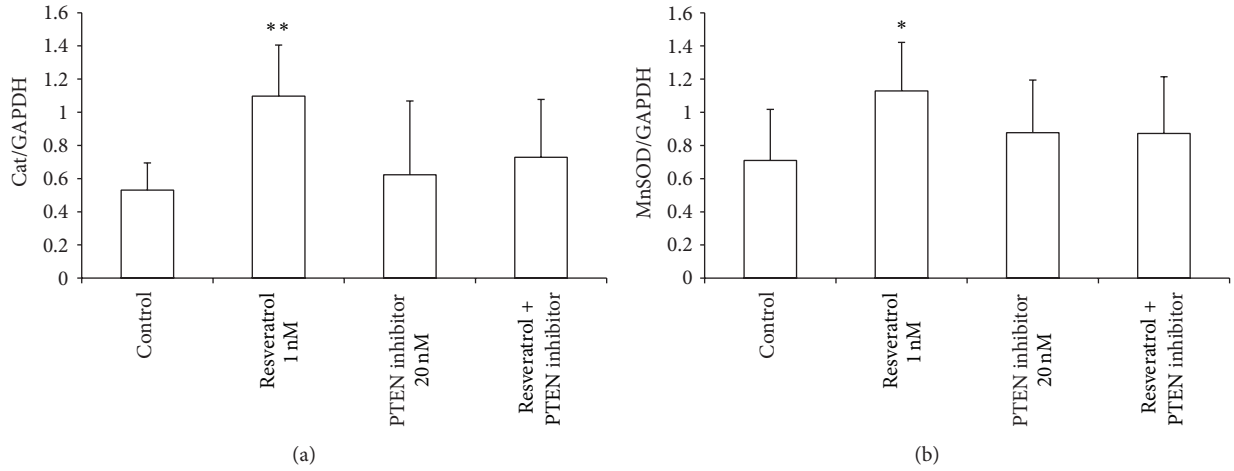

Figure 4: Resveratrol upregulates the expression of catalase (a) and MnSOD (b) in MCF-7 cells. Resveratrol (1 nM) increased mRNA levels of catalase and Mn-superoxide dismutase ($^{*}P < 0.05$; $^{**}P < 0.01$ *versus* control), and these effects were prevented when coincubating with the PTEN inhibitor (20 nM). Data are expressed as means + SD for 3 different experiments.

reaches the maximum within 48 h. Coincubation with an inhibitor of PTEN activity (potassium bisperoxo (bipyridine) oxovanadate (V)) at a dose of 20 nM reverts this effect. Therefore, the decrease in Akt phosphorylation by resveratrol is mediated by PTEN activation.

2.4. Resveratrol Upregulates Endogenous Antioxidant Genes via PTEN Signaling Pathway. Nutritional concentrations of resveratrol upregulate the expression of catalase (Figure 4(a)) and MnSOD (Figure 4(b)) after 48 h of incubation. However, this upregulation is prevented when cells are coincubated with a PTEN inhibitor, suggesting the implication of PTEN in resveratrol-mediated activation of endogenous antioxidant gene expression.

2.5. PTEN Mediates the Antioxidant Effect of Resveratrol in MCF-7 Cells. As stated before, 1 nM resveratrol pretreatment led to a reduction in intracellular hydrogen peroxide levels. However, Figure 5 shows how coincubation with a PTEN inhibitor (20 nM) reverts this antioxidant effect.

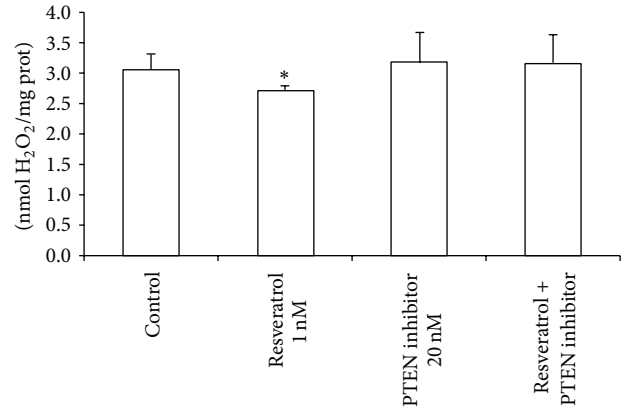

Figure 5: Resveratrol diminishes peroxide levels in MCF-7 cells, and this effect is mediated by the PTEN/Akt signaling pathway. MCF-7 cells were treated with resveratrol (1 nM) alone, potassium bisperoxo (bipyridine) oxovanadate (V) as a PTEN inhibitor alone (20 nM), or both together. Data are expressed as means + SD for 4–8 different experiments; $^{*}P < 0.05$ *versus* control.

3. Materials and Methods

3.1. Cell Culture. Human mammary gland tumor cells (MCF-7) were cultured in Iscove's modified Dulbecco's medium (IMDM) without phenol red, supplemented with 10% (v/v) heat-inactivated fetal bovine serum. Cells were plated in 25 or 75 cm^2 culture flasks and maintained at 37°C with 5% CO_2 in air. All the experiments were performed once cells reached confluence.

3.2. Treatments. Based on previous experiments of our laboratory [21, 22] and on the literature [17, 18], cells were treated for 48 h with either DMSO (for the control group), resveratrol (at concentrations ranging from 1 nM to 1.5 μM), or resveratrol together with 20 nM bisperoxo (bipyridine) oxovanadate (V) as PTEN inhibitor [23]. 0.2 nM estradiol was used as a positive control when measuring hydrogen peroxide levels [21].

3.3. Determination of Peroxide Levels in MCF-7 Cells. Intracellular levels of hydrogen peroxide were determined by fluorimetry using a modification of the method described by Barja [24].

Briefly, cells were washed twice with PBS and then incubated at 37°C with a PBS solution containing 0.1 mM homovanillic acid and 6 U/mL horseradish peroxidase. The incubation was stopped at 5 min with 1 mL of cold 2 M glycine buffer containing 50 mM EDTA and 2.2 M NaOH. The fluorescence of supernatants was measured using 312 nm as an excitation wavelength and 420 nm as an emission wavelength.

The levels of peroxides were calculated using a H_2O_2 standard curve and results were expressed per milligram of protein.

3.4. Immunoblot Analysis of Akt Phosphorylation and PTEN Protein Expression Levels. After 48 h of pretreatment with resveratrol, cells were washed twice with cold PBS and lysed in cold lysis buffer (62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% v/v glycerol), which was supplemented with a protease inhibition cocktail (10 μL per 1 mL of lysis buffer) and sodium orthovanadate 200 mM (10 μL per 1 mL of lysis buffer) to inactivate proteases and phosphatases. Immediately after harvesting, aliquots of whole cell lysates (40 μg, based on previous experiments [21, 22]) were boiled for 10 min, electrophoresed on SDS 10% polyacrylamide gels, and electroblotted (Bio-Rad) onto a PVDF membrane (Bio-Rad). Membranes were blocked at room temperature for 1 hour with 0.05 g/mL nonfat milk or BSA 0.05 g/mL in TBS-0.1% Tween 20 (TBS-T) according to the antibody. Afterwards, membranes were incubated with primary antibodies against phospho-Akt Ser 473, PTEN (1 : 1000, Cell Signaling Technologies, Boston, MA, USA), or α-tubulin as loading control (1 : 1000, Santa Cruz BioTech USA), overnight at 4°C. Blots were then washed again three times for 10 min at room temperature and then incubated for 1 h with a secondary horseradish peroxidase (HRP) linked anti-rabbit IgG antibody (1 : 2000) (Cell Signaling, Boston, MA, USA). After washing three times again, membranes were developed by using the ECL Prime Western Blotting Detection reagent as specified by the manufacturer (Amersham Pharmacia, USA). Autoradiographic bands were assessed using a Fujifilm scanning densitometer (Fujifilm LAS-1000 plus). The densitometric analysis was performed using Image J 1.34s software. For comparison between blots, one aliquot of the same sample was loaded as a standard in each gel to allow data normalization.

3.5. mRNA Gene Expression. Catalase (Cat) and manganese superoxide dismutase (MnSOD) mRNA expression was determined by real-time PCR with glyceraldehyde-3P-dehydrogenase (GAPDH) as the endogenous control, according to previously published results [21, 22].

For this purpose, total RNA was isolated from cultures by extraction with TRIzol Reagent (Invitrogen), according to the manufacturer's instructions. RNA was quantified by measuring the absorbance at 260 nm. The purity of the RNA preparations was assessed by the 260/280 ratio.

cDNA was synthesized from 1 μg total RNA using a reverse transcriptase (RT) system kit of Applied Biosystems (High-Capacity cDNA Reverse Transcription Kits). The reaction was incubated as recommended by the manufacturer, for 10 min at 25°C, followed by 120 min at 37°C, and then for 5 min at 85°C, and finally cooled to 4°C to collect the cDNA and then stored at −20°C prior to the real-time PCR assay.

The quantitative PCR was performed using the detection system 7900HT Fast Real-Time PCR System (Applied Biosystems) with Maxima SYBR Green/ROX qPCR Master Mix (2X) (Fermentas). Target and control were run in separate wells.

Specific primers employed, sense and antisense for each gene, respectively, were MnSOD, 5′-CGT GCT CCC ACA CAT CAA TC-3′ and 5′-TGA ACG TCA CCG AGG AGA AG-3′; Catalase, 5′-ACG TTG GAT GGA GAA GTG CGG AGA TTC AAC-3′ and 5′-ACG TTG GAT GTT CAC ATA GAA TGC CCG CAC-3′; and GAPDH, 5′-CCT GGA GAA ACC TGC CAA GTA TG-3′ and 5′-GGT CCT CAG TGT AGC CCA AGA TG-3′. Target cDNAs were amplified in separated tubes using the following procedure: 10 min at 95°C and then 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 62°C for 1 min per cycle.

The standard curve method was used to evaluate the relative expression levels of catalase and MnSOD in resveratrol pretreated MCF-7 cells. Briefly, the threshold cycle (Ct) was determined and converted to a relative amount through the use of a standard curve prepared from dilutions of cDNA mix of all samples. The logarithmic formula used to transform Ct values was

$$\text{Exp} = \frac{(\text{Ct, sample} - \text{Intercept})}{\text{Slope}}. \quad (1)$$

3.6. Statistical Analysis. Quantitative variables are expressed as means and standard deviation of different experiments. Once the normality of the variables was tested by Kolmogorov-Smirnov test, the statistical analysis was performed using the one-way analysis of variance (ANOVA) test to check any possible statistically significant difference between groups and the adequate post hoc tests. The level of significance was chosen at $P < 0.01$ or $P < 0.05$. All analyses were performed using SPSS statistical software version 19.0.

4. Discussion

Antioxidant supplementation is a common medical practice among the elderly [25]. We report here that the antioxidant effect of low nanomolar concentrations of resveratrol is mediated via the upregulation of antioxidant gene expression, involving activation of PTEN/Akt signaling pathway.

Numerous studies have reported the beneficial antioxidant properties of resveratrol. For example, in human blood platelets treated with peroxynitrite, resveratrol inhibited protein carbonylation and nitration, as well as lipid peroxidation [6]. Resveratrol has also been shown to protect primary hepatocytes in culture against oxidative stress damage by increasing the activities of catalase, superoxide dismutase, glutathione peroxidase, NADPH quinine oxidoreductase, and glutathione-S-transferase [7]. In addition, resveratrol was able to diminish oxidative stress by increasing gastrocnemius catalase activity, MnSOD activity, and MnSOD protein content in young and old rats submitted to a 14-day muscle disuse by hindlimb suspension [9].

In our cellular model, resveratrol also acts as an antioxidant by increasing MnSOD and Cat mRNA levels, which in turn decreases H_2O_2 levels. This H_2O_2decrease is in agreement with studies reporting the ability of resveratrol to protect PC12 cells against H_2O_2-induced cytotoxicity [15, 16], suggesting the potential capacity of resveratrol to prevent oxidative stress-induced cell death. In this regard, we checked

the potential antiapoptotic effect of 1 nM resveratrol on MCF-7 cells by measuring BCL-XL levels by Western Blotting. However, we did not find any difference in BCL-XL levels between control and resveratrol group (data not shown), suggesting that the antioxidant effect of such a low dose of resveratrol does not affect apoptosis.

In any case, the mechanism by which resveratrol can exert its antioxidant effect has not been fully elucidated. Resveratrol is able to act as a phytoestrogen and mimic estrogen biological effects [26, 27]. However, it has also been shown to act as an antagonist [28]. In fact, our first experiments aimed to check if resveratrol was able to behave as estradiol in our model system, thus binding to estrogen receptors. However, we could not inhibit resveratrol antioxidant effect when coincubating cells with resveratrol and tamoxifen (an inhibitor of estrogen receptors) (data not shown). Furthermore, resveratrol did not activate MAPK and NFκB signalling pathways, thus suggesting that the pathway involved in the expression of antioxidant genes is not mediated by estrogen receptors or MAPK and NFκB signalling pathways. Interestingly, these results suggest that resveratrol antioxidant effects may not change between men and women.

Here we show that PTEN, an antagonist to the PI3K/Akt pathway, is involved in resveratrol antioxidant effects. Resveratrol increases PTEN and decreases phospho-Akt levels. In this regard, Waite et al. observed that preincubation with resveratrol or other phytoestrogens for 48 h had been able to increase PTEN levels and decrease phospho-AKT levels in MCF-7, at concentrations ranging from 0.1 nM to 1 μM [17]. Wang et al. also found an increase in PTEN levels when incubating a prostate cancer cell line with 10 μM resveratrol for 24 h [18]. Interestingly, PTEN has been recently shown to increase the activity of antioxidant enzymes, such as glutathione peroxidase (GPx), Cat, and MnSOD in a lung cancer cell line [29]. This led us to hypothesize and finally demonstrate that PTEN/Akt pathway was involved in resveratrol antioxidant effect.

As stated before, resveratrol bioavailability is very low and its absorption is highly variable, depending on the way it is consumed and the kind of food ingested [12, 13]. Two of the first human studies on the absorption and bioavailability of resveratrol used a single oral dose treatment of 25 mg [12, 13]. Despite the use of high sensitivity methods and a specific molecular analysis, the presence of nonmetabolized resveratrol in circulating plasma was difficult to detect. Approximate calculations showed maximal concentrations of <10 ng/mL (≈40 nM), 0.5–2 hours after the oral dose. Estimates of the plasma concentrations of resveratrol plus total metabolites were considerably higher, around 400–500 ng/mL (≈2 μM), indicating a very low oral bioavailability of free resveratrol, but significant one of its metabolites. Vitaglione et al. also studied the bioavailability of resveratrol after red wine consumption and found low micromolar (1–6 μM) or nanomolar concentrations of free *trans*-resveratrol in plasma [11]. Thus, we chose for our experiment the lowest concentration (1 nM) that was able to diminish hydrogen peroxide levels (see Figure 1). This is indeed within the range of nutritionally relevant concentrations found in plasma after moderate wine intake. Consuming normal amounts of resveratrol-rich nutrients, such as grapes, peanuts, blueberries, blackberries, and red wine [30], may result in plasma concentrations of free resveratrol that, as we show here, increase the expression of antioxidant genes and thus may delay the onset of oxidative stress-related conditions. Therefore, our results may have practical importance.

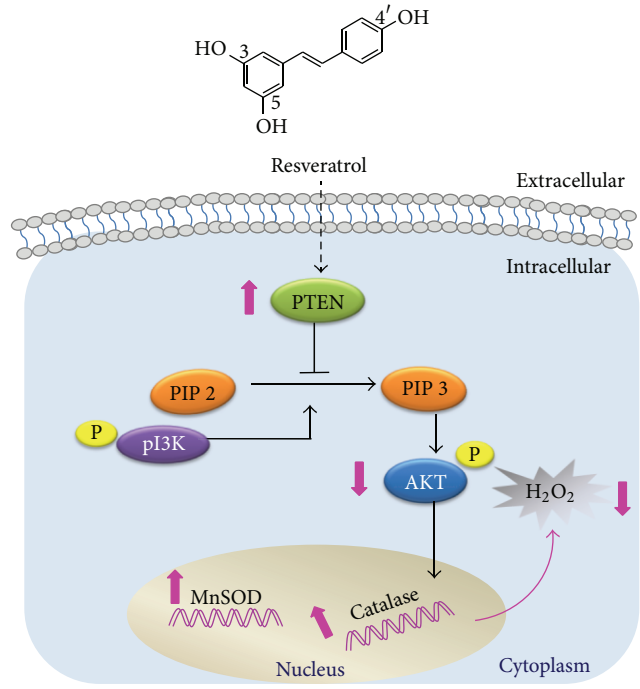

FIGURE 6: Proposed mechanism for resveratrol to upregulate antioxidant gene expression.

5. Conclusions

The major conclusion of the current study is that nutritionally relevant concentrations of resveratrol can decrease oxidative stress within the cell by upregulating antioxidant genes. As illustrated in Figure 6, resveratrol increases PTEN levels, which in turn inhibits phosphoinositide 3-kinase (PI3K) function, leading to a decrease in phospho-Akt levels and, finally, to upregulation of antioxidant genes (Cat and MnSOD). As a consequence, lower levels of hydrogen peroxide can be observed within the cell.

Conflict of Interests

All authors have indicated that they have no financial/commercial conflict of interests.

Acknowledgments

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Review Article

Biology of Ageing and Role of Dietary Antioxidants

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Interest in relationship between diet and ageing is growing. Research has shown that dietary calorie restriction and some antioxidants extend lifespan in various ageing models. On the one hand, oxygen is essential to aerobic organisms because it is a final electron acceptor in mitochondria. On the other hand, oxygen is harmful because it can continuously generate reactive oxygen species (ROS), which are believed to be the factors causing ageing of an organism. To remove these ROS in cells, aerobic organisms possess an antioxidant defense system which consists of a series of enzymes, namely, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR). In addition, dietary antioxidants including ascorbic acid, vitamin A, vitamin C, α-tocopherol, and plant flavonoids are also able to scavenge ROS in cells and therefore theoretically can extend the lifespan of organisms. In this connection, various antioxidants including tea catechins, theaflavins, apple polyphenols, black rice anthocyanins, and blueberry polyphenols have been shown to be capable of extending the lifespan of fruit flies. The purpose of this review is to brief the literature on modern biological theories of ageing and role of dietary antioxidants in ageing as well as underlying mechanisms by which antioxidants can prolong the lifespan with focus on fruit flies as an model.

1. Introduction

Our understanding on aging is still quite limited. As a complex biological process, aging involves a variety of factors. On the one hand, the variation of average lifespan from different regions is believed to be due to the differences in not only genes but also environmental conditions and eating habits. On the other hand, most organisms actually die from age-related diseases rather than aging itself. In modern society, aging-related neurodegenerative diseases have been a rising lethal threat to human beings. WHO has promoted the concept of "healthy lifespan," aiming to increase the ratio of healthy to total lifespan.

The first documented study on aging was conducted in 1532 by Muhammad in his book "Ainul Hayat." Almost 5 centuries have passed, and the mechanism and cause of aging are still not clear. In order to increase both average and maximum lifespans as well as to decrease the occurrence of age-related diseases, the mechanism behind aging needs to be explored at molecular level. Recently, extensive research has attempted to identify mechanisms underlying the links between diets and health. This review will summarize the biological theories of ageing and review the research on role of dietary antioxidants in delaying the aging.

2. Aging Theories

2.1. Stochastic Theories of Aging (STA). STA proposes that aging is the result of inevitable small random changes that accumulate with time and the failure of repairing stochastic damages in cells. The precursor of this concept is the wear and tear theory, initially proposed by August Weismann, who believed that the aging was due to constantly exposed to wounds, infections, and injuries and also from time to time, consuming excessive fat, sugar, and receiving undue UV lights or outsourced stresses. The accumulated damages would cause minor damages to cells and tissues, contributing to the age-related decline of organ functional efficiency. It

has been revealed that animals that are raised in protected environment and do not suffer from those minor exogenous insults, still age. Later on, the theory is modified by incorporating the failure of repair hypothesis. For example, somatic mutation postulates that aging is due to alterations of chromosome number or formations of lesions in existing chromosomes, caused by accumulation of stochastic genetic mutations. Evidence gathered by Hart and Setlow [1] helps to develop the theory of DNA damage and repair. It is claimed that DNA damage contributes to aging process because there is a positive correlation between DNA repair capacity and lifespan. However, nowadays STA is no longer regarded to be the sole potential candidates for the explanation of aging. As a promising modified successor, free radical theory has been becoming one of the most widely accepted aging mechanism hypotheses.

2.2. Free Radical Theory of Ageing (FRTA). FRTA was first proposed by Harman [2], stating that aging is due to accumulation of oxidative damages to tissues and organs caused by free radicals. It has been considered as one of the major theories providing a testable biological mechanism for aging process. Free radicals are any substances with unpaired electrons and readily react with healthy molecules in a destructive way. They can be produced in large quantities in cells by different mechanisms, such as exposure to oxygen, radiation, or environmental toxins, for example, pesticide and herbicide. The three major stages of free radical reactions are initiation, propagation, and termination. No matter how it is initiated, once formed, the free radicals can propagate itself indefinitely in the presence of oxygen until those radicals reach a high concentration to react with each other and produce a nonradical species [3].

Reactive Oxygen Species (ROS), the most abundant free radicals in cells, cover a wider range. Generally speaking, any highly reactive molecules containing oxygen can be classified into this category. ROS are unavoidable products during normal intracellular metabolism. They actually play essential roles in cell differentiation, proliferation, and host defense response [4]. However, their bad reputations are definitely overwhelming. Various cell components are believed to be damaged by oxygen-derived free radicals, of which lipid peroxidation, DNA damage, and protein oxidation are probably the most critical.

ROS can cause the lipid oxidation in cells. Polyunsaturated fatty acids, the main component of cell membranes, are vulnerable to free radical attack because they contain such multiple double bonds, which possess extremely reactive hydrogen atoms. As a result, the structure is susceptible to be attacked by free radicals, especially hydroxyl radicals, which will lead to the destruction of cell membrane permeability, and eventually the cellular dysfunction [5].

ROS can also damage the DNA. The ROS-induced DNA damage mainly includes strand break, cross-linking, base hydroxylation, and base excision. The induction of those DNA damages will result in mutagenesis and consequently transformation, especially if combined with a deficient apoptotic pathway [6, 7].

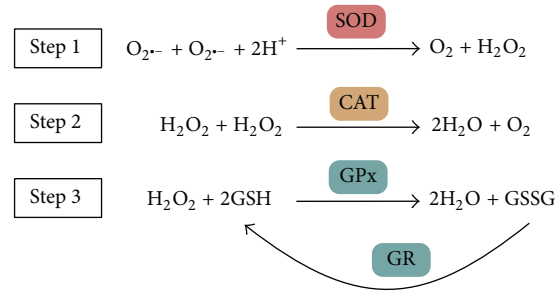


Figure 1: Main enzymatic antioxidant defense system in vivo and their reactions on scavenging free radicals and hydrogen oxide. SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; and GR, glutathione reductase.

ROS can also lead to the oxidation of proteins in vivo. The proteins in cells are also believed to be the main targets of free radicals. Aromatic amino acids, cysteine, and disulphide bonds are susceptible to the attack of free radicals, which will lead to protein denaturation and enzyme inactivation [5]. Furthermore, the reactive protein derivatives generated might act as intermediates to induce propagation of oxidative damages to other cell components [8].

Two main antioxidant systems, namely, enzymatic antioxidants and nonenzymatic ones, act systematically to scavenge the free radicals [9]. The enzymatic antioxidant system consists of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) (Figure 1). This system is the main defense system against ROS in vivo. There are two major types of SOD. One is CuZnSOD (SOD1), which mainly exist in cytoplasm, with copper and zinc being present in the active site. The other one is MnSOD (SOD2), locating in mitochondrial matrix, with manganese being present in the active site. They can catalyze the reaction to decompose superoxide anion radicals into H_2O_2, which will then be converted to water and oxygen by CAT or GPx. CAT is one of the most efficient redox enzymes, with iron being present in its active site, mainly found in peroxisome [10]. It can catalyze the conversion of H_2O_2 into water and oxygen. Otherwise, H_2O_2 would be converted to hydroxyl radical, one of the most active and harmful radicals to living cells. GPx is a selenium-containing enzyme, protecting cells and tissues from oxidative damage by removing H_2O_2 with the oxidization of glutathione. On the other hand, GR can convert the oxidized glutathione to its reduced form. However, the contribution of GPx in insects including fruit flies is relatively low [11].

The nonenzymatic antioxidants system serves as the second defense system against the free radicals. Nonenzymatic antioxidants can not only provide direct protection against oxidative damages but also more importantly enhance the function of endogenous enzymatic antioxidants by synergistically scavenging the reactive free radicals [12]. Vitamins C and E are the most renowned antioxidants in this category. However, recent study revealed that under certain circumstances, they might function as prooxidants [13]. In addition

to vitamins, there are many small molecules which serve as nonenzymatic antioxidants, such as phenolic, flavonoids, and carotenoids naturally present in foods. They can be obtained from daily diets, belonging to a group of food-derived phytochemicals called nutraceuticals [14, 15].

2.3. Mitochondrial Decline Theory of Aging (MDTA). MDTA has for so long been proposed to explain the aging process [16, 17]. Mitochondrial respiratory capacity declines with aging. Cytochrome c oxidase (CcO), the terminal oxidoreductase of mitochondrial electron transport chain (ETC), is consistently reported to decline in both aged invertebrates and vertebrates [18, 19]. Especially, its subunits III and VIb are significantly reduced in aging flies [20]. It has been reported that CcO deficiency would result in reduction of total ETC activity due to the increased production of either superoxide anion radicals or hydrogen peroxide in mitochondria. Therefore, there are solid connections between MDTA and FRTA. Theoretically speaking, enhancing antioxidant defense system will not only lead to reduced amount of free radicals but also ameliorate the functional decline of mitochondria.

2.4. Decline Theory of Ubiquitin Proteasomal System (UPS). Protein misfolding and aggregation are essential factors, contributing significantly to aging process and especially to the formation and development of neurodegenerative diseases, such as Parkinson's disease (PD) and Alzheimer's disease (AD) [21]. They can be cleared mainly by UPS [22, 23]. It is reported that age-related decline is associated with the lower activity of the 26S proteasome. Thus, maintenance of the 26S proteasome activity with age is vital for promoting longevity. The 26S proteasome is a complex of the 20S core chamber attached to two 19S caps on each end. The 20S proteasome itself cannot degrade multiubiquitinated proteins since the pores leading into the catalytic chamber are closed. The opening of the gates is triggered by the 19S attached to the ends of the 20S core chamber [24, 25].

Rpn11 is one lid component of the multiple subunits making up the 19S, which can be divided into two subcomplexes, that is, the base and lid. It is reported that knock down of Rpn11 will reduce 26S proteasome activity, leading to increased age-related accumulation of ubiquitinated proteins and shorter lifespan. On the contrary, overexpressing Rpn11 can reduce age-related accumulation of ubiquitinated proteins and thus extends lifespan [26].

2.5. Genetic Theory of Ageing. The genetic theory of ageing states that longevity is largely determined by the genes. As one of the most complicated biological processes, aging involves factors covering a wide range from genetic to environmental ones. Single gene mutation has been proved to be one of the most useful techniques to understand aging mechanisms at molecular level. Previous studies in *C. elegans*, *Drosophila*, and rodents have revealed dozens of genes, whose mutation would lead to extended lifespan. Those selected genes are named as longevity determined genes [27, 28] (Table 1).

Table 1: Selected longevity determined genes recently recognized in fruit flies, for which allelic variation is associated with extension in longevity.

Gene names	Molecular mechanism
Mth	A P-element insertion at *Mth* increases lifespan by 35% [31]
Indy	P-element insertion at *Indy* shows extended mean lifespan [35]
Chico	Heterozygous for *chico* shows an increase in median lifespan [36]
dTOR	Inhibition of TOR pathway leads to 24–26% lifespan extension [37]
Sirtuins	Gene upregulation of sirtuins increases the lifespan [38]

In *Drosophila*, single P-element insertion mutation lines can be easily generated [29] and the newly inserted locus could be identified by flanking sequence of the inserted transposon [30]. Lin et al. [31] reported that a P-element insertion was identified with an extra 35% longer lifespan, compared to wild type flies (Table 1). At the same time, they found that these *methuselah* (Mth) mutant flies showed higher resistance to various stresses, such as high temperature, starvation, and paraquat [31]. Mth protein belongs to class B of G protein-coupled receptors (GPCRs), a protein family with their iconic, large ligand-binding N-terminal extracellular domains, playing a key role in intracellular signal transduction [32, 33]. To date, the specific function of Mth is still unknown. It has been demonstrated that flies expressing a Mth antagonist peptide live significantly longer [34]. Humans have homologous gene to Mth (APG1), which could be a promising candidate for development of antiageing drugs [32].

Many other genes may be involved in the process of aging. In this connection, it has been shown that decreased expression of Indy gene in fly and worm extends longevity [35]. Indy gene encodes a transporter of Krebs cycle intermediates with the highest rate of uptake for citrate. It is known that cytosolic citrate has a role in energy regulation by affecting fatty acid synthesis and glycolysis [35]. It has been also found that chico gene, encoding an insulin receptor substrate that functions in an insulin/insulin-like growth factor (IGF) signaling pathway, has a role in aging as mutation of chico extends fruit fly median lifespan by up to 48% in homozygotes and 36% in heterozygotes [36]. Some evidence suggests that the fat body in *Drosophila* acts as a nutrient sensor, which uses TOR signaling to generate a humoral signal that modulates insulin signaling and growth in peripheral tissues. Modulation on its activity of gene in the TOR pathway leads to a longer lifespan [37]. Recent work suggests that sirtuins, encoding a conserved family of nicotinamide adenine dinucleotide (NAD+)-dependent protein deacetylases, have been also shown to regulate lifespan in many model organisms including yeast and mice by modulating ROS levels notably during a calorie restriction [38].

3. *Drosophila* and Other Models in Aging Research

It is critical to conduct a study on proper models in order to elucidate the aging mechanisms more thoroughly. Studies on humans are most straightforward. However, the duration of human aging is a limiting factor since researchers themselves also, at the same time, go through the same process. Meanwhile, ethical issues also block many research experiments on human beings. Therefore, it turns to laboratory model systems and then tries to extrapolate laboratory data to clinical value. The selection of models is diverse and under debate [39, 40]. In general, the mainstream model systems to conduct the aging study include cells, yeast (*Saccharomyces cerevisiae*), roundworms (*Caenorhabditis elegans*), fruit flies (*Drosophila melanogaster*), mice (*Mus musculus*), and rats (*Rattus norvegicus*).

Human cells are one of the major model systems in studying aging mechanisms. Researchers can easily focus on human biology when carrying out experiments on human cells. Nevertheless, in vitro data might not be always consistent with in vivo one. Meanwhile, the most widely employed parameters for cellular models on aging study are cell proliferation and stress resistance. However, the correlation of those factors with organismal aging is still under serious debate [41, 42].

Nonmammalian model systems, such as yeast, roundworms, and fruit flies, share a large number of key biological pathways with humans [43], though their physiology and phenotypes are way from alike with mammals. Meanwhile, aging researches are always based on statistical analysis and comparison at the population level. Nonmammalians models are comparatively easier and cheaper to manipulate in large numbers. On the other hand, aging is a complex biological process, involving too many factors at the same time. Nevertheless, it is reasonable and practical to conduct assays on relatively simpler systems first to observe more direct and immediate response after certain treatment. Actually, many genes and signal pathways modulating ageing process have already been identified in yeast [44], worms [45], and fruit flies [46], which serve as basis for further understanding human aging mechanisms.

As to the mammal systems, such as mice and rats, their physiology and daily activities are more parallel to humans, compared to those nonmammalian models. At the same time, as the mainstream animals employed in laboratory for decades, the related experiment protocols are quite mature and stable. However, there is still no solid evidence indicating that those rodents age for the same causes and mechanisms as humans [39]. Therefore, if a particular age-related mechanism is investigated, simpler and easier nonmammalian models might be more preferred choice.

Last but not least, nonhuman primates are recently regarded as a potential alternative for human aging studies [47]. It is claimed that Rhesus monkeys (*Macaca mulatta*) share about 90% of their genome with human beings [48]. In addition, age-related changes in neurological structure and function of monkeys also share great similarity with those of humans [49, 50]. Nevertheless, the actual employment of this nonprimate model in aging study is still quite rare for the following reasons. First of all, those primates have comparatively long lifespan, which is a practical problem for laboratory manipulation. Secondly, the costs to conduct experiments on those animals are relatively high [47]. Thirdly, a serious issue is the moral concern by animal rights groups.

Drosophila model has been widely used for biological researches, especially in the field of genetics and developmental biology. In light of the study on genetics of longevity in fruit flies, specific genes regulating lifespan have been revealed during the past decades, which involved in stress response, antioxidant system, insulin signaling pathway, and TOR pathway. It is reported that SOD or CAT mutant flies (partially knock out either SOD or CAT genes) will lead to much shorter lifespans along with a greater sensitivity to oxidative stress [51, 52]. On the contrary, transgenic flies with additional copies of CAT and SOD show median lifespan increase ranging from 6% to 33%, overexpression of SOD increases mean lifespan up to 40% [53–55].

Studies on the relation between diet supplements and lifespan of fruit flies have been continuously producing inspiring results. Experiments conducted by Bonilla et al. [56, 57] demonstrated that melatonin in diet could significantly increase the lifetime and the resistance to paraquat challenge in *Drosophila*. Similarly, resveratrol had been proved to be effective in lifespan extension in fruit flies by Bauer et al. [58] and Wood et al. [59]. We had demonstrated that green tea and broccoli could extend the median lifespan of fruit flies [60, 61].

Serving as an efficient model in aging research for decades, fruit flies possess unique advantages over other organism models. First, fruit flies and humans share many conserved physiological pathways, such as superoxide metabolism, insulin-like signaling, many of which have been proposed as vital elements for ageing regulation [43]. Second, more than 70% of known disease-causing genes in humans are conserved in fruit flies and 50% of fly protein sequences have mammalian homologs [62, 63]. Third, the technique of genetic manipulation in *Drosophila* is now quite mature. There are a wide variety of transgenic flies available, which simplify the exploration for the targets [64]. Meanwhile, fly strains with longer life span are reported to have no reduction in metabolic rate [65]. Fourth, *Drosophila* have complex nervous system with a relatively weak blood-brain barrier, which makes it a suitable model system for screening and evaluation of effects of drugs and functional compounds on neurodegenerative diseases [66, 67]. Fifth, fruit flies are comparatively easier and cheaper to maintain in large numbers, which is essential for a cohort study. The short life cycle, tiny body size, high fecundity, and known sequence of full genome make it an ideal model for aging research at population level [68]. In addition, the effects of diet supplements on aging of fruit flies have been investigated, providing promising results in the last 20 years, which not only construct practical bench methods to do related analysis, but also package powerful statistical protocols to systematically estimate and assess the effects of certain supplement compounds on aging [69]. By and large, *D. melanogaster* model is more than simple and

valid to be employed in the study of universal aging mechanisms.

4. Energy Restriction (ER) Prolongs the Lifespan

ER is to reduce moderately nutrient availability without malnutrition. ER has been shown to extend the lifespan of diverse organisms including rodents, yeast, *Drosophila,* and *C. elegans* [40, 70–72]. The mechanisms of the lifespan-prolonging activity of ER in *Drosophila* were widely investigated at molecular levels. Up to date, the most recognized mechanisms for ER are related to its effect on the metabolic rate, the nutrient sensing insulin/IGF-1 like pathway, the TOR pathway, apoptotic pathway, sirtuin pathway, and olfactory and gustatory system [73]. In addition, ER has been proposed to be associated with lesser damage of cellular macromolecules such as DNA [74], proteins [75], and lipids [76].

We have studied the gene expression of SOD1, SOD2, CAT, Rpn11, and Mth in fruit flies fed one of the three diets, namely, energy restriction diet (ER, 0.39 kcal/mL diet), standard energy diet (SE, 0.78 kcal/mL diet), and high energy diet (HE, 2.35 kcal/mL diet). Results showed that ER increased the mean lifespan by 16% compared with the control flies. It was demonstrated that ER group had a greater activity and gene expression of SOD1 and SOD2 than other two groups of flies. The elevated expression of Rpn11 induced by ER was observed at some time points, suggesting that the interaction of ER with Rpn11 may also mediate the lifespan-prolonging activity of ER. However, ER had no effect on the gene expression of CAT and Mth. The lifespan prolonging activity of ER was at least partially mediated by its effect on gene expression of SOD and possible Rpn11 but unlikely on the gene expression of CAT and Mth. It is also possible that the lifespan prolonging activity of ER is not due to its effect on a single gene rather than on a cluster of genes involved in oxidative stress, IIS pathway, apoptotic pathway, programmed autophagy, and the olfactory system.

5. Antiageing Nutraceuticals and Functional Foods

The term "nutraceutical" is actually a combined form of "nutrition" and "pharmaceutical." The generally accepted definition is "a food or part of a food which provides health benefits, including the prevention and/or treatment of a disease." Most nutraceuticals are dietary supplements. Studies both in vitro and in vivo reveal that consumption of nutraceuticals, especially the ones with high antioxidant capacity, has an inverse relationship with cardiovascular diseases, various cancers, and diabetes. However, their antiageing activity is yet to be proven. On the basis of FRTA, it is postulated that any substance with a great antioxidant capacity can be a potential candidate for delaying the aging.

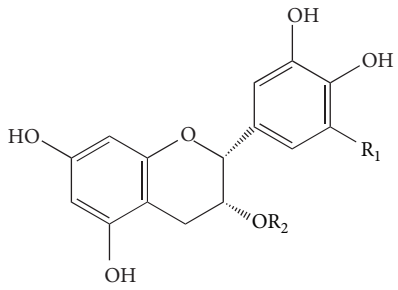
5.1. Tea Catechins and Theaflavins. Tea, next to water, is the second most popular beverage consumed by humans in the world. Black tea is more widely consumed in Western countries while green tea is preferred in the Eastern world. Black tea extracts mainly contain catechins and theaflavins (Figure 2). Evidences from clinical trials suggest that consumption of tea has various health benefits. Leenen et al. [77] demonstrated that drinking either green tea or black tea would lead a significant increase in plasma antioxidant potential by ferric-reducing antioxidant power (FRAP) assay. Furthermore, it has been reported in different population studies that consumption of green tea or black tea could significantly reduce DNA oxidation and lipid peroxidation [78, 79].

As to the antiaging activity of tea, previous studies conducted in this laboratory revealed that green catechins and black theaflavins could extend mean lifespan of *Drosophila* by 10–16%. This was accompanied by greater expression of the endogenous antioxidant enzymes SOD and CAT [60, 61, 80] (Table 2). Studies on *C. elegans* also showed similar results, indicating that treatment of epigallocatechin gallate (EGCG), an active ingredient in tea, would lead to a significant longer lifetime [81, 82]. In mice, consuming tea polyphenol, starting from 13 month till death, could increase the average lifespan by more than 6% [83].

5.2. Apple Polyphenols. A proverb says "one apple a day keeps doctors away." Apple has been recognized as a healthy fruit in many cultures. It contains a large number of phytochemicals, mainly polyphenols with strong antioxidant activities, including chlorogenic acid, phloretin, proanthocyanidin B2, epicatechin, catechin, and rutin (Figure 2).

Consumption of apple has been inversely associated with the risk of cardiovascular disease, hypercholesterolaemia, and various cancers. The Women's Health Study, involving almost 40,000 women with a 6.9-year follow-up, examined the correlation between flavonoids and cardiovascular disease, finding an inverse correlation between cardiovascular disease and consumption of apples [84]. The Iowa Women Study on nearly 35,000 women revealed that apple consumption was inversely related to the death caused by coronary heart diseases in postmenopausal women [85]. Furthermore, several clinical studies have linked apple consumption with a lower risk of cancers, especially lung cancer. It was found that eating apples would reduce the risk of lung cancer, with being more effective in women than in men [86, 87].

Experiments on animals showed similar results and revealed some potential mechanisms of the beneficial effects of apple. It was reported that, in cholesterol-fed rats, there was a significant reduction of plasma and liver cholesterol level along with increased amount of high density lipoproteins (HDL) [88]. Another study conducted by Leontowicz et al. [89] has demonstrated that apples have much better cholesterol lowering effects than pears and peaches, suggesting that, having similar amount of fiber content, apples' superior activity might be due to its larger quantity of phenolic components. Apple has been proved effective in inhibiting low-density lipoprotein (LDL) oxidation while the greatest inhibitory effect comes from apple peels [90]. In addition, apple can greatly inhibit the growth and proliferation of liver and colon cancer cells [91, 92]. Moreover, apple juice concentrate has been demonstrated to be effective in neuroprotection in both genetically compromised and normal aged



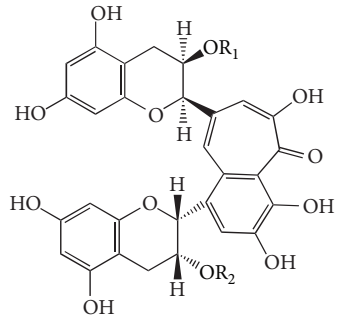
(−)-Epicatechin: $R_1 = R_2 = H$

(−)-Epigallocatechin: $R_1 = OH$, $R_2 = H$

(−)-Epicatechin gallate: $R_1 = OH$, $R_2 =$ gallate

(−)-Epigallocatechine gallate: $R_1 = OH$, $R_2 =$ gallate

(1)



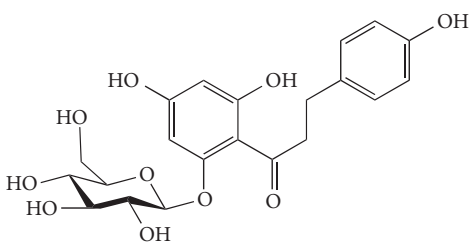
Theaflavin: $R_1 = R_2 = H$

Theaflavin-3-gallate-A: $R_1 =$ galloyl, $R_2 = H$

Theaflavin-3′-gallate-B: $R_1 = OH$, $R_2 =$ galloyl

Theaflavin-3, 3′-digallate: $R_1 = R_2 =$ galloyl

(2)



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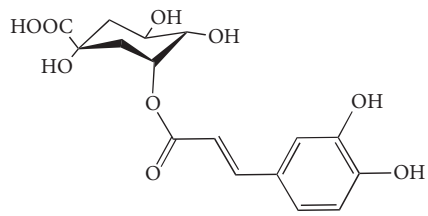
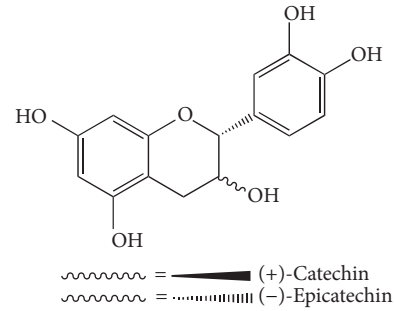


Chlorogenic acid

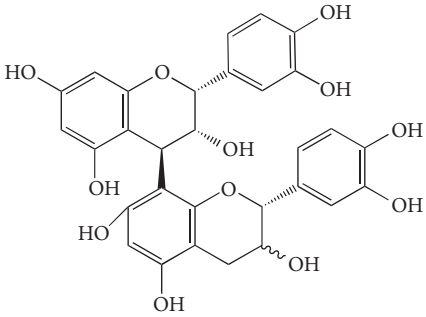
(4)



(+)-Catechin

(−)-Epicatechin

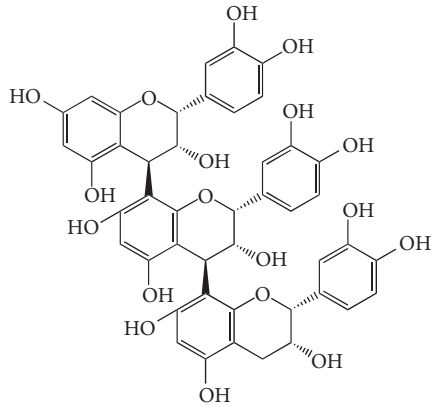
(5)



Procyanidin B_1

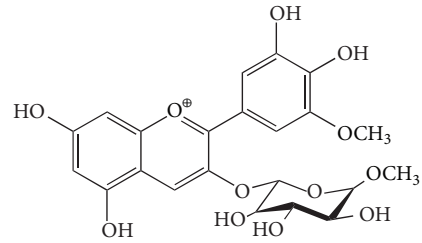
Procyanidin B_2

(6)



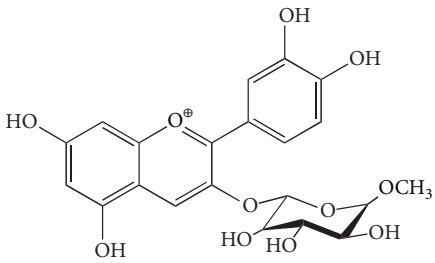
Procyanidin C1

(7)



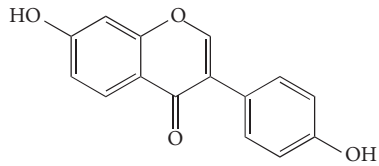
Petunidin-3-glucoside

(8)



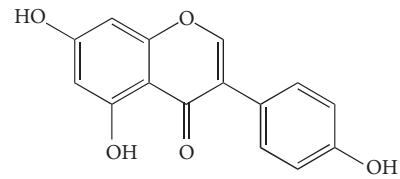
Cyanidin-3-glucoside

(9)



Daidzein

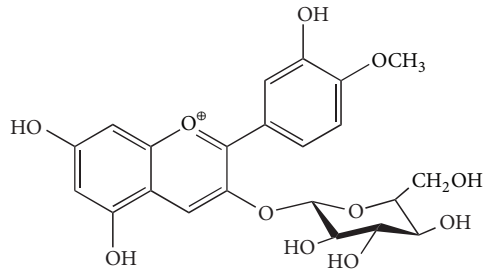
(10)



Genistein

(11)

Figure 2: Continued.



Peonidin-3-glucoside
(12)

Figure 2: Chemical structures of (1) green tea catechins, (2) black tea theaflavins, (3–7) polyphenols in apple, (8, 9) blueberry anthocyanins, (10, 11) soybean isoflavones, and (9, 12) black rice anthocyanins.

Table 2: Effect of selected nutraceuticals or functional foods on ageing and the possible underlying mechanisms.

Phytochemical antioxidants	Dose	Mean lifespan extension	Molecular mechanism	Reference
Apple polyphenols	10 mg/mL	10%	Upregulate SOD1, SOD2, Cat, and Rpn11 genes. Downregulate MTH gene	[96]
Blueberry anthocyanin extract	5 mg/mL	10%	Upregulate SOD1, SOD2, Cat, and Rpn11. Downregulate MTH gene	[97]
Black rice anthocyanin extract	30 mg/mL	14%	Upregulate SOD1, SOD2, Cat, and Rpn11 genes. Downregulate MTH gene	[98]
Green tea catechin extract	10 mg/mL	16%	Upregulate CuZnSOD, MnSOD, and Cat genes	[60, 61]
Black tea theaflavins	5 mg/mL	10%	Increase CAT activity. Upregulate SOD1 and Cat genes	[80]
Sesamin	2 mg/mL	12%	Upregulate SOD1, SOD2, and Rpn11 genes.	[99]
Curcumin	100 μM	19%	Downregulate the expression of several aging-related genes, including TOR, *InR, Hep*, *sun*, and *mth*	[100]
Marine microalga DHA-rich extract	10 mg/mL	10%	Upregulate SOD1 and SOD2 genes. Downregulate MTH gene	[101]
Nectarine extract	4%	14–22%	Reduce the transcript level of phosphoenolpyruvate carboxykinase (*PEPCK*), iron regulatory protein 1B (*Irp-1B*), *4E-BP*. Influence the redox status and reduce oxidative damage indirectly through modulate the JNK signaling pathway.	[102]

mice [93–95]. However, antiageing activity of apple and the underlying mechanisms remain elusive.

We have studied the effect of apple polyphenols (AP) on the lifespan of fruit flies and its interaction with gene expressions of SOD, CAT, Mth, Rpn11, CcO subunits III, and VIb [96]. Results showed that AP could extend the mean lifespan by 10% in fruit flies. This was accompanied by upregulation of gene SOD1, SOD2, and CAT while downregulation of Mth in the aged fruit flies. Chronic paraquat exposure could shorten the maximum lifespan from 68 to 31 days and reduce the climbing ability by 60%, while supplementation of AP into diet could partially reverse the paraquat-induced mortality and decline in climbing ability. AP could upregulate Rpn11 while it appeared to have no significant effect on gene expression of ubiquitinated protein, CcO subunits III and VIb. It was therefore concluded that the antiaging activity of AP was, at least in part, mediated by its interaction with genes SOD, CAT, Mth, and Rpn11 [96].

5.3. Blueberry Extracts. Blueberries, containing large amounts of polyphenols, possess a greater antioxidant capacity than most other fruits and vegetables [103, Figure 2]. It has been reported that consumption of natural compounds in blueberries can retard the age-related physiological and functional deficits [104]. Krikorian et al. [105] have evaluated the health benefits of blueberry supplementation, revealing that daily consumption of wild blueberry juice for 12 weeks would improve memory function in older adults with early memory decline. However, larger sample size and more consistent clinical data are lacking to draw a conclusion.

Studies in vitro and in vivo on experimental animal models also provide solid and inspiring results. Galli et al. [106] claimed that blueberry supplemented diet could reverse age-related decline in hippocampal heat shock protein (HSP) in rats. Similarly, blueberries are also suggested effective in enhancing cognitive and motor behavior as well as attenuating cognitive declines in object recognition memory in

aged rats [107]. Furthermore, age-related deficits in NMDAR-dependent long-term potentiation, a cellular substrate for learning and memory, are also reported to be ameliorated by blueberry enriched diet [108].

We have investigated the lifespan-prolonging activity of blueberry extracts in fruit flies and explored its underlying mechanism. Results revealed that blueberry extracts at 5 mg/mL in diet could significantly extend the mean lifespan of fruit flies by 10% [97]. Result was in agreement with that of Wilson et al. [109], who demonstrated that blueberry extract, mainly the fraction enriched in proanthocyanidin compounds, in diet could increase lifespan and slow ageing related declines in *C. elegans*. In our study, it was found that the mean lifespan extension was accompanied by upregulating gene expression of SOD, CAT, and Rpn11 and downregulating Mth gene [97]. Intensive H_2O_2 and paraquat challenge tests showed that lifespan was only extended in Oregon-R wild type flies but not in SOD^{n108} (deficiencyin SOD) or Cat^{n1} (deficiency in Cat) mutant strains, indicating that the prolongevity activity of blueberry was mediated by its enhancement on endogenous antioxidant system. Chronic paraquat exposure shortened the maximum survival time from 73 to 35 days and decreased the climbing ability by 60% while blueberry extracts at 5 mg/mL in diet could significantly increase the survival rate and partially restore the climbing ability with upregulating SOD, CAT, and Rpn11. It is clear that blueberry extract could affect the gene expression of Mth, Rpn11, and endogenous antioxidant enzymes SOD and CAT, thus leading to the mean lifespan extension (Table 2).

5.4. Soybean Isoflavones. Soybeans are considered as a great source of complete protein, which contains all the essential amino acids in sufficient amounts for human use [110]. They can serve as a good alternative to animal proteins for vegetarians. Daidzein and genistein, the main isoflavones in soybeans, possess the antioxidant activity.

The notion that consumption of soy protein could offer health benefits has been popular during the past decades. Soy protein in diet has been inversely associated with hypercholesterolaemia, bone loss, and various cancers. According to Food and Drug Administration (FDA), "25 grams of soy protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease." The meta-analysis conducted by Anderson et al. [111, 112] demonstrates that consumption of soy protein can decrease serum total cholesterol, LDL cholesterol, and triacylglycerol concentrations. Meanwhile, it is claimed that the decreasing effect is at least partially related to subjects' initial cholesterol concentrations and isoflavones might account for at least 60% of the cholesterol-lowering effects of soy protein [111]. More than 50 trials since then, investigating health benefits of isoflavones, have been conducted [113, 114]. It has been further demonstrated that LDL reduction induced by soy protein without isoflavones is mild, indicating that isoflavones might be the main active compounds, contributing to the cholesterol-lowering effects [115, 116]. Besides that, evidences from clinical studies reveal that consumption of soy foods, especially isoflavones, leads to higher femoral/lumbar spine bone mineral density in postmenopausal women [117]. It is also reported that in Asian countries where soy foods are more prevalent, the incidence of breast and endometrial cancer is relatively low. Actually plasma genistein in Japanese can reach 4 μM while the one can be as low as 40 nM in people consuming a typical western diet [118, 119]. Moreover, the case-control studies carried out by Shu et al. [120] and Wu et al. [121] have proved that high amount of soy intake are associated with low risk for breast cancer. However, epidemiological findings on its anticancer activity are not as consistent as the ones on its cholesterol-lowering effect.

Though the underlying mechanisms for the efficacy of soybean isoflavones are still not fully understood, studies on cells, isolated arteries, and animals provide insightful clues. It is stated that isoflavones are able to activate endothelial nitric oxide synthase, exerting vasodilatory effect [118]. Moreover, studies on isoflavones' effects on vascular smooth muscle cells (VSMC) reveal that isoflavones can inhibit cell proliferation and DNA synthesis [122]. Generally speaking, it is believed that actions of isoflavones largely overlap with those of estrogens, especially for its influence on cardiovascular diseases [123].

We have investigated the soybean isoflavones extract on the mean lifespan and expression levels of genes SOD, CAT, and Mth in fruit flies. Results demonstrated that soybean isoflavones extract in diet could significantly increase mean life span of fruit flies with upregulation of endogenous antioxidants SOD1, SOD2, and CAT on both mRNA and protein level in selected time points with no effect on MTH (unpublished data). Result was in agreement with that of Borrás et al. [124], who showed that antioxidant activity of genistein was mediated via the upregulation of antioxidant gene expression, such as increased mRNA levels of MnSOD and activation of NFκB, suggesting that supplementation of isoflavones may be beneficial in decreasing oxidative stress, thus contributing to lifespan extension. However, Altun et al. [125] recently found that genistein would decrease the maximum lifespan of female *D. melanogaster*.

5.5. Black Rice Anthocyanins. Black rice is an excellent source of dietary antioxidants. It is widely consumed in China. Supplementation of black rice confers some health benefits including anticancer, anti-inflammation, antidiabetes, and anti-Alzheimer's disease. Composition analysis shows that black rice is rich in fiber, vitamin E, and polyphenols. The antioxidant activity of black rice is mainly ascribed to the high content of anthocyanins, two majors of which are cyanidin-3-O-glucoside and peonidin-3-glucoside [126], with cyanidin-3-O-glucoside accounting for more than 80% of total anthocyanins [127, Figure 2]. Wang et al. [128] compared the antioxidant capacities of 14 anthocyanins using the automated oxygen radical absorbance capacity (ORAC) assay, and the result showed that cyanidin-3-O-glucoside has the highest ORAC activity, which was 3.5 times stronger than Trolox (vitamin E analogue). The further indepth insight into the antioxidative mechanism of black rice demonstrated that anthocyanins were inhibitors of xanthine oxidase, one of the generators of superoxide anion radicals [129].

We have investigated the lifespan-prolonging activity of black rice anthocyanins extracts and its effect on gene expressions of SOD1, SOD2, CAT, Mth, and Rpn11 [98]. Results demonstrated that black rice anthocyanins at 30 mg/dL could prolong the mean lifespan of fruit lies by 14% accompanied with upregulation of mRNA SOD1, SOD2, CAT, and Rpn11 and with downregulation of Mth. In addition, black rice anthocyanins at 30 mg/dL increased the survival time of Alzheimer transgenic line Aβ42 33769 with chronic exposure to paraquat. Huang et al. [130] found that black rice possessed antiaging, antihypoxia and, antifatigue effects in subacute ageing model mice.

6. Conclusion

Many natural antioxidants, nutraceuticals, and functional foods have been identified as free radical or active oxygen scavengers. Functional foods and nutraceuticals which possess the antioxidant activity may play an important role in delaying the aging (Table 2). Development and research on these functional foods and nutraceuticals are of interest to both public and scientific community. To better understand their antiaging activity, it is essential to identify the active ingredients and underlying mechanisms. On the basis of limited research, it appears that dietary antioxidants have the antiageing activity at least in fruit fly model, most likely by enhancing endogenous enzymatic defense capacity via upregulation of SOD and catalase and suppression on formation of free radicals.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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Review Article

Effect of Antioxidants Supplementation on Aging and Longevity

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If aging is due to or contributed by free radical reactions, as postulated by the free radical theory of aging, lifespan of organisms should be extended by administration of exogenous antioxidants. This paper reviews data on model organisms concerning the effects of exogenous antioxidants (antioxidant vitamins, lipoic acid, coenzyme Q, melatonin, resveratrol, curcumin, other polyphenols, and synthetic antioxidants including antioxidant nanoparticles) on the lifespan of model organisms. Mechanisms of effects of antioxidants, often due to indirect antioxidant action or to action not related to the antioxidant properties of the compounds administered, are discussed. The legitimacy of antioxidant supplementation in human is considered.

1. Introduction

Aging is an unavoidable, universal, biological phenomenon affecting all multicellular organisms (with few apparent exceptions) and probably common also among unicellular organisms, including protozoa, yeast, and bacteria [1, 2]. Although different hypotheses have been put forward to explain the cellular and molecular mechanisms of aging, recent studies made it increasingly clear that aging is due to accumulation of molecular damage, giving rise to a unified theory of aging [3–8]. Among reactions contributing to this damage, reactions of free radicals and other reactive oxygen species are the main reason, apart from reactions of metabolites such as sugars and reactive aldehydes and spontaneous errors in biochemical processes [9].

From a thermodynamic point of view, all aerobic organisms are subject to the action of common oxidant, that is, oxygen. The redox potential of the $O_2/2H_2O$ redox system (approximately + 0.8 V at pH 7) is more positive than those of most other biologically relevant redox systems. Therefore, the oxidation by O_2 of organic compounds will have a negative free enthalpy and should proceed spontaneously. In other words, organic compounds and structures composed of them are thermodynamically unstable in an oxygen-containing atmosphere. Molecular oxygen, in its triplet basal state, is rather unreactive due to the spin restriction. However, formation of oxygen free radicals and other reactive oxygen species (ROS) opens the gate for potentially deleterious oxidative reactions of oxygen [7]. Seen from that perspective, the "Free Radical Theory of Aging" (FRTA) [10], now more commonly termed the oxidative damage theory of ageing, seems to address a key facet of intrinsic biological instability of living systems [11, 12]. The basic idea of the FRTA is that free radicals and other ROS, formed unavoidably in the course of metabolism and arising due to the action of various exogenous factors, damage biomolecules, and accumulation of this damage are the cause of age-related diseases and aging.

If FRTA is true, antioxidants should slow down aging and prolong lifespan. This apparently obvious conclusion has stimulated enormous number of studies aimed at finding a relationship between levels of endogenous antioxidants and lifespan of various organisms on the effects of addition of exogenous antioxidants on the course of aging and lifespan of model organisms. Pubmed provides more than 13300 hits for conjunction of terms "antioxidant" and "aging or ageing." However, in spite of the plethora of studies, the answer to the question if exogenous antioxidants can prolong life is far from being clear.

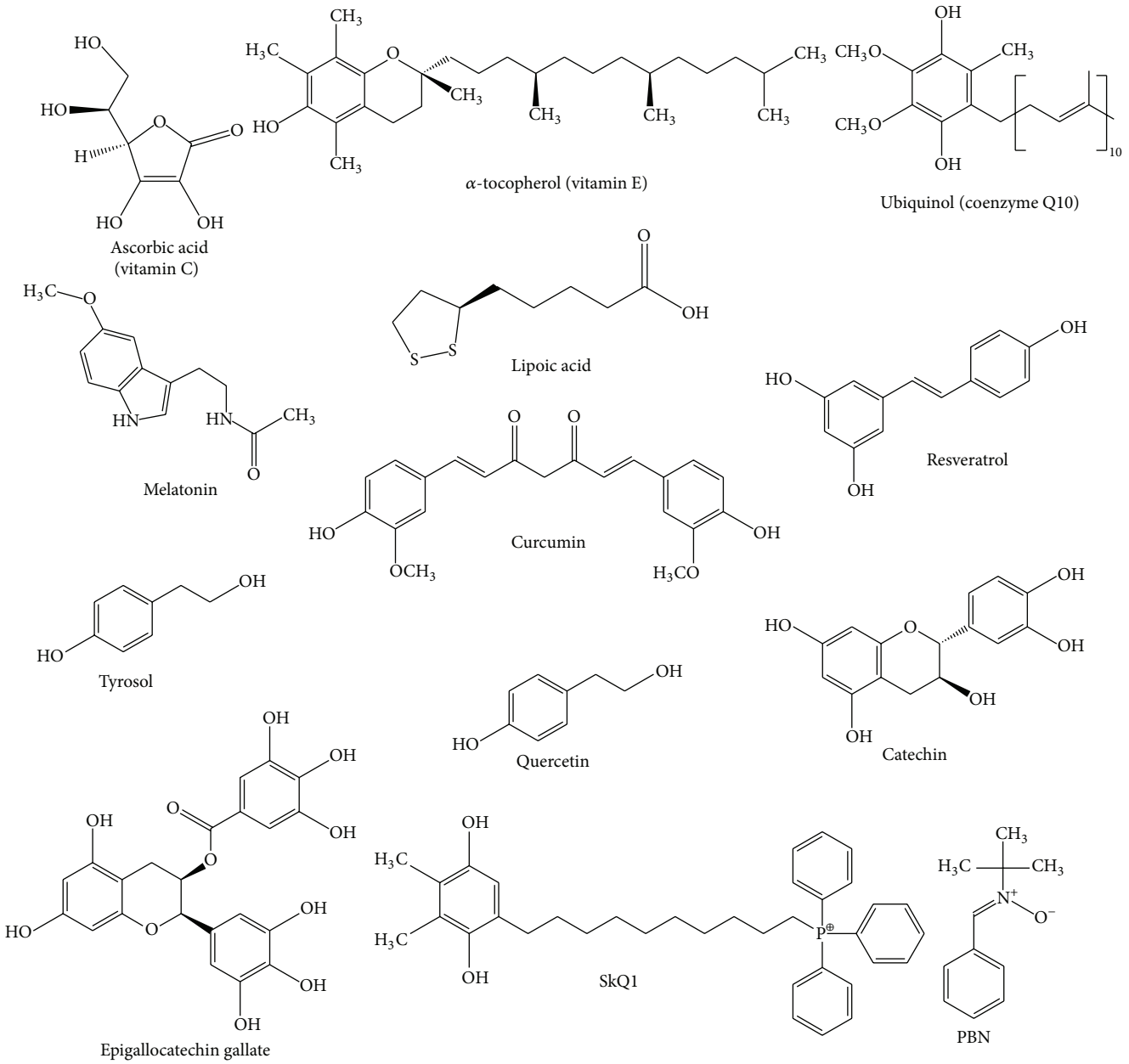

Figure 1: Some antioxidants studied as antiaging agents.

2. Effect of AOs on the Lifespan of Model Organism

Many studies have addressed the question of supplementation with antioxidant vitamins, especially vitamins C and E, and synthetic compounds can prolong the lifespan of model animals. Vitamin C (ascorbic acid) is the major hydrophilic antioxidant and a powerful inhibitor of lipid peroxidation. In membranes, this molecule rapidly reduces α-tocopheroxyl radicals and LDL to regenerate α-tocopherol and inhibit propagation of free radicals. Vitamin E (α-tocopherol) is the main hydrophobic antioxidant in cell membranes and circulating lipoproteins. Its antioxidant function is strongly supported by regeneration promoted by vitamin C. Vitamin E is thought to prevent atherosclerosis through inhibition of oxidative modification. Coenzyme Q (ubiquinol, CoQ) and lipoic acid in their reduced forms and melatonin (Figure 1) are also efficient antioxidants.

Novel endogenous indole, indolepropionamide, another endogenous antioxidant, is similar in structure to melatonin, binds to the rate-limiting component of oxidative phosphorylation in complex I of the respiratory chain, and acts as a stabilizer of energy metabolism, thereby reducing ROS production [13].

Epitalon is a synthetic tetrapeptide Ala-Glu-Asp-Gly, showing antioxidant activity [14]. (S,S)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carbonyl-beta-alanyl-L-histidine (*S*, *S*-Trolox-carnosine) is a synthetic analogue of carnosine

containing a Trolox (water-soluble analog of vitamin E) residue [15].

Recently, the antiaging effect of resveratrol (RSV) has been a hotly discussed topic. RSV was first isolated from the roots of white hellebore (*Veratrum grandiflorum, O. Loes*) in 1940 and later in 1963 from the roots of *Polygonum cuspidatum* (or *Fallopia japonica*), a plant used in traditional Chinese and Japanese medicine [16]. This polyphenolic compounds are a phytoalexin that stimulates cell defenses in plants. RSV is synthesized in many plants, such as peanuts, blueberries, pine nuts, and grapes, which protects them against fungal infection and ultraviolet irradiation. It mainly accumulates in a glycosylated state (piceid). Some dimethoxylated RSV derivatives (pterostilbene) are also present as well as RSV oligomers (ε-viniferin, a dimer, and hopeaphenol, a tetramer). Interestingly, RSV plays a number of protective roles in animals, although it is rapidly metabolized in a conjugated form (glucorono- or sulfo-) [17]. Since the early 1990s, it has been suggested that RSV could be the molecule responsible for the French paradox, that is, the low occurrence of coronary heart diseases and cardiovascular diseases in South-Western France, despite the consumption of a high saturated fat diet. The French paradox was correlated to some extent with the regular consumption of red wine, which contains high levels of RSV [18].

Curcumin [1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] (diferuloylmethane, CUR), the main component of the yellow extract from the plant *Curcuma longa* (turmeric, a popular Indian spice), is a main bioactive polyphenol, which has been used widely as a spice, food additive, and a herbal medicine in Asia [19]. Tetrahydrocurcumin (THC) is an active metabolite of CUR. Orally ingested CUR is metabolized into THC by a reductase found in the intestinal epithelium. THC possesses extremely strong antioxidant activity compared to other curcuminoids. The antioxidant role of THC has been implicated in recovery from renal injury in mice and in anti-inflammatory responses [20]. Tyrosol is a main phenol present in extra virgin olive oil [21].

Some researchers hope that development of new means of introduction of antioxidants into cells or construction of new antioxidants can make a breakthrough in antioxidant modulation of aging and longevity. If mitochondria are the main source of ROS in the cell, mitochondrially targeted antioxidants could be more effective than traditional ones. This idea was the basis of synthesis of positively charged derivatives of plastoquinone and other antioxidants which are retained in the mitochondria due to the high negative potential at the inner mitochondrial membrane [22]. SkQ1 is a mitochondria-targeted, plastoquinone-containing [10-(6′-plastoquinonyl) decyltriphenylphosphonium] [23].

Results of studies on the supplementation of model organisms with antioxidant vitamins and other antioxidants are divergent. Examples of recent studies devoted to this question are summarized in Table 1 and these data are only commented in this section.

Ascorbic acid partially rescued the lifespan of superoxide dismutase (SOD)-deficient yeast *Saccharomyces cerevisiae* which was considerably reduced as a result of lack of this vital antioxidant enzyme [12]. However, this effect should be seen rather as a partial restoration of the redox status seriously deranged in these cells rat compared to life extension of normal yeast cells. Another study, using but D-erythroascorbic acid (ascorbic acid homologue produced in the yeast) showed little effect of this antioxidant on the replicative lifespan of wild-type yeast [13]. Similar reports have been published for multicellular organisms, in which antioxidants had life-prolonging effects on mutants deficient in antioxidant defense or were subjected to oxidative stress but did not affect the lifespan of healthy wild type animals.

Supplementation of the growth medium of *S. cerevisiae* with the lipophilic antioxidants α-tocopherol and CoQ alone, or in combination with α-tocopherol, increased oxidative stress and decreased cellular lifespan [24]. It should be recalled, however, that *S. cerevisiae* is unable to produce polyunsaturated fatty acids [25] so lipid oxidative damage may be of lower importance and lack of protective effects of hydrophobic antioxidants, located mainly in cell membranes [24], maybe not surprising in this species.

Effect of vitamin C on the lifespan of several multicellular model organisms (*Caenorhabditis elegans, Drosophila melanogaster,* mice, rats, and guinea pigs) has been recently reviewed by Pallauf et al. No consistent picture emerges from the summary of data, some studies demonstrating prolongation of lifespans and others showing no effect [26]. Ernst et al. conducted a comprehensive literature review regarding the effect of vitamin E on lifespan in model organisms including single-cell organisms, rotifers, *C. elegans*, *D. melanogaster,* and laboratory rodents. The findings of their review suggest that there is no consistent beneficial effect of vitamin E on lifespan in model organisms, which corresponds to results of meta-analysis of mortality in human intervention studies [27].

While most of the studies concerning mammals have been done on mice, an interesting study has addressed the effect of dietary supplementation with either vitamin E or vitamin C (ascorbic acid) on a wild-derived animal, short-tailed field vole (*Microtus agrestis*). Antioxidant supplementation for nine months reduced hepatic lipid peroxidation, but DNA oxidative damage to hepatocytes and lymphocytes was unaffected. Surprisingly, antioxidant supplementation significantly shortened lifespan in voles maintained under both cold ($7 \pm 2^{\circ}$C) and warm ($22 \pm 2^{\circ}$C) conditions [28].

Hector et al. (2012) quantified the current knowledge of life extension of model organisms by RSV. These authors used meta-analysis techniques to assess the effect of RSV on survival, using data from 19 published papers, including six species: yeast, nematodes, mice, fruit flies, Mexican fruit flies, and turquoise killifish. While the lifespan of the turquoise killifish was positively affected by the RSV treatment, results are less clear for flies and nematodes, as there was important variability between the studies [29].

The rapid expansion of nanotechnology provided a huge assortment of nanoparticles (NPs) that differ in chemical composition, size, shape, surface charge and chemistry, and coating and dispersion status. Antioxidant delivery can be

Table 1: Effect of supplementation with natural and synthetic antioxidants on the lifespan of model organisms.

Organism	Additive	Parameter studied	Effect reported	Reference
Saccharomyces cerevisiae, budding yeast	Ascorbic acid	Replicative lifespan of SOD-1 deficient mutant	Partial restoration of normal replicative lifespan	[128]
Saccharomyces cerevisiae	Erythroascorbic acid	Replicative lifespan of wild-type yeast	Little effect	[24]
Saccharomyces cerevisiae	α-tocopherol, CoQ alone, or with α-tocopherol	Replicative lifespan	Decrease increased oxidative stress	[24]
Paramecium tetraurelia	Vitamin E	Clonal lifespan	Increase maximal (382 versus 256 fissions) at 1000 mg/L medium	[129]
Paramecium tetraurelia	Melatonin	Clonal lifespan		[130]
*Asplanchna brightwellii,*rotifer	Vitamin E (25 ug/mL)	Lifespan	Increase limited to the prereproductive stage [15]	[131]
Philodina acuticornis odiosa, rotifer	Indolepropionamide	Lifespan	Increase up to 3-fold	[13]
Caenorhabditis elegans, nematode	CoQ Vitamin E	Lifespan	Prolongation	[132]
Caenorhabditis elegans	200 μg/mL vitamin E from hatching to day 3	Survival	Increase (17–23%)	[133]
Caenorhabditis elegans	γ-Tocopherol	Lifespan	Slight extension, no effect of α-tocopherol	[134]
Caenorhabditis elegans	γ-, or α-tocopherol	Lifespan	No effect	[134]
Caenorhabditis elegans	Polydatin, resveratrol-3-O-β-mono-D-glucoside	Mean lifespan	Increase by up to 31% and 62% under normal and acute stress conditions, respectively	[56]
Caenorhabditis elegans	Curcumin	Lifespan	Increase in *mev*-1 and *daf*-16 mutants	[135]
Caenorhabditis elegans	Quercetin, isorhamnetin, and tamarixetin	Lifespan	Increase by 11–16%	[136]
Caenorhabditis elegans	Quercetin-3-O-glucoside	Lifespan	Increase by low concentrations, decrease by high concentrations	[137]
Caenorhabditis elegans	Myricetin, quercetin, kaempferol, and naringenin	Lifespan	Increase	[90]
Caenorhabditis elegans	Caffeic acid, and rosmarinic acid	Lifespan	Increase	[138]
Caenorhabditis elegans	Catechin	Mean lifespan, median lifespan	Increase by 9 and 13%, respectively, at 200 μM	[86]
Caenorhabditis elegans	(−)-Epicatechin	Lifespan	No effect	[87]
Caenorhabditis elegans	Epigallocatechin gallate (220 nM)	Mean lifespan	Increase by 10%	[139]
Caenorhabditis elegans	Epigallocatechin gallate	Mean lifespan	Increase under stress conditions but not under normal conditions	[140]
Caenorhabditis elegans	Ferulsinaic acid (0.5–100 μM)	Lifespan	Increase	[141]
Caenorhabditis elegans	Procyanidins from apples (*Malus pumila,*65 μg/mL)	Mean lifespan	Increase	[87]
Caenorhabditis elegans	Tyrosol	Lifespan	Increase	[21]
Caenorhabditis elegans	Mn-N,N′-bis(salicylidene) ethylenediamide chloride (EUK-8), an SOD mimetic	Lifespan	Extension but only after specific culture conditions	[142]
Caenorhabditis elegans	KPG-7, a herb complex	Lifespan	Prolongation	[143]
Caenorhabditis elegans	EGb 761, extract of *Ginkgo biloba* leaves	Mean lifespan	Prolongation	[94]

Table 1: Continued.

Organism	Additive	Parameter studied	Effect reported	Reference
Caenorhabditis elegans	Royal gelly	Lifespan	Prolongation	[34]
Caenorhabditis elegans	Pt nanoparticles (a SOD/CAT mimetic)	Lifespan	Prolongation	[31]
Drosophila melanogaster	Lipoic acid	Lifespan	Increase	[144]
Drosophila melanogaster	Melatonin	Lifespan	Increase	[145]
Drosophila melanogaster	Epitalon	Lifespan	Increase by 11–16%	[14]
Drosophila melanogaster	Carnosine	Average lifespan	Increase in males, no effect on females	[15]
Drosophila melanogaster	*S,S*-Trolox-carnosine	Average lifespan	Increase in males (by 16%) and in females (by 36%)	[15]
Drosophila melanogaster	Curcumin, 1 mg/g of medium	Lifespan	Increase	[146]
Drosophila melanogaster	Curcumin, 0.5 and 1.0 mg/g	Lifespan	Increase by 6% and 26% in females and by 16% and 13% in males	[65]
Drosophila melanogaster	Curcumin	Lifespan	Extension, gender- and genotype-specific	[64]
Drosophila melanogaster	*Aloe vera* extract	Lifespan	Extension	[147]
Drosophila melanogaster	Extract of black rice	Lifespan	Increase by ca 14%	[89]
Drosophila melanogaster	Cacao	Lifespan	Increase	[88]
Drosophila melanogaster	Black tea extract	Mean lifespan	Increase by 10%	[148]
Drosophila melanogaster	EUK-8 Mn 3-methoxy-N,N′-bis(salicyldene) ethylenediamine chloride (EUK-134), mitoquinone	Lifespan	No effect on wild type flies	[149]
Anastrepha ludens, Mexican fruit fly	γ-, or α-Tocopherol	Lifespan	No effect	[134]
Mus musculus, mouse, strain C57BL/6	Vitamin E, lifelong	Median lifespan	Increase by 15%	[150]
Mus musculus	Vitamin E	Lifespan	No effect	[151]
Mus musculus, C3H/He and LAF1	Vitamin E, 0.25% w/w	Lifespan	Increase in mean lifespan, no effect on maximum lifespan	[152]
Mus musculus, SAMP8 (senescence-acceleration prone)	Lipoic acid	Lifespan	Decrease	[45]
Mus musculus, SAMP8	Melatonin	Lifespan	Increase	[153]
Mus musculus	Tetrahydrocurcumin, 0.2% from the age of 13 m	Average lifespan	Increase	[20]
Mus musculus, males from the age of 12 m	LGcombo, complex mixture of botanical extracts, vitamins, and nutraceuticals	Lifespan	No effect	[33]
Mus musculus	A fullerene mimetic of SOD	Lifespan	Prolongation	[32]
Mus musculus	Royal gelly	Lifespan	Increased mean lifespan, no effect on maximal lifespan	[35]
Rattus rattus, rat, Wistar	Epigallocatechin gallate	Median lifespan	Increase by 8–12 weeks (control: 105 weeks)	[77]
Microtus agrestis, field vole	Vitamin C or vitamin E, for 9 m	Lifespan	Decrease	[28]
Mus musculus, Ellobius talpinus (mole vole), *Phodopus campbelli* (dwarf hamster)	SkQ1 (mitochondrially targeted plastoquinone derivative)	Survival	Increase	[23]

significantly improved using various NPs [30]; some NPs possess antioxidant properties and are able to efficiently attenuate oxidative stress by penetrating specific tissues or organs, even when administered at low concentrations and found to increase the lifespan of model organisms [31, 32]. Nevertheless, there is an increasing concern about the toxicity, especially genotoxicity of NPs, and this question field requires thorough studies.

It has been argued that antioxidant mixtures, such as those found in natural products, are better than simple antioxidant formulas, that is, due to synergism between antioxidants. KPG-7 is a commercially available herb mixture containing *Thymus vulgaris*, *Rosmarinus officinalis*, *Curcuma longa*, *Foeniculum vulgare*, *Vitis vinifera* (polyphenol), silk protein, *Taraxacum officinale,* and *Eleutherococcus senticosus*, which have been reported to include a variety of antioxidant, antitumoral, and anti-inflammatory bioactivities. Positive effects of such extracts on the lifespan of model organisms have been reported but other studies showed no significant effects. For example, administration of a complex mixture of vitamins, minerals, botanical extracts, and other nutraceuticals, rich in antioxidants and anti-inflammatories, to male mice starting from the age of 12 m, failed to affect their lifespan [33].

In the honeybee *Apis mellifera* L., queens live and reproduce for 1–4 years but hive workers, which are derived from the same diploid genome, live for only 3–6 weeks during the spring and summer. Queens are fed throughout their lives with royal jelly, produced by the hypopharyngeal, postcerebral, and mandibular glands of the worker bees. In contrast, workers are fed royal jelly for only a short period of time during their larval stages. It suggests that royal contains longevity-promoting agents for queens which may perhaps affect the longevity of other species if it affects the "public" mechanisms of aging [34]. However, the effect of royal jelly on the maximal lifespan of mice was rather disappointing [35]. Moreover, the action of complex preparations including plant extracts is difficult to interpret because, apart from antioxidants, they contain various biologically active products [36].

3. Reversal of Age-Related Changes by Antioxidants

Apart from the effect of prolongation of lifespan by antioxidant administration throughout most of the lifetime (long-lasting experiments), another approach to study antiaging effect of antioxidants consists in short-time experiments, in which functional tests compare the status of experimental animals before and after supplementation. An experiment of this type consisted in administration of N-*tert*-butyl-α-phenylnitrone (PBN) to aged Mongolian gerbils for 2 weeks. Such a treatment reduced the amount of protein carbonyls in brain, augmented the activity of glutamine synthetase, and decreased the number of errors in radial arm maze patrolling behavior, normalizing the values to those typical for young animals. However, these changes were reversible after cessation of PBN treatment [37]. Similarly, relatively old mice (17.5 months) fed high-CoQ diet (2.81 mg/g) for 15 weeks improved special performance in Morris water maze test and reduced protein oxidative damage [38].

4. How Do "AOs" (Do Not) Act? Possible Explanations

Generally, the effects of antioxidant supplementation in model organisms are disappointing. Many studies showed no effect or even negative effects on the lifespan. Only in some cases considerable prolongation of lifespan was obtained and in organisms which are evolutionarily quite distant from mammals. In some cases, mean but not maximal lifespan was affected, which may be caused by reduction of mortality due to diseases rather than interference with the aging process itself. An apparently obvious conclusion from the plethora of studies could be that antioxidants cannot be expected to prolong significantly the lifespan, especially of mammals, which does not support the FRTA.

However, perhaps such a simple conclusion would be precocious, not taking into account experimental setup employed in different studies. One of the questions is the relevance of use of model organisms if understanding human aging is aimed. The basic biochemical mechanisms are so common in all living world that there are good reasons to expect that the mechanisms governing aging are also universal. It may not always be true. It has been suggested that there are "public" and "private" mechanisms of aging [39]. Seemingly, the mechanisms of aging of *S. cerevisiae*, used as a model organism in biogerontology, may be rather private than public. This refers to both "chronologic" aging where yeast survival is limited by exhaustion of nutrients and/or accumulation of toxic products of metabolism and to "replicative" aging which seems to be a measure of fecundity rather than longevity and is limited by other factors compared to those relevant to aging of multicellular organisms [40, 41]. Somatic cells of *C. elegans* and *D. melanogaster* are postmitotic, which only partly reflects the situation in mammalian tissues.

It should be taken into account that ascorbic acid, which is a vitamin for primates, is synthesized by other organisms including mice and rats [42]. It does not preclude the antioxidant action of ascorbate in these organisms but administration of exogenous ascorbic acid may inhibit its endogenous synthesis.

Sometimes the administered antioxidants may be not fully taken up especially when added to complex media. Numerous studies using *C. elegans* have used a protocol, in which chemicals are orally delivered by incorporating them into the nematode growth media or mixing with the food bacteria. However, actual exposure levels are difficult to estimate. The use of liposomes loaded with water-soluble substances resulted in successful oral delivery of chemicals into the intestines of *C. elegans*. When using liposomes, oral administration of hydrophilic antioxidants (ascorbic acid, N-acetyl-cysteine, reduced glutathione, and thioproline) prolonged the lifespan of the nematodes, whereas the conventional method of delivery showed no longevity effects [43]. It is also difficult to estimate the amount of ingested food in many model organisms, such as *C. elegans* or *D.*

melanogaster, so the effects of admixture of high doses of antioxidants may lead feeding rejection and thus starvation [44].

The life-prolonging effect of antioxidants may be limited to a more or less narrow "therapeutic window". This window may be different for various organisms, that is, due to differences in the uptake rate and metabolism. Not always, the experimental conditions may hit the therapeutic window.

Introduction of antioxidants in the diet may affect the endogenous antioxidant system and the effect is not always advantageous. Farr et al. reported that supplementation with lipoic acid reduced indices of oxidative stress increasing glutathione level and decreasing the concentration of lipid peroxidation products and glutathione peroxidase activity. However, this treatment actually decreased the lifespan of SAMP8 mice [45].

The life-prolonging effect can be correlated with antioxidant properties of an additive in some but not in other cases. For example, onion flavonoids, quercetin, quercetin 3′-O-β-D-glucopyranoside, and quercetin 3-O-β-D-glucopyranoside-(4 → 1)-β-D-glucopyranoside increased the lifespan of *C. elegans* but no direct correlation was found between antioxidative activity and antiaging activity [46]. Similarly, no correlation was found between the antioxidant activities of 6 plant extracts and their lifespan benefits in *C. elegans* [47].

It should be remembered that (i) the effects of an antioxidant may be not due to its direct antioxidant action but to its indirect antioxidant effects (induction of endogenous antioxidant mechanisms) and (ii) compound called "antioxidant" may have a plethora of other effects *in vivo*, not related at all to its antioxidant action.

Antioxidants can have deleterious effects on traits that, as a consequence, increase longevity. For instance, thioproline was reported to increase longevity of *D. melanogaster* which might be ascribed to its direct antioxidant action; however, it decreased also the metabolic rate, mean weight at eclosion, and development speed of the fruit flies which might be more relevant for its life-prolonging effect [44].

Similarly, RSV reduced acute oxidative damage; however, it did not extend the normal life span of *C. elegans* indicating that antioxidant properties of this compound were probably not adequate to affect ageing [48]. Howitz and colleagues proposed that RSV is capable of increasing the deacetylase activity of human sirtuin 1 (SIRT1) [49]. SIRT1, the closest homolog of the yeast silent information regulator (sir)2 protein, functions as an NAD^+-dependent histone and nonhistone protein deacetylase in several cellular processes, like energy metabolism, stress responses, and so forth. It has been found that RSV activates SIRT1 by increasing its binding with lamin A, thus aiding in the nuclear matrix localization of SIRT1. Ghosh et al. suggested that rescue of adult stem cell decline in laminopathy-based premature aging mice by RSV is SIRT1-dependent [50]. Besides SIRT1 activation, RSV inhibits SIRT3, and it can mimic calorie restriction/dietary restriction (DR) effects [51]. DR with adequate nutrition is the only nongenetic and the most consistent nonpharmacological intervention that extends lifespan in model organisms from yeast to mammals and protects against the deterioration of biological functions, delaying or reducing the risk of many age-related diseases. It has already been known since the 1930s that a severe lowering of calorie intake dramatically slows the rate of ageing in mammals and lowers the onset of numerous age-related diseases, including cancer, cardiovascular disease, diabetes and neurodegeneration. It is found that DR induced an 80% increase in the lifespan of unicellular organisms and some invertebrates and a 20–40% increase in small mammals [52]. The biological mechanisms of DR's beneficial effects include modifications in energy metabolism, redox status, insulin sensitivity, inflammation, autophagy, neuroendocrine function, and induction of hormesis/xenohormesis response. The molecular signalling pathways mediating the antiaging effect of DR include not only sirtuins, but also AMP-activated protein kinase (AMPK), insulin/insulin growth factor-1, and target of rapamycin (TOR/mTOR), which form a complex interacting network. Rascón et al. reported that the lifespan extension effects of RSV are conserved in the honeybee and may be driven by a mechanism related to DR. In contrast, hyperoxic stress abolished the RSV life-extension response [53].

Although RSV has been found to extend the lifespan of many model organisms including yeast, nematodes, and fruit flies in the Sir2 or (Sirtuin 2)-dependent manner, some other groups have questioned the importance of the Sir2 pathway for ageing and could not confirm a beneficial effect of RSV on the lifespan of *D. melanogaster.* A *Drosophila* strain with ubiquitous overexpression of dSir2 using the UAS-GAL4 system was long-lived relative to wild-type controls but was neither long-lived relative to the appropriate transgenic controls nor a new line with stronger overexpression of dSir2. These findings underscore the importance of controlling for genetic background and for the mutagenic effects of transgene insertions in studies of genetic effects on lifespan [48]. Burnett et al. found that DR increased fly lifespan independently of dSir2 but these findings do not necessarily rule out a role for sirtuins in determination of metazoan lifespan [54].

Marchal et al. reviewed the beneficial effects of RSV in different mammalian species, including humans, and concluded that they generally reflect the effects observed during chronic DR without malnutrition. Although most of these effects have been observed in individuals without age-associated pathology, in those, which were overweight or obese, they indicate the role of RSV in metabolic regulation and the antiaging efficacy of this intervention. One explanation is the positive and rapid changes induced by RSV, which lead to adaptive metabolic response associated with an energy balance regulation and maintenance of overall health. Moreover, data on the effects of this molecule on longevity in healthy but nonobese mammals are rare, and these authors recommend that longitudinal studies on experimental models close to humans, such as nonhuman primates, multiply [18].

Recent studies have indicated that at equivalent and diet-achievable doses pterostilbene is a more potent modulator of cognition and cellular stress than RSV, likely driven by increased peroxisome proliferator-activated receptor alpha expression and increased lipophilicity due to substitution of

hydroxy with methoxy group in pterostilbene [55]. Wen et al. investigated polydatin and its role in extending lifespan, improving oxidative stress resistance and the possible regulation mechanism involved in the insulin/IGF-1 signaling (IIS) pathway. Polydatin protected against oxidative stress. It improved the expression of the inducible oxidative stress protein (GST-4) and corresponding stroke frequencies in the transgenic CL2166 strain but not due to its direct antioxidant action by mainly increased SOD-3::GFP expression in CF1553 worms and translocation of DAF-16 to the nucleus in worm cells [56].

Similarly, although CUR is a directly acting antioxidant, its lifespan-prolonging effects seem to be dependent mainly on its indirect antioxidant action (induction of antioxidant proteins) or interference with cellular signaling. CUR regulates the expression of inflammatory cytokines (e.g., TNF, IL-1), growth factors (e.g., VEGF, EGF, and FGF), growth factor receptors (e.g., EGFR, HER-2, and AR), enzymes (e.g., COX-2, LOX, MMP9, MAPK, mTOR, and Akt), adhesion molecules (e.g., ELAM-1, ICAM-1, and VCAM-1), apoptosis related proteins (e.g., Bcl-2, caspases, DR, and Fas), and cell cycle proteins (e.g., cyclin D1). CUR modulates the activity of several transcription factors (e.g., NF-κB, AP-1, and STAT) and their signaling pathways [57]. Recent studies performed in both invertebrate and vertebrate models have been conducted to determine whether CUR was also neuroprotective [58]. A compelling new body of literature is also mounting to support the efficacy of CUR in stress and mood disorders. Current understanding of the biological basis for antidepressant-relevant biochemical and behavioral changes shows convergence with some mechanisms known for standard antidepressants [59].

Recently, Xiang et al. reported that THC regulates the oxidative stress response and aging via the O-type forkhead domain transcription factor (FOXO). In NIH3T3 cells, THC induced nuclear accumulation of FOXO4, a member of the FOXO family of transcription factors, by inhibiting phosphorylation of protein kinase B (PKB)/Akt. FOXO factors act as sensors in the insulin/IGF-1 (IIS) pathway and influence mammalian longevity. Overall, the totality of the evidence supports a potential role of FOXO3A in human health, aging, and longevity. The association of FOXO with diverse aging phenotypes, including insulin sensitivity, CHD, cancer, type 2 diabetes, and longevity, is suggestive of a "gatekeeper" role in the IIS pathway. An important downstream mechanism whereby FOXO3A might influence human aging is through modification of oxidative stress. In *D. melanogaster*, THC attenuated the oxidative stress response, an effect that was blocked in a FOXO mutant background. THC extended the life span of *Drosophila* under normal conditions, and loss of either FOXO or Sir2 activity eliminated this effect. Based on these results, it seems that THC may regulate the aging process via an evolutionarily conserved signaling pathway that includes both FOXO and Sir2 [60].

Pu et al. tested the hypothesis that dietary CUR, which has an antioxidant effect, can improve aging-related cerebrovascular dysfunction via mitochondrial uncoupling protein 2 UCP2 upregulation. Dietary CUR administration for one month remarkably restored the impaired cerebrovascular endothelium-dependent vasorelaxation in aging Sprague Dawley rats. In cerebral arteries from aging Sprague Dawley rats and cultured endothelial cells, CUR promoted eNOS and AMPK phosphorylation, upregulated UCP2, and reduced ROS production. These effects of CUR were abolished by either AMPK or UCP2 inhibition. Chronic dietary CUR significantly reduced ROS production and improved cerebrovascular endothelium-dependent relaxation in aging wild type mice but not in aging $UCP2^{-/-}$ mice. CUR supplementation ameliorated age-associated large elastic artery stiffening, nitric oxide-mediated vascular endothelial dysfunction, oxidative stress, and increase in collagen and AGEs levels in mice [61].

Yanase et al. examined the effects of PAK1-deficiency or downregulation on a few selected functions of *C. elegans*, including reproduction, expression of HSP16.2 gene, and lifespan. They found that PAK1 promotes reproduction, whereas it inactivates HSP16.2 gene and shortens lifespan, as do PI-3 kinase (AGE-1), TOR, and insulin-like signalling/ILS (Daf-2) in this worm. These findings not only support the "trade-off" theory on reproduction versus lifespan, but also suggest the possibility that the reduced reproduction (or HSP16.2 gene activation) of this worm could be used as the first indicator of extended lifespan for a quick *in vivo* screening for PAK1-blockers [62]. Yu et al. examined the modulation of oxidative-stress resistance and associated regulatory mechanisms by CUR also in a *C. elegans* model. CUR-treated wild-type *C. elegans* exhibited increased survival during juglone-induced oxidative stress compared to the control treatment. In addition, CUR reduced the levels of intracellular ROS in *C. elegans*. CUR induced the expression of the gst-4 and hsp-16.2 stress response genes. Lastly, their findings from the mechanistic study in this investigation suggest that the antioxidant effect of CUR is mediated via regulation of age-1, akt-1, pdk-1, osr-1, unc-43, sek-1, skn-1, sir-2.1, and mev-1 [63].

In *D. melanogaster*, CUR, which extended the lifespan of *D. melanogaster*, also modulated the expression of several aging-related genes, including mth, thor, InR, and JNK [64]. Shen et al. found that lifespan extension by CUR in *Drosophila* was associated with the upregulation of Mn-SOD and CuZn-SOD genes and the downregulation of dInR, ATTD, Def, CecB, and DptB genes. These authors suggested that CUR increases mean lifespan of *Drosophila* via regulating gene expression of the key antioxidant enzyme SOD and reducing lipid peroxidation [65].

However, not always overexpression of antioxidant enzymes may be relevant for the lifespan. In particular, the overexpression of major antioxidant enzymes, which decrease the steady-state level of ROS, does not extend the lifespan of mice. Overexpression of SODs protects against oxidative stress but has little or no effect on the lifespan of *C. elegans* [66, 67]. The lifespan of sod-2 mutant of *C. elegans* was not decreased but even extended suggesting that ROS toxicity does not play a major role in lifespan regulation in these animals [68]. One possible explanation of why deletion of individual SOD genes failed to shorten lifespan is compensation by additional SOD genes. However, a recent

report from the Hekimi lab demonstrates that worms lacking all five SOD genes are viable and have normal lifespan, despite significantly increased sensitivity to multiple stresses [69]. These observations indicate that oxidative damage caused by superoxide radical does not contribute to worm aging. It should be expected that species with weak antioxidant defense, accumulating oxidative damage, should be short lived, which is definitely not true for the longest living rodent, the naked mole rat *Heterocephalus glaber* [70].

The term "green tea" refers to the product manufactured from fresh tea leaves by steaming or drying at elevated temperatures with the precaution to avoid oxidation of the polyphenolic components known as catechins. The natural product EGCG accounts for 50–80% of catechins in green tea, representing 200–300 mg in a brewed cup of green tea. Several other catechins such as (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epicatechin (EC) are found in lower abundance in green tea. EGCG is defined as a major green tea catechin that contributes to beneficial therapeutic effects, including antioxidant, anti-inflammatory, anticancer, and immunomodulatory effects [71].

EGCG binds strongly to many biological molecules and affects a variety of enzyme activities and signal transduction pathways at micromolar or nanomolar levels [72]. Most of the medicinal properties of green tea are associated with the "epicatechins" (2R, 3R) rather than the catechins (2S, 3R). The green tea catechins have been shown to be more effective antioxidants than Vitamins C and E, and their order of effectiveness as radical scavengers is ECG < EGCG < EGC < EC < catechin. The metal-chelating properties of green tea catechins are believed to be also important contributors to their antioxidative activity [73]. EGCG acts as a powerful hydrogen-donating radical scavenger of ROS and RNS and chelates divalent transition metal ions (Cu^{2+}, Zn^{2+} and Fe^{2+}), thereby preventing the Fe^{2+}-induced formation of free radicals *in vitro*. Among 12 polyphenolic compounds, EGCG most potently inhibited Fe^{2+}-mediated DNA damage and iron ascorbate-promoted lipid peroxidation of brain mitochondrial membranes. During ageing, total Fe^{2+} concentration increases in some brain regions that are involved in the pathogenesis of degenerative diseases, such as Alzheimer's, Parkinson's, and Huntington's disease. This Fe^{2+} accumulation obviously fosters the production of the highly reactive hydroxyl radicals ($OH^{\cdot}$), which attacks a large number of functional groups of the biomolecules in neurons. By chelating redox-active transition metal ions, the gallate groups of EGCG are thought to inhibit the Fenton-like-reaction mechanism [74]. Thus, the formation of $OH^{\cdot}$ is inhibited. Consequently, polyunsaturated fatty acids in, for example, mitochondrial membranes are protected from lipid peroxidation [75].

Results obtained by Weinreb et al. shed some light on the antioxidative-iron chelating activities of EGCG underlying its neuroprotective/neurorescue mechanism of action, further suggesting a potential neurodegenerative-modifying effect for EGCG. Their study sought a deeper elucidation of the molecular neurorescue activity of EGCG in a progressive neurotoxic model of long-term serum deprivation of human SH-SY5Y neuroblastoma cells. In this model, proteomic analysis revealed that EGCG (0.1–1 μM) affected the expression levels of diverse proteins, including proteins related to cytoskeletal components, metabolism, and heat shock. EGCG induced the levels of cytoskeletal proteins, such as beta tubulin IV and tropomyosin 3, playing a role in facilitating cell assembly. Moreover, EGCG increased the levels of the binding protein 14-3-3 gamma, involved in cytoskeletal regulation and signal transduction pathways in neurons. EGCG decreased protein levels and mRNA expression of the beta subunit of the enzyme prolyl 4-hydroxylase, which belongs to a family of iron-oxygen sensors of hypoxia-inducible factor (HIF) prolyl hydroxylases that negatively regulate the stability and degradation of several proteins involved in cell survival and differentiation. Accordingly, EGCG decreased protein levels of two molecular chaperones that were associated with HIF regulation, the immunoglobulin-heavy-chain binding protein, and the heat shock protein 90 beta [76]. *In vivo*, EGCG increased expression and activity of antioxidant enzymes, such as glutathione peroxidase, glutathione reductase, SOD, and CAT and inhibited prooxidative ones, such as monoamine oxidase (MAO)-B. The rat lifespan extension by EGCG was due to reduction of liver and kidney damage and improving age-associated inflammation and oxidative stress through the inhibition of transcription factor NF-κB signaling by activating the longevity factors: forkhead box class O 3A (FOXO3A) and SIRT1 [77]. FOXO genes are the closest human homologues of *C. elegans* DAF-16. In *C. elegans*, DAF-16 increases the expression of manganese superoxide dismutase (SOD2), which converts superoxide to less damaging hydrogen peroxide and is a potent endogenous protector against free radicals, among other "antiaging" effects. *In vivo* studies show that oxidative lesions in DNA, proteins, and other tissues accumulated with age and feeding calorically restricted diets (a potent insulin sensitizer) to rodents and humans mitigate this damage [78]. Brown et al. showed that 25 μM EGCG does not provoke a significant change in the intracellular ROS level of *daf-16* mutant *C. elegans*, while in the wild type strain ROS levels are significantly reduced by the flavonoid. This indicates that EGCG decreases ROS levels in the nematode in a DAF-16 dependent manner [79].

Meng et al. examined EGCG for its antiaging effect on human diploid fibroblasts. Fibroblasts treated with EGCG at 25 and 50 μM for 24 h considerably increased CAT, SOD1, SOD2, and glutathione peroxidase gene expressions and their enzyme activities, thus protecting the cells against H_2O_2-induced oxidative damage, accompanied by decreased intracellular ROS accumulation and well-maintained mitochondrial potential. Moreover, fibroblasts treated with EGCG at 12.5 μM for long term showed less intracellular ROS with higher mitochondrial potential, more intact mitochondrial DNA, much elevated antioxidant enzyme levels, and more juvenile cell status compared to those of the untreated group [80]. Davinelli et al. investigated the combined effect of L-carnosine and EGCG on the activation of two stress-responsive pathways: heme oxygenase (HO)-1 and Hsp72 (the inducible form of Hsp70), which play an important role in cytoprotection against oxidative stress-induced cell damage.

They demonstrated that the neuroprotective effects of EGCG and L-carnosine are achieved through the modulation of HO-1/Hsp72 systems. Moreover, the combined action of both compounds resulted in a synergistic increase of HO-1 expression which suggests a crosstalk between the HO-1 and the Hsp72-mediated pathways [81]. Rodrigues et al. analyzed the neuroprotective effects of prolonged consumption of a green tea extract rich in catechins but poor in EGCG and other green tea bioactive components that could also afford benefit. Theses authors demonstrated that the consumption of an extract rich in catechins rather than EGCG protected the rat hippocampal formation from aging-related declines contributing to improving the redox status and preventing the structural damage observed in old animals, with repercussions on behavioral performance [82]. Feng et al. investigated the protective effects of EGCG on hydrogen peroxide (H_2O_2)-induced oxidative stress injury in human dermal fibroblasts. The incubation of human dermal fibroblasts with EGCG markedly inhibited the human dermal fibroblast injury induced by H_2O_2. The assay for 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity indicated that EGCG had a direct, concentration-dependent antioxidant activity. Treatment of human dermal fibroblasts with EGCG significantly reversed the H_2O_2-induced decrease of SOD and glutathione peroxidase and the inhibition of malondialdehyde levels. These authors suggested that EGCG should have the potential to be used further in cosmetics and in the prevention of aging-related skin injuries [83].

In addition to the plethora of evidence that catechins are cytoprotective via antioxidant and antiapoptotic effects, recent observations suggest that the catechins may also contain prooxidant properties, particularly at high concentrations. Thus, at low concentrations *in vitro* (1–50 μM), they are antioxidant and antiapoptotic, whereas at higher concentrations (100–500 μM), the reverse is true. DNA isolated from humans was exposed to 200 μM of EGC and EGCG, which induced oxidative damage due to the production of hydrogen peroxide. Green tea extract (10–200 μg/mL) and EGCG (20–200 μM) exacerbated oxidant activity, oxidative stress, genotoxicity, and cytotoxicity induced by hydrogen peroxide in RAW 264.7 macrophages [84]. Catechins, particularly EGCG (100 μM), have also been shown to increase the oxidative damage incurred after exposure of DNA to 8-oxo-7,8-dihydro-2′-deoxyguanosine [85].

The lifespan-prolonging effect of catechin *in C. elegans* may be related to a significant reduction in body length and modulation of energy-intensive stress response [86]. The lifespan extension of *C. elegans* by apple procyanidins is dependent on SIR-2.1 as treatment with procyanidins had no effect on the longevity of SIR-2.1 worms, which lack the activity of SIR-2, a member of the sirtuin family of NAD+-dependent protein deacetylases [87].

Extension of lifespan of *D. melanogaster* by black tea extract seems to be at least partly due to increased expression of SOD and catalase (CAT) [88]. The analogous effect of black rice extract is most likely due to upregulating the genes of SOD1, SOD2, CAT, Mth, and Rpn11 at the transcriptional level [89]. The effects of flavonoids (myricetin, quercetin, kaempferol, and naringenin) on the lifespan of *C. elegans* involved an increased DAF-16 translocation and sod-3 promoter activity [90].

Longevity-promoting regimens, including DR and inhibition of TOR with rapamycin, RSV, or the natural polyamine spermidine, have often been associated with autophagy and in some cases were reported to require autophagy for their effects. Seemingly, clearing cellular damage by autophagy is a common denominator of many lifespan-extending manipulations [91].

Maintenance of optimal long-term health conditions is accomplished by a complex network of longevity assurance processes that are controlled by vitagenes, a group of genes involved in preserving cellular homeostasis during stressful conditions. Vitagenes encode for heat shock proteins (Hsp) Hsp32, Hsp70 the thioredoxin and the sirtuin protein systems. Dietary antioxidants, such as polyphenols, have been demonstrated to be protective through the activation of hormetic pathways, including vitagenes and proteasomal activity degrading oxidatively modified proteins [92, 93].

The life-prolonging effects of complex extracts are usually ascribed to the antioxidants present in these extracts but they may contain also toxins produced by plants against insects and microorganisms which may induce a hormetic effect [36]. Such a hormetic mechanism of action has been reported for the effects of *Ginkgo biloba* extract EGb 761 on the lifespan of *C. elegans* [94]. But perhaps antioxidants can also act via hormetic mechanisms and can belong to hormesis-inducing compounds (hormetins) [93]. Like toxins, they act in some concentration range, their high concentrations being usually toxic. A hormetic action of quercetin and other flavonoids on *C. elegans* has been documented [95]. It is debatable whether hormesis, which undoubtedly increases longevity of invertebrates, can be of relevance as an aging-delaying factor in mammals and especially in human but there are reasons to assume that it modulates "public" mechanisms of aging and delay aging of mammals even if these effects are not of a large magnitude [36].

Paradoxically, the effect of hormesis may be mediated by increased formation of ROS, especially by the mitochondria believed to be the main source of ROS in the cell. In the concept of mitochondrial hormesis (*mitohormesis*), increased formation of ROS within the mitochondria evokes an adaptive response that culminates in subsequently increased stress resistance assumed to ultimately cause a long-term reduction of oxidative stress. Mitohormesis was claimed to provide a common mechanistic denominator for the physiological effects of physical exercise, reduced calorie uptake, and glucose restriction [96]. This idea questions the FRTA and rather suggests that ROS act as essential signaling molecules to promote metabolic health and longevity [97].

The glycolytic inhibitor lonidamine (5 μM) was found to extend both median and maximum lifespan of *C. elegans* by 8% each. This compound promotes mitochondrial respiration and increases formation of (ROS). Extension of lifespan is abolished by coapplication of an antioxidant, indicating that increased ROS formation is required for the extension of lifespan by lonidamine [98]. The same effects were found in *C. elegans* for low concentrations of arsenite [99], a cytotoxic and

antimalarial quassinoid glaucarubinone [100], and glucose restriction [101].

In summary, complex effects of exogenous antioxidants in model organisms are compatible with the current understanding of the role of ROS, which are not only damaging agents but also take part in the signaling pathways and may mediate beneficial response reactions on the basis of hormetic mechanisms [102–104]. The direct antioxidant action of antioxidant supplements seems thereby to be much less important than induction of endogenous antioxidants, especially via the Nrf-2 dependent pathway [105].

5. Antioxidant Supplementation in Humans: Does It Make Sense?

The changes in the structure of contemporary human populations are characterized by an increase in the fraction of people who are 65 years and older, a phenomenon of significant importance from demographic, political, social, and health points of view [106]. Nutrition has been recognized to have an important impact on overall mortality and morbidity; and its role in extending life expectancy has been the object of extensive scientific research. Dietary supplementation with antioxidants has become more and more popular. However, their biochemical mechanisms of protection against oxidative stress and antiaging effects are not fully understood. The Mediterranean diet (MeDi), a heart-healthy eating plan that emphasizes fruits, vegetables, whole grains, beans, nuts, seeds, healthy fats, and red wine consumption rich in antioxidants like RSV which have been shown to have protective effects against oxidative damage [107]. The Mediterranean lifestyle has been for many millennia a daily habit for people in Western civilizations living around the Mediterranean sea who worked intensively and survived with very few seasonal foods. A high adherence to the traditional MeDi is associated with low mortality (higher longevity) and reduced risk of developing chronic diseases, including cancer, the metabolic syndrome, depression, and cardiovascular and neurodegenerative diseases [108]. Recently, several foodstuffs have been claimed as "antiaging", principally on the basis of their anti-inflammatory and antioxidative properties: berries; dark chocolate; beans (due to their high concentration in low-fat protein, protease inhibitors, fibrins, genistein, and minerals); fish; vegetables; nuts; whole grains; garlic (due to the high amount of garlic-derived polysulfides that undergo catabolism to hydrogen sulfide promoting vasodilatation); and avocados (as a great source of monounsaturated fat, vitamins, and antioxidants) [109]. These authors reviewed the pathophysiological mechanisms that potentially link aging with diet and the scientific evidence supporting the antiaging effect of the traditional MeDi, as well as of some specific foods. Recently, five places [Okinawa (Japan), Sardinia (Italy), Loma Linda (California), Ikaria (Greece), and Nicoya (Costa Rica)] have been recognized as having a very high prevalence of octogenarians and have joined the Blue-Zones, a National Geographic project. Among the lifestyle habits that are common to those populations are high levels of daily physical activity (e.g., gardening and walking), positive attitude (e.g., an ability to articulate a sense of purpose and enriching their day with periods of calm and midday siesta), and a wise diet-high consumption of fruit, wild plants and vegetable, and low consumption of meat products. That diet is similar to the MeDi [110]. MeDi may not only reduce the risk for Alzheimer's disease [111], but also lower mortality rates and speed of disease progression in those already afflicted [112]. On the other hand, in a prospective cohort study of 1410 older adults, a higher adherence to MeDi did not lower the risk for incident dementia [113]. In another study, a higher adherence to MeDi failed to delay the transition from a cognitively healthy status to mild cognitive impairment [114]. Titova et al. suggested that one possible reason for these contrasting findings could be that the MeDi score, which is commonly used to explore correlations between MeDi and health outcomes in elderly cohorts, may mask health-related effects of certain dietary components by including others that are not relevant for the health domain of interest [115].

Most recently, Bacalini et al. discussed the potential impact of so-called "epigenetic diet" on age-related diseases, focusing on cardiovascular disease, highlighting the involvement of epigenetic modifications rather than DNA methylation, such as microRNA [116]. Epigenetic modifications may delay the aging process and impact diverse health benefits by activating numerous intracellular pathways. One leading theory suggests that bioactive phytochemicals including 1-isothiocyanato-4-(methylsulfinyl) butane (sulforaphane), (2R, 3R)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)-3-4-dihydro-2H-chromen-3-yl, 3,4,5-trihydroxybenzoate (epigallocatechin gallate), RSV, and CUR play significant roles as epigenetic modifiers [117, 118].

In recent years, the wealth of basic science research supporting RSV's potential to treat, delay, and even prevent age-related chronic diseases has led to a number of human clinical trials. As research in nonclinical populations becomes more common, disparity in dosing protocols and clinical endpoints will likely continue to cause conflicting findings. The range of daily RSV dosage used in clinical trials for healthy individuals (75 to 5000 mg) [110] would be expected to result in different clinical responses [119, 120]. Brown et al. confirmed this, demonstrating 2500 mg to be more effective than both lower (500 mg and 1000 mg) and higher dosages (5000 mg) in reducing plasma insulin-like growth factor-1 (IGF-1) concentrations [119]. Though 1000 mg RSV did not alter IGF-1 concentrations, it was sufficient to reduce insulin-like growth factor binding protein-3 (IGFBP-3) concentrations. This demonstrates that there may not be a single optimal dose of RSV, but rather the ideal dose may vary depending on the target outcome measures, which is not uncommon for various drugs. Further research is warranted to increase our understanding of the physiological responses of RSV before widespread use in humans can be promoted. Furthermore, chronic studies are an absolute must, as it is still unclear if RSV supplementation on the longer term is beneficial for overall health status [111]. A synthetic analogue of RSV, HS-1793, may be a new potent chemopreventive agent against human prostate and breast cancer cells [121, 122]. HS-1793 showed more potent anticancer effects in several aspects compared to RSV in MCF-7 (wild-type p53) and MDA-MB-231 (mutant p53) cells [122]. Moreover, HS-1793 may inhibit

human prostate cancer progression and angiogenesis by inhibiting the expression of hypoxic condition induced HIF-1α protein and vascular endothelial growth factor (VEGF). HS-1793 showed also more potent effects than RSV on the cytotoxic effects on PC-3 cells [120, 122].

Gnetum gnemon is an arboreal dioecious plant that is cultivated in Indonesia. The seeds of this species mainly contain dimeric stilbenoid compounds [gnetin C, gnemonoside A, and gnemonoside D along with trans-RSV] the active form of RSV. Recent data show showed that the ethanolic extract of *G. gnemon* seeds inhibits endothelial senescence, suggesting that trans-RSV plays a critical role in the prevention of endothelial senescence [123]. Fleenor et al. suggested that gnetin may be a novel therapy for treating arterial aging in humans [124].

It should be noted that status elderly people are a very heterogeneous group. The nutrition situation of "young" seniors does generally not differ from the situation of working-age adults while institutionalized elderly people and those in need of care often show signs of a global malnutrition. The critical nutrients in the nutrition of the elderly particularly include vitamins B12 and D. Six percent of all elderly have a manifest and 10 to 30% a functional vitamin B12 deficiency. The main cause is vitamin B12 malabsorption resulting from a type B atrophic gastritis. The functional vitamin B12 deficiency and the associated hyperhomocysteinemia are risk factors for neurodegenerative diseases and accelerate bone loss. With increasing age, the vitamin D status is deteriorating. About 50% of the elderly living in private households is deficient in vitamin D; in geriatrics vitamin D, deficiency is more the rule than an exception. This is caused by a reduced endogenous biosynthesis, low UVB exposure, and a diet low in vitamin D. A vitamin D deficiency increases the risk for falls and fractures as well as the risk for neurodegenerative diseases. Also the overall mortality is increased [125].

On the other hand, up till now no prospective clinical intervention studies have been able to show a positive association between antioxidant supplementation and increased survival. More studies are needed to understand the interactions among single nutrient modifications (e.g., protein/amino acid, fatty acids, vitamins, phytochemicals, and minerals), the degree of DR, and the frequency of food consumption in modulating antiaging metabolic and molecular pathways and in the prevention of age-associated diseases. Meta-analysis of mortality data from 57 trials with a supplementation period of at least one year was published between 1988 and 2009, with sample sizes ranging from 28 to 39.876 (median = 423), yielding 246.371 subjects, and 29.295 all-cause deaths indicating that supplementation with vitamin E has no effect on all-cause mortality at doses up to 5.500 IU/d [79]. The last meta-analysis of randomized controlled human trials, and studies performed with rodents also do not support the idea that the consumption of dietary supplements can increase the lifespan of initially healthy individuals [91].

Most recently, Macpherson et al. reported that multivitamin-multimineral treatment has no effect on mortality risk [126]. Bjelakovic et al. noted that antioxidant supplements do not possess preventive effects and may be harmful with unwanted consequences to our health, especially in well-nourished populations. The optimal source of antioxidants seems to come from our diet, not from antioxidant supplements in pills or tablets. Even more, beta-carotene, vitamin A, and vitamin E may increase mortality. Some recent large observational studies now support these findings [127].

In summary, while beneficial effects of antioxidant supplements seem undoubtful in cases of antioxidant deficiencies, additional studies are warranted in order to design adapted prescriptions in antioxidant vitamins and minerals for healthy persons.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Estimating Glomerular Filtration Rate in Older People

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We aimed at reviewing age-related changes in kidney structure and function, methods for estimating kidney function, and impact of reduced kidney function on geriatric outcomes, as well as the reliability and applicability of equations for estimating glomerular filtration rate (eGFR) in older patients. CKD is associated with different comorbidities and adverse outcomes such as disability and premature death in older populations. Creatinine clearance and other methods for estimating kidney function are not easy to apply in older subjects. Thus, an accurate and reliable method for calculating eGFR would be highly desirable for early detection and management of CKD in this vulnerable population. Equations based on serum creatinine, age, race, and gender have been widely used. However, these equations have their own limitations, and no equation seems better than the other ones in older people. New equations specifically developed for use in older populations, especially those based on serum cystatin C, hold promises. However, further studies are needed to definitely accept them as the reference method to estimate kidney function in older patients in the clinical setting.

1. Introduction

Chronic kidney disease (CKD) is an important epidemic and public health problem, resulting in end-stage renal disease (ESRD) and increased risk of morbidity and mortality [1]. Hence, early identification and management of CKD patients may delay the progression of renal disease. CKD is currently classified into five stages based on glomerular filtration rate (GFR) as recommended by many professional guidelines (Table 1) [2]. CKD is defined as a GFR below 60 mL/min/1.73 m^2 or by the presence of kidney damage for 3 or more months. Conversely, individuals with a GFR from 60 to 89 mL/min/1.73 m^2 without kidney damage are classified as "decreased GFR." In UK, the prevalence of CKD stages 3–5 (GFR < 60 mL/min/1.73 m^2) is estimated to be 8.5%, and based on a review of 26 studies, a prevalence of CKD of 7.2% in patients aged >30 years and of 8% in patients aged >64 years was reported [3]. The most important Italian studies about CKD prevalence are the GUBBIO and INCIPE studies. The first one included 4,574 subjects aged 18–95 years from Umbria district (Central Italy) and showed a prevalence of CKD stages 3–5 of about 5.7% in men and 6.2% in women [4]. The INCIPE study [5] included subjects aged ≥ 40 years and showed a prevalence of CKD (stages 1–4) that is equal to 12.7%. After adjusting the analysis for age and gender, the prevalence of stage 3 CKD was lower

Table 1: The kidney disease outcomes quality initiative (KDOQI) stages of kidney disease.

Stage	GFR*	Description
1	90+	Normal kidney function
2	60–89	Mildly reduced kidney function
3A	45–59	Moderately reduced kidney function
3B	30–44	
4	15–29	Severely reduced kidney function
5	<15 or on dialysis	Very severe or endstage kidney failure

*All GFR values are normalized to an average body surface area (BSA) of 1.73 m^2.

in Italy (13.2%) compared to the US population enrolled in the National Health and Nutrition Examination Survey (NHANES) (20.3%).

In USA, the prevalence of CKD based on data from the third NHANES (NHANES III) was 11% (3.3% with stage 1; 3.0% with stage 2; 4.3% with stage 3; 0.2% with stage 4; and 0.2% with stage 5). In this study, a graded increase in the prevalence of CKD was shown at older age groups [6]. In a related analysis using data from NHANES III, the highest prevalence (45%) was found among subjects aged 80 years or more [7]. The lower prevalence in Italy was related to a lower rate of the main risk factors for kidney disease, such as diabetes, obesity, and metabolic syndrome.

The association between age and incident CKD was investigated among community-dwelling participants who were part of the Framingham Offspring Study (mean age at baseline: 43 years). For every 10-year increase in age, the odds ratio for developing incident CKD was 2.56 [8]. CKD is associated with adverse outcomes such as disability [9], cachexia [10], cardiovascular disease (CVD) [11], diabetes mellitus [12], hospitalization, and death [11]. Accurate estimation of GFR is important for detecting and staging CKD, determining drug dosages, and stratifying risk. Creatinine clearance and other reference procedures, such as clearance of inulin, ^{125}I-iothlamate, ^{51}Cr-EDTA, or iohexol, are used to determine GFR. In the oldest subjects, the above mentioned techniques are not easily applicable and a 24-hour urine collection for creatinine clearance is often impracticable [13]. For these reasons, some equations based on serum creatinine, age, race, and sex are used to obtain an estimation of GFR (eGFR). Therefore, the aim of this review was to summarize age-related changes in kidney structure and function, methods for estimating kidney function, and the impact of reduced kidney function on geriatric outcomes, as well as the reliability and applicability of eGFR in older patients.

2. Age-Related Changes in Kidney Structure and Function

The aging process determines important modifications of kidney structure and function, such as kidney vasculature, filtration, and tubule-interstitial function (Figure 1). Overall, aging is associated with a loss of renal mass by about 20–25% from 30 to 80 years of age [14], and the length of the kidney decreases by 15% from 17 to 85 years of age [15]. At the microscopic level, the aging human kidney is characterized by increased fibrosis, tubular atrophy, and arteriosclerosis [16, 17]. In the autopsy study by Neugarten et al. [18], older age was associated with increased numbers of sclerotic glomeruli and interstitial fibrosis, with a loss of about 20 to 30% of the glomeruli present in younger adults. The aging kidney exhibits an increase of mesangium, as well as the obliteration of some juxtamedullary nephrons followed by the formation of a direct channel between afferent and efferent arterioles (i.e., aglomerular circulation). Small arteries and arterioles present intimal thickening and atrophy of the media which contribute to dysfunction of the autonomic vascular reflex [19]. The most relevant age-related tubule-interstitial changes are the formation of tubular diverticuli, atrophy, fat degeneration, interstitial fibrosis, and medullary hypotonicity [19].

Such morphological changes determine important functional alterations and make the aging kidney more vulnerable to the development of kidney disease (Figure 1). Indeed, it has shown an age-related reduction in the clearance of inulin (13%–46%) [16, 20–23]. In the Baltimore Longitudinal Study of Aging (BLSA), a decrease in creatinine clearance of 0.75 mL/min/year was observed in subjects aged 30 to 90 years, although one-third of them had no decrease in renal function for up to 25 years. Since older hypertensive patients were not excluded from the BLSA sample, it was not possible to disentangle the effect of aging on kidney function from that of hypertension [24]. Decreasing filtration during aging is accompanied by a decrease in creatinine production due to the age-related loss of muscle mass (sarcopenia), and consequently serum creatinine does not necessarily increase with the progressive decrease in filtration [25]. For this reason, GFR measurement is generally adjusted for body surface area.

Aging kidney progressively loses its ability to maintain sodium/potassium balance due to the reduced tubular sodium secretion and potassium absorption [19]. Sodium output and fractional excretion of sodium are increased in old subjects, due to a diminished response of the ascending loop of Henle to renin and aldosterone. However, reduced filtration and tubular secretion significantly slow the elimination of a salt load in older people. Additionally, medullary hypotonicity significantly contributes to reduced ability of aging kidney to concentrate the urine. Finally, both potassium secretion and urea reabsorption are reduced during aging [19].

The kidney also plays an important role in synthesis, metabolism, and elimination of different hormones. While CKD naturally progresses with hormonal disorders [26], the aging process *per se* seems to play a minor role in endocrine kidney functions.

Kidneys are the primary sources of erythropoietin (EPO). Peritubular fibroblasts in the renal cortex are the main site of EPO synthesis, which is controlled by hypoxia-inducible transcription factors (HIFs) [27]. EPO is an essential factor for the viability and proliferation of erythrocytic progenitors. Proximal tubular function is preserved in healthy older

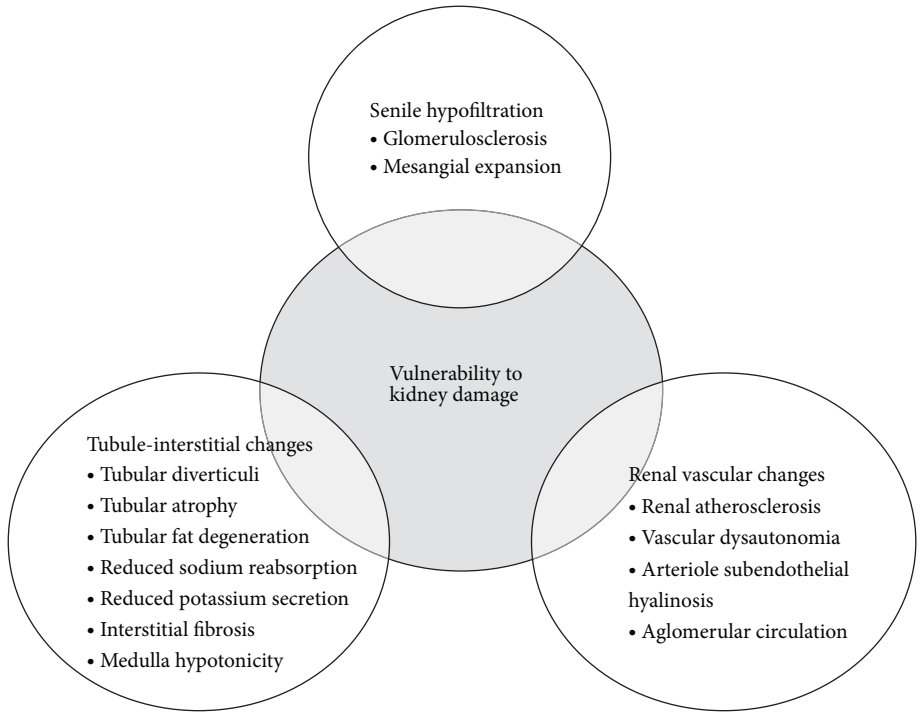

Figure 1: Summary of age-related changes in kidney structure and function. Data reported from Musso & Oreopoulos, Nephron Physiology 2011.

people and their serum EPO levels are usually normal [19] or slightly increased perhaps as a compensatory response to age-related subclinical blood loss, increased erythrocyte turnover, or increased EPO resistance. However, EPO levels are unexpectedly lower in anemic older patients compared to younger ones, suggesting a blunted response to low hemoglobin [28].

Vitamin D is necessary to maintain calcium homeostasis and optimal bone health [29]. The main circulating form of vitamin D is 25-hydroxyvitamin D (25[OH]D) (calcidiol), which requires activation by renal 1α-hydroxylase to form the metabolically active form of vitamin D, 1,25-dihydroxvitamin D (1,25$[OH]_2$D) (calcitriol). Parathyroid hormone (PTH) increases activity of the renal 1α-hydroxylase in response to low calcium levels. Age *per se* does not affect PTH and active vitamin D levels, which are usually normal in healthy older people [19], whereas CKD results in decreased kidney mass and compensatory elevation in PTH [30].

A progressive reduction in renal function is linked to alterations in thyroid hormone levels and/or metabolism, resulting in high prevalence of subclinical hypothyroidism and the low T3 syndrome [31]. This syndrome is mainly characterized by a decrease in total (T3) and free triiodothyronine (fT3) plasma concentration, whilst thyroid-stimulating hormone (TSH) and T4 remain in the normal range. Recent studies suggest that as many as 80% of patients with ESRD present low T3 levels and as many as 20 to 25% are subclinically hypothyroid [32].

Kidney is the major site of insulin clearance from the systemic circulation, removing approximately 50% of insulin in the peripheral circulation. Insulin clearance by the kidney is accomplished by glomerular filtration and proximal tubular uptake and degradation [33]. The age-related decline in kidney function leads to reduced insulin clearance, which is partly offset by diminished glucose tolerance due to defective insulin secretion and action during aging.

Finally, alterations of sex steroid production and metabolism (leading to primary hypogonadism and disturbances of the hypothalamic-pituitary axis) are observed when moderate GFR reductions arise. As many as 40 to 60% of CKD stage-5 men have been reported to be hypogonadal on the basis of low concentrations of total and free testosterone [34].

3. Methods for Estimating Kidney Function

Measurement of renal function is important in the diagnosis and management of renal diseases. GFR is the standard measure of renal function. GFR is the rate at which substances are filtered from the blood of the glomeruli into Bowman's capsules of the nephrons. Any substance freely filtered by the glomerulus and not subsequently secreted, reabsorbed, or metabolized by the distal parts of the renal system has a clearance equivalent to the GFR. It correlates with renal damage in the kidneys of patients with chronic kidney disease, and it therefore reflects overall renal functional capacity. In addition, most functions of the kidney, including endocrine ones (i.e., 1,25-dihydroxyvitamin D and erythropoietin synthesis), are directly related to GFR. In addition, appropriate dosing of drugs excreted by the kidney depends on accurate estimation of GFR. For these reasons, GFR is the

most widely accepted measurement for assessing the overall function of the kidney [35].

Conventional techniques for estimating GFR use the principle of renal clearance of various markers of GFR, including creatinine, cystatin C, inulin, and radiocontrast agents (e.g., iothalamate and iohexol). Renal clearance techniques involve measuring blood and urine concentrations of either endogenous (e.g., creatinine, cystatin C) or exogenous (e.g., inulin, radiocontrast agents) substances and calculating GFR from the ratio of urine to plasma concentrations of the marker, multiplied by the urine flow rate. These methods however are not always easily suitable in clinical practice. For this reason, equations for estimating GFR (estimated GFR, eGFR) were developed. In this section, we will discuss feasibility and reliability of these methods, with special attention to eGFR equations in older people.

3.1. Clearance of Exogenous Markers

3.1.1. Inulin. Inulin is an inert polyfructose sugar that does not bind to plasma protein, is freely filtered by the kidney, does not undergo metabolism, tubular secretion, or absorption, and is therefore rapidly excreted into the urine by glomerular filtration only. Measuring inulin clearance needs an indwelling intravenous cannula, and urinary catheter must be in place. The technique involves oral water loading (15–20 mL/kg) and intravenous loading dose of inulin of 30 to 50 mg/kg followed by a continuous infusion until establishing a steady-state plasma concentration of 15 to 20 mg/dL. The bladder is usually flushed with air to eliminate any pooled urine. After a 1 h equilibration period, three to four 30-min urine collections with midpoint (or flanking) blood specimens are obtained for measurement of blood and urine concentration of inulin [35]. Clearance is computed for each urine collection period, and the results are averaged [36]. It is generally accepted that inulin clearance provides the most accurate available determination of GFR [37]. However, it is rarely used in clinical setting because it is cumbersome for several reasons: inulin is expensive, its commercial sources are limited, it must be dissolved by boiling before use, and the laboratory assay is complex and expensive [35]. Moreover, it is extremely uncomfortable for older patients and increases the risk of urinary tract infections from urinary catheter. When spontaneous voiding is used, incontinence or retention may increase the risk of error due to incomplete urine collections [38].

3.1.2. Iothalamate. Iothalamate is commonly administered as a radioactive iodine label for ease of assay after small doses; it is most commonly administered using bolus subcutaneous injection [38]. ^{125}I-Iothalamate has been widely adopted for measurement of GFR. To block thyroidal uptake, cold iodine is administered at the time of ^{125}I-iothalamate administration, thus precluding its use in people with known allergies to iodine [38].

3.1.3. Iohexol. In order to avoid the use of radioactive compounds, techniques have been developed aimed at detecting low levels of iodine compounds in the urine. This has allowed the use of nonradiolabeled iodinated contrast agents, such as iohexol. The assay of these agents can be obtained by high performance liquid chromatography (HPLC) [39], which is unfortunately a very expensive and time consuming method [40–43]. For these reasons, a new technique has been proposed based on X-ray fluorescence. However, such method is less sensitive than HPLC, necessitating the administration of significantly larger doses of iohexol, thus leading to increased risk of nephrotoxicity and adverse reactions [38, 40, 44, 45]. As the iothalamate, iohexol cannot be used in patients with allergy to iodine.

3.1.4. Other Exogenous Markers. Among other markers, ^{51}Cr-EDTA consistently underestimates inulin clearance, probably because of tubular reabsorption [46]. Diethethylenetriaminopentaacetic acid (DTPA), an analog of EDTA, usually labeled with ^{99m}Tc, may undergo extrarenal elimination and can bind to plasma proteins to a nonpredictable extent, leading to imprecision and bias [38].

3.2. Clearance of Endogenous Markers.

Measurement of the clearance of endogenous filtration markers, such as creatinine, is widely used in clinical setting.

3.2.1. Creatinine and Creatinine Clearance. Creatinine is a metabolic product of creatine and phosphocreatine arising from the muscle compartment. Thus creatinine is directly related to muscle mass and undergoes little daily change [47]. However, its production may change over time if considering longer periods in which major changes occur in body composition [48]. Creatinine has a low molecular weight (113 D), does not bind to plasma proteins, and is freely filtered by the glomerulus. There is also a little, but not negligible, quote of secretion of creatinine by the renal tubule. The proportion of creatinine excreted by tubular secretion increases with the reduction of renal function [49], and this has important clinical implications because the GFR may decrease more rapidly than creatinine clearance, which may therefore overestimate kidney function. A small extrarenal elimination has been demonstrated likely linked to the degradation of creatinine by intestinal bacteria and therefore influenced by antibiotics [50].

Current recommendation for creatinine measurement suggests the use of a standardized method based on modified Jaffé reaction, able to separate creatinine from noncreatinine chromogens [51, 52]. Unfortunately, creatinine alone is not very sensitive. Indeed, a 50% reduction in GFR is necessary so that the values of creatinine begin to rise [53]. Additionally, circulating creatinine may be falsely low in patients with reduced muscle mass: older patients often have decreased renal function with normal circulating levels of creatinine, which has been referred to as concealed renal failure [54].

To obtain creatinine clearance, a long urinary collection period—6 to 24 h—is used to avoid the requirement for water loading and, in the steady state, a single blood sample obtained either at the beginning or the end of the collection period may be assumed to represent the average serum

concentration during the urine collection. Timed collections are subject to errors in older patients, due to inaccurate record of time and incomplete urine collection in patients with incontinence [38]. The tubular secretion of creatinine is extremely variable and does not allow the use of a constant correction factor [55]. Variability in creatinine clearance measurement also depends on age, gender, and muscle mass [56]. Even the dietary intake is a source of variability: creatine derived from ingested meat is converted into creatinine and may result in increases up to 30 per cent of its total excretion [57].

3.2.2. Cystatin C. Cystatin C (CysC) is a single chain basic protein with low molecular weight (13 kD) produced by all nucleated human cells, whose circulating concentrations can be easily determined by an automated particle-enhanced immunoturbidimetric method [58]. CysC is mainly filtrated by the kidney [59, 60], and renal clearance of CysC is 94% of the renal clearance calculated using the Cr51EDTA clearance [61, 62]. However, CysC also undergoes tubular catabolism and reabsorption. Other factors affecting the production of CysC include the use of systemic glucocorticoids [63] and thyroid dysfunction [64, 65]. CysC was proposed as a marker of GFR potentially superior to serum creatinine [60]. A meta-analysis of 46 cross-sectional studies including adults and children suggested the superiority of CysC compared to serum creatinine and to creatinine-based equations in the estimation of GFR [60]. CysC and microalbuminuria are considered early markers of kidney damage [66]. Finally, CysC was the best predictor of kidney failure and death from cardiovascular disease in a longitudinal cohort study of 4637 older people [67]. Nevertheless, even CysC could be affected by changes in body composition. Indeed, fat-free mass, a parameter inversely related to age, affects CysC level, and in older patients with chronic kidney disease CysC-based GFR estimation improves when fat-free mass is taken into account [68].

3.3. GFR Equations. Equations have been proposed in order to provide the physician an easy way of calculation and an accurate estimation of kidney function (Table 2). The Cockcroft and Gault equation [69] was the first published and still widely used. However, it does not take into account the variability of creatinine production [69, 70], and it systematically overestimates the GFR in obese or edematous patients [70].

The Modification of Diet in Renal Disease Study (MDRD) equations were derived on the basis of measured ^{125}I-iothalamate clearance and were normalized to 1.73 m^2 body surface area (BSA) [71]. Their use has been endorsed by national professional health care organizations [52]. Additionally, the original MDRD equations were reexpressed to account for the difference resulting from the standardization of serum creatinine measurements to the isotope dilution mass spectrometry (IDMS) reference method [72]. Nevertheless, MDRD equations were found to lose accuracy in selected subgroups of patients, such as those with normal renal function, type 1 diabetes, elderly, and kidney transplant recipients (i.e., subgroups not included in the MDRD study population) [73–75]. Indeed the MDRD Study equations were developed in people with CKD, and as such their major limitations are imprecision and systematic underestimation of measured GFR at higher levels of kidney function [76].

The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equations were developed in an attempt to improve the estimation of GFR in patients older than 70 years [77]. Creatinine-based CKD-EPI equation is based on standardized serum creatinine [77, 78]. It was found to be as accurate as the MDRD equation at GFR less than 60 mL/min/1.73 m^2 and more accurate than the MDRD equation at higher GFR values [77, 79]. To overcome the bias due to variability of serum creatinine, equations based on CysC alone (CKD-EPI$_{CYS}$) or in combination with creatinine (CKD-EPI$_{CR\text{-}CYS}$) have been developed. Overall, the CKD-EPI$_{CR\text{-}CYS}$ equation had better precision and accuracy than that based on creatinine alone or CysC alone [80]. Nevertheless, even CKD-EPI study population included relatively few participants older than 70 years of age.

Recently, two eGFR equations were developed and validated in a population of older adults aged 70 years or more enrolled in the Berlin Initiative Study (BIS): the BIS1 equation based on serum creatinine alone and the BIS2 equation based on both serum creatinine and cystatin C [81]. BIS equations showed excellent agreement with directly measured GFR [81]. A study comparing the performances of BIS-1, MDRD, and CKD-EPI equations in estimating GFR in older patients showed that BIS-1 was the most reliable for assessing renal function in older white patients, especially in those with CKD stages 1 to 3 [82]. In old people (mean age: 85 years), CKD-EPI$_{CR\text{-}CYS}$ and BIS2 equations showed better accuracy compared to MDRD, CKD-EPI, and BIS1 estimates.

4. Estimated GFR and Outcomes Relevant to Older Patients

Although the overall prevalence of CKD is roughly 10% in the general population, it increases with age [2]. Additionally, CKD is associated with several comorbid conditions in older people, such as cardiovascular disease and disability, which in turn increase the risk of hospitalization and death [3, 4].

Several studies have demonstrated that a reduction of eGFR is associated with lower scores in subjective physical function and physical activity scales [83]. In an elderly population study, declining eGFR was associated with increasing risk of worsening disability, defined as the loss of ≥ 1 Activities of Daily Living (ADL) over the 6-year follow-up, at GFR below 60 mL/min [84]. In the Cardiovascular Health Study cohort, including 5,888 persons aged 65 years or older [85], the cross-sectional prevalence of a limitation in ADL was 12% among participants with CKD compared to 7% among participants without CKD. Finally, frailty, a condition characterized by a decline in physical function and functional capacity predisposing to disability, is highly prevalent among patients with CKD [86].

Osteoporosis and CKD are both common conditions in older adults and may be associated with substantial

Table 2: Equations for estimating renal function.

Cockcroft and Gault [69]	eCCr = (140−age) × weight in kg/(72 × Scr), ×0.85 in females
Six-variable MDRD [71]	$170 * [\text{Scr}]^{-0.999} * [\text{age}]^{-0.176} * [\text{BUN}]^{-0.170} * [\text{serum albumin}]^{0.318}$, ∗0.762 in females, ∗1.180 if black
Four-variable MDRD [71]	$[186.3 \times (\text{Scr})^{-1.154} \times (\text{age})^{-0.203}]$, ×0.742 in females, ×1.212 if black
CKD-EPI (creatinine) [77]	Female (Scr ≤ 0.7), eGFR = $144 \times (\text{Scr}/0.7)^{-0.329} \times (0.993)^{\text{Age}}$ (Scr > 0.7), eGFR = $144 \times (\text{Scr}/0.7)^{-1.209} \times (0.993)^{\text{Age}}$ Male (Scr ≤ 0.9), eGFR = $141 \times (\text{Scr}/0.9)^{-0.411} \times (0.993)^{\text{Age}}$ (Scr > 0.9), eGFR = $141 \times (\text{Scr}/0.9)^{-1.209} \times (0.993)^{\text{Age}}$
CKD-EPI (cystatin C) [80]	(Scys ≤ 0.8), eGFR = $133 \times (\text{Scys}/0.8)^{-0.499} \times 0.996^{\text{Age}}$ [×0.932 if female] (Scys > 0.8), eGFR = $133 \times (\text{Scys}/0.8)^{-1.328} \times 0.996^{\text{Age}}$ [×0.932 if female]
CKD-EPI (cystatin C-creatinine) [80]	Female (Scr ≤ 0.7) (Scys ≤ 0.8), eGFR = $130 \times (\text{Scr}/0.7)^{-0.248} \times (\text{Scys}/0.8)^{-0.375} \times 0.995^{\text{Age}}$ [×1.08 if black] (Scr ≤ 0.7) (Scys > 0.8), eGFR = $130 \times (\text{Scr}/0.7)^{-0.248} \times (\text{Scys}/0.8)^{-0.711} \times 0.995^{\text{Age}}$ [×1.08 if black] Female (Scr > 0.7) (Scys ≤ 0.8), eGFR = $130 \times (\text{Scr}/0.7)^{-0.601} \times (\text{Scys}/0.8)^{-0.375} \times 0.995^{\text{Age}}$ [×1.08 if black] (Scr > 0.7) (Scys > 0.8), eGFR = $130 \times (\text{Scr}/0.7)^{-0.601} \times (\text{Scys}/0.8)^{-0.711} \times 0.995^{\text{Age}}$ [×1.08 if black] Male (Scr ≤ 0.9) (Scys ≤ 0.8), eGFR = $135 \times (\text{Scr}/0.9)^{-0.207} \times (\text{Scys}/0.8)^{-0.375} \times 0.995^{\text{Age}}$ [×1.08 if black] (Scr ≤ 0.9) (Scys > 0.8), eGFR = $135 \times (\text{Scr}/0.9)^{-0.207} \times (\text{Scys}/0.8)^{-0.711} \times 0.995^{\text{Age}}$ [×1.08 if black] Male (Scr > 0.9) (Scys ≤ 0.8), eGFR = $135 \times (\text{Scr}/0.9)^{-0.601} \times (\text{Scys}/0.8)^{-0.375} \times 0.995^{\text{Age}}$ [×1.08 if black] (Scr > 0.9) (Scys > 0.8), eGFR = $135 \times (\text{Scr}/0.9)^{-0.601} \times (\text{Scys}/0.8)^{-0.711} \times 0.995^{\text{Age}}$ [×1.08 if black]
BIS1 [81]	$3736 \times \text{creatinine}^{-0.87} \times \text{age}^{-0.95} \times 0.82$ (if female)
BIS2 [81]	$767 \times \text{cystatin C}^{-0.61} \times \text{creatinine}^{-0.40} \times \text{age}^{-0.57} \times 0.87$ (if female)

eCCr: estimated creatinine clearance; Scr: serum creatinine; BUN: blood urea nitrogen; Scys: serum cystatin C; MDRD: Modification of Diet in Renal Disease; CKD-EPI: Chronic Kidney Disease Epidemiological Collaboration; BIS: Berlin Initiative Study.

morbidity [87]. In the NHANES III study, a double risk of hip fractures was observed among persons with eGFR below 60 mL/min/1.73 m^2 compared to the general population [88]. Additionally, several studies have shown that hypocalcemia, hyperphosphatemia, hyperparathyroidism, vitamin D deficiency (both 25-OH and 1,25-OH vitamin D), and metabolic acidosis play a key role in increasing the risk of fractures in older people [89]. Disorders of mineral-bone metabolism leading to abnormal bone architecture and fracture may in part explain the relationship between CKD and low physical function.

Cognitive impairment has been frequently observed in patients with CKD especially in older subjects [90]. CKD is related to a wide range of deficits in cognitive functioning, including verbal and visual memory and organization, and components of executive functioning and fluid intellect. Small vessel disease, which is known to contribute to the pathophysiology of CKD [91], can lead to cerebral ischemic lesions, both in the form of silent or subclinical cerebral infarcts or white matter lesions, increasing the risk of cognitive decline and dementia [92]. In the Cardiovascular Health Study, a 37% higher risk of incident dementia was found among older adults with CKD during a median follow-up period of 6 years [93]. Patients in all stages of CKD have a higher risk for development of cognitive impairment and this may be a major determinant in their quality of life and cognitive impairment is associated with an increased risk of death in dialysis patients [94].

Stroke is the most frequent neurological disease and represents a continuously evolving medical and social problem [95]. A Taiwanese study showed that CKD itself may represent a causal risk factor for stroke beyond traditional cardiovascular risk factors. Indeed, patients primarily affected by CKD have higher risk of stroke compared to the general population [96]. Thus, patients with chronic renal failure should be carefully monitored for prevention of stroke regardless of the presence and severity of traditional cardiovascular risk factors. MRIs studies have demonstrated that patients with CKD have a higher prevalence of subclinical brain infarcts and deep white matter lesions, even after adjusting to traditional risk factors such as smoking, hypertension, and diabetes [97]. There are only few data on the impact of renal dysfunction on mortality in patients suffering from stroke. In the study of Hojs Fabjan et al. [65], decreased eGFR was associated with higher short-term (in-hospital) mortality among patients with ischemic stroke.

Depression is frequently observed in elderly patients with CKD [98]. This is of great importance because the presence of depression in CKD is associated with poorer outcomes such as hospitalizations, progression to dialysis, and increased morbidity and mortality [99]. About 20% to 30% of patients with CKD have clinical depression [98]. In a retrospective study of elderly patients with non-dialysis-dependent CKD stages 1–5 followed up for 4.7 years about 30% had depression, which was associated with significantly higher age-adjusted mortality rates [100].

Anemia is commonly related to CKD, and it is typically normocytic, normochromic, and hypoproliferative [101]. In prospective randomized controlled trials, anemia is related to mortality, nonfatal cardiovascular events, left ventricular hypertrophy, hospitalizations, and progression of kidney disease [102]. Data from Longitudinal Aging Study Amsterdam with a follow-up of 3 years indicate that anemia in older adults doubles the risk of recurrent falls [103]. This comorbidity can

be particularly dangerous in older adults, since it may also lead to other negative outcomes such as impaired physical function and cognitive decline [104].

Obstructive sleep apnea (OSA) is an important and common comorbidity in patients with CKD [105]. OSA increases the risk of systemic hypertension and vascular disease [106], both of which are common complications of CKD. OSA may also accelerate the deterioration of renal function in patients with CKD directly through the effect of hypoxia on the kidney [107] or indirectly by increasing systemic blood pressure, inflammatory cytokines, and sympathetic nervous system activity [108]. This condition leads to an impairment of sleep quality and daytime function [109].

Chronic obstructive pulmonary disease (COPD) dramatically increases with age and it is a progressive, debilitating respiratory condition [110]. COPD is characterized by typical symptoms including lung airflow limitations, cough, and difficulty breathing. This condition may lead to emphysema and chronic bronchitis and plays a pivotal role in conditioning the health status and having major prognostic implication. An important study of Antonelli Incalzi et al. showed that COPD is significantly associated with CKD [111].

Cachexia is an important cause of death in elderly CKD patients, even if it is not completely clear whether malnutrition is part of a cause-effect relationship. In adult CKD patients, decreased appetite plays a major role in wasting. Wasting has also been linked to high levels of leptin and proinflammatory cytokines [112]. Malnutrition is considered to be a uremic risk factor for cardiovascular disease, leading to an increased cardiovascular mortality. Moreover, atherosclerosis is considered an inflammatory disease, and chronic inflammation may reduce the patient's appetite and increase the rate of protein depletion, wasting, and hypercatabolism [113].

When renal function declines, many drugs or their active metabolites that depend on renal excretion may accumulate with an increase of potential toxicity, and patients with renal disease may be more vulnerable to a given drug effect [114]. This necessitates dosage adjustment in order to prevent adverse drug reactions (ADRs) [115]. This is especially important in older people, who are more vulnerable to adverse drug reactions due to an increased prevalence of renal impairment (partly due to structural and functional changes in the kidney as a result of aging), polypharmacy, and frailty [116]. It is worth noting that reduced eGFR is associated with increased risk of adverse drug reactions from water-soluble drugs even when serum creatinine levels are within the normal range [25]. High risk combination of drugs in people treated with complex polypharmacy regimens deserves to be mentioned. An example of high risk combination is the simultaneous use of diuretics, nonsteroidal anti-inflammatory drugs (NSAIDs), ACE inhibitors (ACEI), and/or angiotensin receptor blockers (ARBs) (the so-called triple whammy) that may impair kidney function [117]. Older patients are at greater risk of experiencing this triple whammy effect, and although medications for hypertension and heart failure have the important potential to reduce the likelihood of stroke and myocardial infarction, much care must be taken to ensure that this is not achieved at the price of inducing renal failure, especially in the elderly [118].

5. Potential Issues in Estimating GFR in Older Patients

Despite the huge amount of findings described above, eGFR has some important limitations when applied to older and frail patients. Indeed, whatever is the equation used to estimate GFR, the interpretation of results obtained in older people may not be so easy.

An example of how it can be difficult to understand the meaning of these measures in frail and older patients comes from studies showing the existence of a U-shaped relationship between eGFR and mortality. By using MDRD equation, Cox et al. showed for the first time that all-cause mortality risk increased in subjects with eGFR higher than 89 mL/min/1.73 m^2 enrolled in the Hull and East Yorkshire renal and diabetes registers [119]. An increased risk of cardiovascular events and a near significant increase in total and cardiovascular mortality were observed in octogenarians with CKD-EPI-based eGFR values of 75 mL/min/1.73 m^2 or more enrolled in the HYVET trial [120]. All-cause mortality, but not mortality from myocardial infarction or stroke, was significantly increased in patients with MDRD-based eGFR greater than or equal to 90 mL/min/1.73 m^2 in the large population study of the Alberta Kidney Disease Network repository [121]. Finally, a similar U-shaped relationship between eGFR and mortality was observed in the Cardiovascular Health Study with creatinine-based CKD-EPI, but not with cystatin-C-based CKD-EPI equation [122]. There are at least three potential mechanisms which could be invoked to explain this apparent discrepancy in the relationship between eGFR and mortality [123]: (i) a direct harmful effect exerted by high eGFR on kidney hemodynamics, as it has been demonstrated in obese and diabetic individuals [124, 125] (however, with this phenomenon one would expect higher eGFR to associate more strongly with progression of kidney disease than with mortality, which has not been demonstrated [123]); (ii) an effect due to unmeasured or residual confounding from other detrimental pathophysiological processes, such as excess body weight as observed in obesity [123]; (iii) high eGFR which may not only be a reflection of GFR, but also reflects inflammation, frailty, and/or muscle loss which may contribute to reducing serum creatinine. Though not definitely demonstrated, this latter mechanism is more likely to explain the observed U-shaped relationship between eGFR and mortality. Indeed, both low serum creatinine and low 24 h urine creatinine are associated with adverse outcomes [126]. Interestingly, such a U-shaped relationship was not observed with CysC-based eGFR [122]. Thus, despite the findings showing that even CysC could be affected by changes in body composition [68], CysC-based eGFR likely represents the most meaningful and reliable method to estimate kidney function in older patients.

Another potential issue is represented by the difference among kidney function estimates obtained with different equations. Structural differences between equations likely

account for discrepancy. Indeed, the Cockcroft-Gault equation intends to measure the creatinine clearance, whereas all other equations listed in Table 2 are proxies of the GFR. Creatinine clearance is influenced by tubular secretion and extrarenal clearance of creatinine as well as by drugs affecting the renal handling of creatinine [127]. For this reason, creatinine clearance usually exceeds GFR, whereas Cockcroft-Gault equation usually provides lower values than GFR equations, and age and weight are main sources of discrepancy [128]. This makes Cockcroft-Gault and GFR equations not interchangeable in the estimation of renal function. Implications of the above findings are straightforward; dosing requirement of a given drug cleared by the kidney will dramatically change depending upon the equation used to obtain an estimate of kidney function. This is especially true for the Cockcroft-Gault versus MDRD- or CKD-EPI-based values and might result in underdosing and, then, lack of efficacy or overdosing and, then, risk of ADRs [129]. As a general rule, it seems reasonable to suggest adjusting kidney cleared drugs dosing according to the recommendation provided by the manufacturer and if no equation is recommended, refer to the one proved more reliable in the reference population [129]. Further studies using newly available equations specifically developed in older patients (BIS equations) [81] are needed to verify whether the greater accuracy in estimating GFR could be translated into recommendations for drug dosing.

6. Conclusions

The prevalence of CKD increases with age, and CKD is often associated with several comorbid conditions and adverse outcomes in older patients. Thus, the availability of an accurate method for estimating kidney function in this highly vulnerable population would be of paramount importance.

Methods based on clearance of radiolabelled or non-radiolabelled exogenous markers are expensive and not easy to apply in clinical settings. Kidney function estimate based on simple determination of serum creatinine level is hardly reliable in older patients because of the frequent loss of muscle mass secondary to age itself and aging-related conditions. Additionally, the clearance of creatinine is often biased in older patients due to inaccurate or incomplete urine collection. GFR equations may facilitate the estimation of kidney function in older patients. However, all of them have their own limitations, and no equation proved to be better than the other ones. New equations specifically developed for use in older people, especially those based on serum cystatin C, hold promises. However, few studies have been carried out to definitely accept them as the reference method to estimate kidney function in older patients in clinical practice. Further research is needed to verify whether these new equations can overcome the above described issues in prognostic stratification and dosing of kidney cleared drugs.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Catechol-O-methyltransferase (COMT) Genotype Affects Age-Related Changes in Plasticity in Working Memory: A Pilot Study

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Objectives. Recent work suggests that a genetic variation associated with increased dopamine metabolism in the prefrontal cortex (catechol-O-methyltransferase Val158Met; COMT) amplifies age-related changes in working memory performance. Research on younger adults indicates that the influence of dopamine-related genetic polymorphisms on working memory performance increases when testing the cognitive limits through training. To date, this has not been studied in older adults.*Method*. Here we investigate the effect of COMT genotype on plasticity in working memory in a sample of 14 younger (aged 24–30 years) and 25 older (aged 60–75 years) healthy adults. Participants underwent adaptive training in the *n*-back working memory task over 12 sessions under increasing difficulty conditions. *Results*. Both younger and older adults exhibited sizeable behavioral plasticity through training ($P < .001$), which was larger in younger as compared to older adults ($P < .001$). Age-related differences were qualified by an interaction with COMT genotype ($P < .001$), and this interaction was due to decreased behavioral plasticity in older adults carrying the Val/Val genotype, while there was no effect of genotype in younger adults.*Discussion*. Our findings indicate that age-related changes in plasticity in working memory are critically affected by genetic variation in prefrontal dopamine metabolism.

1. Introduction

Working memory performance declines with normal aging [1], and evidence from animal models [2], human imaging [3], and pharmacological challenge studies [4] suggests that age-related declines in working memory are associated with decreased dopaminergic neurotransmission. The enzyme catechol-O-methyltransferase (COMT; EC 2.1.1.6) degrades the neurotransmitters dopamine, epinephrine, and norepinephrine, and a functional polymorphism in the COMT gene (Val158Met) accounts for a fourfold variation in enzyme activity, resulting in a substantial decrease in dopamine availability in carriers of the Val/Val genotype [5]. The prefrontal cortex, a region which is frequently associated with executive function and working memory, seems to be particularly prone to an increased COMT activity. It has been argued that this is due to the lack of the dopamine transporter (DAT) and the resulting particular importance of COMT for the degradation of dopamine [6]. There is initial experimental evidence that the COMT polymorphism affects working memory performance as well as associated brain activation in healthy older adults [7, 8], and recent evidence suggests that COMT effects are amplified in older as compared to younger adults [9]. Overall, however, evidence is conflicting: a population-based study detected the effects of COMT on maintenance and updating in working memory using a letter-number sequencing task [10], while recent cross-sectional studies in younger [11, 12] and older adults [13] detected no

effect of COMT genotype on working memory functions and general cognitive ability "*g*", respectively.

It has been assumed that the influence of polymorphisms in genes related to the dopamine system on working memory performance may be more pronounced in a training context than in single-assessment performance scores, possibly because the influence of confounding factors is reduced [14]. Indeed, two recent working memory training studies conducted with younger adults (range 20–31 years) revealed post- but not pretraining differences between carriers of different DAT [15] and LIM homeobox transcription factor 1, alpha (LMX1A) [16] genotypes.

The testing-the-limits approach [17, 18] has been introduced in cognitive aging research as a method to use adaptive training procedures in order to estimate behavioral (cognitive) plasticity, as measured, for example, in individual differences in the maximum gain that can be induced during behavioral training in a cognitive task. However, effects of dopaminergic neurotransmission on age-related changes in behavioral plasticity in working memory, that is, training-related increases using adaptive training studies, have not hitherto been studied. Here we investigate the effect of COMT genotype on plasticity in working memory in younger and older healthy adults, using an adaptive (testing-the-limits) training procedure in the *n*-back working memory task over 12 sessions. We hypothesized that (i) behavioral plasticity in younger adults would be larger than in older adults, (ii) carriers of the Val/Val genotype of the COMT gene would show decreased behavioral plasticity in working memory, as compared to carriers of a Met allele, and (iii) the effect of COMT genotype on plasticity would be larger in older than in younger adults. Given reports that effects of COMT genotype on working memory performance may be moderated by gender [19] and educational attainment [20], we controlled for these variables in our study.

2. Methods

2.1. Subjects. 15 younger (range 24–30 years) and 33 older (range 60–75 years) healthy adults were recruited from the community. One participant in the younger group and 6 participants in the older group denied blood sampling, and 2 older participants dropped out of the training program. Therefore, the final analysis sample consisted of 14 younger and 25 older participants (see Table 1). All older participants performed within the age-related normal range (±1 SD) for tests of processing speed, executive functions, memory, and attention from the CERAD (Consortium to Establish a Registry for Alzheimer's Disease [21]) neuropsychological battery. Participants were task naïve (i.e., they never performed the task prior to our study) and had no history of psychiatric or neurological diseases. The study was approved by the local Ethical Committee and conducted in accordance with Declaration of Helsinki principles (1964). Written informed consent was obtained from all participants.

2.2. Working Memory Assessment. All participants completed 12 sessions (45 minutes each) of adaptive *n*-back working memory training over a period of 4 consecutive weeks (three sessions per week). Before the first training session, 20 mL of blood was collected and neuropsychological testing was conducted. The training task consisted of a computerized numerical version of the *n*-back paradigm [22], during which white digits ranging from 0 to 9 were visually presented for the duration of 500 ms in the center of an otherwise black screen in a random sequence. Each block consisted of 20 to 28 trials including 5 to 7 targets. Task difficulty was adaptively increased by reducing the interstimulus interval and by increasing the memory load from 2-back up to 5-back (for specific details of the training procedure please see [23]). Participants began training at session 1 with difficulty level 1 (0-, 1-, and 2-back; interstimulus interval = 1800 ms). If a subject successfully completed the first run (that is 3 blocks of 0-back, 3 blocks of 1-back, and 3 blocks of 2-back) with a hit rate of 80% or above within each block and with no false alarms, the next difficulty level was introduced in the following run. From level 1 to level 5, interstimulus interval gradually decreased from 1800 to 1000 ms in steps of 200 ms. At level 6, the next *n*-level was introduced (3-back) and 1-back was removed; that is, participants completed 3 blocks of each 0-, 2-, and 3-back. In addition, interstimulus interval was set back to 1800 ms. This procedure continued until 5-back was introduced at level 16.

2.3. Neuropsychological Assessment. For neuropsychological screening, neuropsychological tests were selected for measuring short-term memory (Digit Span Forward, Digit Span Backward), processing speed (Digit Symbol), episodic memory (CERAD Delayed Recall), executive functions (Verbal Fluency), and reasoning (Raven's SPM, Figural Relations).

Short-Term Memory Tasks. To obtain an estimate of each participant's short-term memory capacity, Digit Span Forward and Backward from the Wechsler Adult Intelligence Scale (WAIS, [24]) were administered. Two trials of each list length were presented. If participants failed to repeat both trials of a certain list length, the assessment of this task was terminated. The score used in the following analyses was determined by the length of the longest correctly repeated trial.

Processing Speed Task. The Digit Symbol Substitution subtest (Digit Symbol) of the WAIS [24] was included to assess mental processing speed and attention. In Digit Symbol, participants were asked to copy symbols as quickly as possible into empty boxes located below a random sequence of numbers ranging from 1 to 9 according to a specific coding key. The score used for analyses was the number of correct symbols completed within 60 seconds.

Episodic Memory Task. As a measure of episodic memory, all participants performed the memory task from the neuropsychological test battery of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD; [21]). Participants were asked to remember 10 words that were presented to them sequentially three times in varying order and recall the words after a delay. For further analyses, the number of correctly

Table 1: Characteristics of the sample at baseline assessment. Unless otherwise indicated, values represent means ± standard deviations.

	Younger ($N = 14$)			Older ($N = 25$)		
	Val/Val ($N = 3$)	Any Met ($N = 11$)	$T_{(12)}$ (P)	Val/Val ($N = 11$)	Any met ($N = 14$)	$T_{(23)}$ (P)
Age	26.00 ± 1.00	26.09 ± 2.17	0.069 (0.946)	67.36 ± 4.34	64.64 ± 3.37	1.77 (0.090)
Gender N (female/male)	1/2	8/3	$\chi^2(14.1) = 1.59,\ P = 0.224$	5/6	7/7	$\chi^2(25.1) = 0.05, P = 0.821$
Years of education	18.67 ± 0.58	18.18 ± 1.74	0.47 (0.651)	15.46 ± 3.15	16.88 ± 3.62	1.10 (0.281)
Minimental state examination total score	30.00 ± 0.00	29.91 ± 0.30	0.51 (0.621)	29.27 ± 1.01	29.64 ± 0.84	1.00 (0.328)
n-back performance (% correct)	96.23 ± 2.39	97.73 ± 1.81	1.17 (0.266)	88.47 ± 5.96	91.53 ± 5.29	1.36 (0.188)
Digit Span Forward	7.67 ± 0.58	6.91 ± 0.83	1.46 (0.169)	6.64 ± 0.81	7.14 ± 1.03	1.34 (0.194)
Digit Span Backward	6.67 ± 0.58	6.00 ± 0.89	1.20 (0.252)	5.27 ± 1.42	5.21 ± 1.25	0.11 (0.914)
Digit Symbol	46.00 ± 5.29	46.00 ± 7.59	0.00 (1.00)	32.73 ± 6.80	32.79 ± 6.66	0.02 (0.983)
CERAD Delayed Recall	9.00 ± 1.73	9.63 ± 0.50	1.16 (0.269)	7.91 ± 1.87	8.07 ± 1.77	0.22 (0.826)
Verbal Fluency	18.33 ± 1.53	20.55 ± 4.25	0.86 (0.404)	18.00 ± 6.99	18.14 ± 5.48	0.06 (0.955)
Figural Relations	26.67 ± 2.52	24.45 ± 2.70	1.27 (0.227)	18.36 ± 3.93	20.00 ± 3.82	1.05 (0.305)
Raven's SPM	22.00 ± 2.65	22.36 ± 1.24	0.14 (0.889)	14.91 ± 4.18	17.79 ± 3.26	1.94 (0.065)

recalled items after a delay of 15 minutes was used (CERAD Delayed Recall).

Executive Functions Task. Verbal Fluency requires the ability to generate words while monitoring previously recalled words and following specific rules. Verbal Fluency was assessed by a German version of the Controlled Oral Word Association Test (COWAT; [25]). Participants were asked to generate as many words as possible starting with the letter "S" within 60 seconds (not including proper names or names of places and cities).

Abstract Reasoning Tasks. Abstract reasoning abilities were measured by Raven's Standard Progressive Matrices (Raven's SPM; [26]) and by the Figural Relations subtest of a German intelligence test (Leistungspruef system; [27]). To solve these tasks, participants were required to identify patterns of nonverbal symbols. In Raven's SPM, they were instructed to find a matching item to complete a pattern, while in the Figural Relations they had to mark the nonmatching item of a pattern of symbols. Both reasoning tasks were timed and the scores were derived from the number of correct items accomplished within 7.5 minutes (Raven's SPM) or 3 minutes (Figural Relations), respectively.

2.4. Blood Sampling and Analyses. Blood was sampled and stored at a temperature below −20°C. Genotype analyses were conducted using commercially available kits (Biologis, Frankfurt, Germany). Specifically, whole blood was thawed, DNA was extracted, and exon 4 of the COMT gene was amplified using reverse PCR and genotyped using pyrosequencing. Overall, there were 5 carriers of Met/Met, 20 heterozygotes, and 14 carriers of the Val/Val genotype. The distribution was not significantly different from Hardy-Weinberg equilibrium ($\chi^2 = .27$, $P = .603$). Carriers of either Val/Met or Met/Met COMT genotype were classified into one group (any Met) and contrasted with Val/Val carriers (see [9, 10] for a similar approach).

2.5. Statistics. Statistical analyses were performed with SPSS version 17.0 (SPSS Inc., Chicago, IL). For our hypotheses, we performed a 2 (older versus younger) × 2 (Val/Val versus any Met) × 12 (training session 1 through 12) mixed model between-group within-subjects analysis of variance with α set to .05 and β set to .8. An a priori power analysis with a minimum cell size of $N = 3$, a minimum detectable difference of two n-back levels for main effects (hypotheses 1 and 2), and one level for interaction effects (hypothesis 3) revealed a power of >.80 to detect main effects and of >.60 to detect within-subjects interactions (PASS software, NCSS Inc., Kaysville, Utah).

3. Results

3.1. Differences between Genotype Groups at Pretest. Demographic characteristics and neuropsychological test performance of the study sample, as a function of age and genotype, are reported in Table 1. Within the younger group, there were no significant differences between carriers of the Val/Val COMT genotype and Met allele carriers with respect to age, gender distribution, years of education, initial n-back performance, mental status, and performance in neuropsychological tests (all Ps > .16).

Within the older group, no significant differences between Val/Val and any Met COMT genotypes were found with respect to age, gender distribution, years of education, mental status, and initial n-back performance (all Ps > .09). Met allele carriers showed a trend towards better performance in Raven's SPM compared to Val/Val carriers ($T_{23} = 1.94$, $P = .065$). In all other neuropsychological tests, no significant differences were found between the genotype groups (all Ps > .18).

3.2. Training Gains, Age, and COMT Genotype. After 12 sessions of n-back training, younger carriers of the Val/Val genotype achieved n-back difficulty level 14 on average ($M = 14.00$, SD = 4.36), Met allele carriers reached level 12 ($M = 11.55$, SD = 2.94). T-tests showed that this difference was not significant ($T_{12} = 1.17$, $P = .265$). Older Val/Val carriers accomplished level 4 ($M = 4.45$, SD = 1.57) and Met allele carriers reached level 6 ($M = 6.07$, SD = 2.06, $T_{23} = 2.16$, $P = .042$).

To investigate the influence of age and COMT genotype on the level progression through adaptive training, a 2 (older versus younger) × 2 (Val/Val versus any Met) × 12 (training session one through 12) mixed model between-group within-subjects analysis of variance was conducted and revealed a main effect of age group ($F_{1,35} = 44.71$, $P < .001$) but not of genotype ($F_{1,35} = .95$, $P = .337$). There was a large within-subjects effect for training session ($F_{11,385} = 151.31$, $P < .001$), which was qualified by both a training × age group ($F_{11,385} = 37.42$, $P < .001$) and a training × age group × genotype interaction ($F_{11,385} = 4.53$, $P < .001$). As shown in Figure 1, these interactions were due to an increased overall plasticity in younger as compared to older adults and a decreased plasticity in older carriers of the Val/Val genotype as compared to older carriers of any Met allele.

To control for effects of gender and education, a mixed model between-group within-subjects analysis of covariance was conducted. Again, a main effect of age group was revealed ($F_{1,33} = 35.79$, $P < .001$), indicating that younger adults achieve higher training gains in n-back compared to older adults irrespective of gender and education. No main effects of genotype ($F_{1,33} = .88$, $P = .357$), gender ($F_{1,33} = 1.03$, $P = .318$), and education ($F_{1,33} = .47$, $P = .500$) were found. Again, significant training × age group ($F_{11,363} = 29.73$, $P < .001$) and training × age group × genotype interactions were revealed ($F_{11,363} = 2.96$, $P = .001$).

A separate 2 (genotype) × 12 (training session) analysis of variance within the older group revealed a significant training × genotype effect ($F_{11,253} = 2.57$, $P = .004$), suggesting that the slope of level progression is less steep in older Val/Val carriers compared to older Met allele carriers.

In older adults, post hoc t-tests showed that genotype-related differences in the level progression were only significant in the last quarter of the training program (session 10:

T_{23} = 2.37, P = .027; session 11: T_{23} = 2.19, P = .039; session 12: T_{23} = 2.16, P = .042, see Figure 1). In younger participants, no significant genotype-related differences in the level progression were revealed by post hoc t-tests (all Ps > .26).

4. Discussion

Our results indicate that differences in dopamine metabolism, as related to a polymorphism (Val158Met) of the COMT gene, may affect working memory plasticity in older adults. Both younger and older adults exhibited sizeable behavioral plasticity, which was larger in younger as compared to older adults. However, these age-related differences were qualified by an interaction with COMT genotype. This interaction was due to decreased behavioral plasticity in older adults homozygous for the Val allele, as compared to older Met allele carriers. Specifically, our data suggest that older Val/Val carriers can increase their n-back performance at a lower rate (less steep slope in level progression; see Figure 1) and reach their maximum performance at a lower difficulty level compared to Met allele carriers. We did not detect a similar effect in younger adults. Our findings are consistent with previous results [9], which showed that effects of the COMT genotype are larger in older than in younger adults. These age-related differences have been interpreted within the framework of a nonlinear (inverse U-shaped) relationship between prefrontal dopamine availability and executive cognitive performance. Due to an age-related decrease of dopaminergic neurotransmission, older adults represent the left and relatively steep section of this proposed dopamine/performance inverse U-curve. Therefore, an additional reduction in dopamine availability caused by an increased enzymatic activity of the Val/Val COMT genotype seems to have a stronger impact on working memory performance in older compared to younger adults. The testing-the-limits approach [17, 18] employed in our study suggests that the behavioral malleability of working memory functions in older adults critically depends on dopamine metabolism.

No differences in neuropsychological test performance were detected at baseline assessment. However, the sensitivity of the testing-the-limits approach [17] may have enabled us to detect differences even in a small sample since adaptive training has been suggested to magnify individual differences in cognitive performance [28]. Therefore, divergent nonfindings from other studies may be due to the one-time assessment of cognitive function and also due to the wide age range typically present in these samples [10, 11, 13].

Even though significant effects of age, training, and genotype were detected in the current study, sample size is a limitation and further independent replication is needed. Another point to consider is that the effects of the COMT genotype have been shown to interact with certain variants of other genes associated with dopaminergic neurotransmission, such as the DAT gene [15, 29, 30] and the D-amino acid oxidase activator (DAOA [G72]) gene [31]. Furthermore,

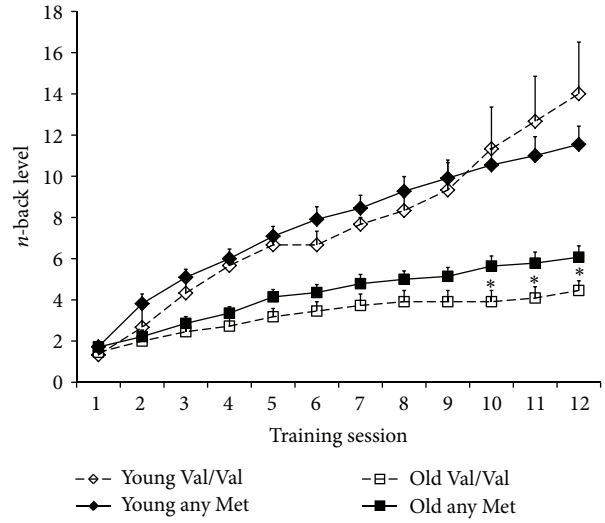

Figure 1: Behavioral plasticity (level attained in each session) in younger (diamonds) and older adults (squares) as a function of genotype (dotted line represents Val/Val; continuous line represents any Met). Error bars reflect standard errors of the mean. Significant differences in n-back levels within age groups are marked by asterisks.

interactions between genotypes and phenomena of age-related cognitive decline (see [32] for review), such as loss of gray and white matter volume [33] and changes in brain functioning and connectivity [34], could be investigated in regard to working memory plasticity in future research. Another valuable extension of the current study would be to include additional samples of different age groups within the age range of below 20, between 30 and 60, and above 75 years to gain a more profound understanding of dopamine-related genetic influences on plasticity across the entire lifespan. Finally, as suggested by Slagter [35], future working memory training studies would benefit from longer training periods (e.g., 25 training sessions) and follow-up measurements to further understand the different shapes of learning curves across different age and genotype groups and to test the stability of training effects over time.

5. Conclusion

Working memory declines with age [1], and age-related decline of, for example, cortical D1 receptors [3] has been shown to correlate with decline in cognitive function. Focusing on a genetic variation in dopamine metabolism, we detected a potential link between differences in dopaminergic neurotransmission and behavioral plasticity in working memory in the elderly. Further understanding of the influences of genetic variations on working memory plasticity could have strong implications on designing individually tailored training programs in healthy and cognitively impaired older adults.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Arterial Elasticity, Strength, Fatigue, and Endurance in Older Women

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Arterial health may influence muscle function in older adults. Study purpose was to determine whether arterial elasticity is related to strength, central and peripheral fatigue, fatigue at rest, and treadmill endurance. Subjects were 91 healthy women aged >60. Treadmill endurance and maximal oxygen uptake (VO_2 max) were measured. Peripheral and central fatigue for the knee extensors were evaluated using two isometric fatigue tests (one voluntary and one adding electrical stimulation). Arterial elasticity was determined using radial artery pulse wave analysis. Linear multiple regression was used in statistical analysis. Large artery elasticity was associated with central fatigue ($P < 0.01$) and treadmill endurance ($P < 0.02$) after adjusting for VO_2 max and knee extension strength. Subjective fatigue at rest was related to large artery elasticity after adjusting for ethnic origin (<0.02). Strength was significantly related to small artery elasticity after adjusting for ethnic origin, leg lean tissue, age, and blood pressure. Arterial elasticity is independently related to strength and fatigue in older women, especially in the central nervous system where arterial elasticity is independently related to perceptions of fatigue at rest and central fatigue. These results suggest that arterial health may be involved with the ability of the central nervous system to activate muscle in older women.

1. Introduction

A large proportion of older adults commonly report fatigue [1]. In addition, older adults who complain of fatigue have poor functional status compared with those who report less fatigue [2]. Finally, central fatigue (a brain mediated decreased ability to activate muscle neurons) is a poorly understood phenomena that contributes to fatigue in both young and old. For example, central fatigue is not increased predictably with elevated brain temperature or decreased glucose levels [3]. Although it might be considered a safety mechanism, exercise training reduces central fatigue during fatiguing intense exercise.

The brain receives 15% of cardiac output and 20% of oxygen uptake at rest [4]. Cerebral blood flow decreases after the age of 30 years [5] with adults aged 71 having cerebral blood flows 20–30% less than those aged 27. Endothelial dysfunction is one of the earliest indicators of structural and functional changes in the aging vascular system and arterial elasticity seems to be related to endothelial dysfunction [6]. Reduced elasticity may alter steady state blood flow to the brain which may affect the delivery of sufficient nutrients, impacting brain function and increasing feelings of fatigue.

To date, there is limited information available concerning the relationship between arterial elasticity, fatigue, and muscle function. Recently, Fahs et al. [7] reported that muscular strength was inversely related to aortic stiffness independent of aerobic fitness, whereas Heffernan et al. [8] found a significant relationship between vascular function and muscular power but not strength. However, it appears

neither study adjusted for the potential confounder muscle size. Some [9] but not all [10] studies find that aerobic exercise training improves arterial stiffness, while older aerobic athletes have lower pulse wave velocity than sedentary peers [9]. In addition, reduced gate performance [11] and poor physical function [12] are related to arterial stiffness in healthy older adults. Increased aortic stiffness is associated with reduced exercise capacity in patients with hypertrophic cardiomyopathy [13] while increased pulse wave velocity is associated with reduced walking distance in patients with peripheral arterial disease and arterial stiffness is associated with reduced walking ability in end stage renal disease [14]. Few of these studies have determined whether the relationships are confounded by variation in aerobic fitness, strength, body composition, or age.

Understanding muscle function and fatigue is particularly important in older women since they have reduced muscle function compared to older men and are therefore vulnerable at earlier ages for loss in muscular function [15]. In addition, fatigue increases with age and increases more rapidly in older women than older men [2]. The purpose of this study is to determine whether arterial elasticity is related to (1) strength; (2) central and peripheral fatigue during a fatiguing maximal isometric contraction protocol; (3) subjective measures of fatigue; and (4) treadmill endurance independent of potential confounders such as muscle size, % fat, aerobic fitness, and age. We hypothesize that arterial elasticity will be negatively related to fatigue and positively related to strength and exercise endurance in older women after adjusting for appropriate confounders.

2. Materials and Methods

2.1. Experimental Approach to the Problem. In order to test these hypotheses, healthy and sedentary older women were recruited to participate in exercise testing and measures of aerobic capacity, body composition, and fatigue. Central and subjective fatigue measures were chosen to obtain objective and subjective reports. Variables such as strength, age, percent body fat, and aerobic fitness were considered potential confounders in exploring the relationship between arterial elasticity and fatigue.

2.2. Subjects. Ninety-one healthy, sedentary (self-reporting no exercise training to the Program Coordinator at initial screening), postmenopausal African American ($n = 15$) and American women of European decent (European American, $n = 76$) who are 60 or older volunteered to participate in this study designed to determine factors that may influence fatigue in older women. Eighteen of the 91 subjects reported taking hormone replacements.

Exclusion criteria included heart disease, abnormal EKG (both at rest and during exercise), smoking, diabetes mellitus, participation in regular physical activity (more than once/week), or medications that affected energy expenditure, insulin levels, thyroid status, or heart rate. Methods and procedures were approved by the appropriate institutional review board, and all subjects gave verbal and written informed consent.

2.3. Procedures

2.3.1. Resting Systolic Blood Pressure (SBP). Resting blood pressure was taken by automatic auscultation (Omron Blood Pressure Monitor, model HEM-780; Omron Healthcare, Inc 1200 Lakeside Dr. Bannockburn, IL) while lying in a supine position. Readings were taken after 12 hours of fast between 7 : 00 and 8 : 00 AM.

2.3.2. Maximal Exercise Testing. It was physician-supervised and conducted using the modified Balke treadmill test protocol. A metabolic cart, calibrated prior to testing (Vmax Spectra29, SonsorMedics, Inc, Yorba Linda, CA), was used to evaluate ventilatory expired gases. Monitoring consisted of 12-lead electrocardiogram and BP measurements taken every two minutes (Omron Blood Pressure Monitor, model HEM-780; Omron Healthcare, Inc 1200 Lakeside Dr. Bannockburn, IL). The testing was commenced with treadmill walking at two mph for two minutes. Treadmill grade was increased by 3.5% every 2 minutes until minute 12 at which time grade was decreased to 12% and speed was increased to 3 mph. The grade then increased by 2.5% each minute until exhaustion. Blood pressure, heart rate, and oxygen uptake were recorded during the last 20 seconds of each level. Participants were encouraged to exercise to fatigue. Termination criteria for testing followed American Heart Association/American College of Cardiology guidelines [16]. Maximum oxygen uptake (VO_2 max in $mL \cdot kg^{-1} \cdot min^{-1}$), maximum respiratory exchange ratio (RER), and maximum heart rate were defined as the highest 20-second averaged value. Criteria for obtaining maximum oxygen uptake were heart rate within 10 beats of age predicted maximum, plateauing of oxygen uptake, and RER of 1.1 or larger. Only those subjects achieving at least two of these criteria were included in the analysis of VO_2 max and treadmill endurance (77 subjects). Treadmill endurance was considered the time spent on the treadmill until voluntarily stopping the test.

2.3.3. Torque Measures. Torque measurements were performed in the knee extensor muscle group using a custom-built chair with the hip and knee secured at approximately 90 degrees of flexion. The leg was secured to a rigid lever arm in an effort to ensure that the knee extensors would perform only isometric contractions. A load cell was attached via a steel rod to the rigid lever arm on one end and an immovable support on the other. The line of pull on the load cell was perpendicular to the rigid lever arm. Thus, the torque produced about the knee was calculated as the product of the load cell force and the length of the lever arm between axis of rotation and the load cell attachment. Prior to data collection, subjects were allowed to perform several warm-up contractions and were familiarized with performing maximum isometric contractions. Maximal voluntary isometric contraction (MVIC) was defined as the peak isometric torque achieved during 3 consecutive maximal

efforts (~5 second contraction separated by 120 seconds of rest). Contraction intensity for subsequent neuromuscular electrical stimulation (NMES) testing was calculated relative to each subjects' MVIC.

2.3.4. Central Activation Ratio (CAR). Bipolar self-adhesive neuromuscular stimulation electrodes (7 × 10 cm) were placed over the distal-medial and proximal-lateral portion of the quadriceps muscle group, as reported previously [17]. Stimulation pulses were delivered using a Grass S88 stimulator with a Grass Model SIU8T stimulus isolation unit (Grass Technologies, West Warwick, RI). Stimulation pulses were 450 μs in duration and delivered at 100 Hz for 100 ms (10 pulses); voltage was set at 125 V. Participants were familiarized with maximum voluntary isometric contraction (MVIC). During MVIC testing, the burst of stimulation was applied. CAR was calculated: MVIC torque/(MVIC torque + Stimulation). The 30-repetition fatigue test (CAR30) was considered central fatigue. CAR was measured on only 40 of the subjects because data was collected on only the last 50 subjects entered in the study and because of inability of 10 subjects to complete protocol.

2.3.5. Fatigue Tests. Fatigue tests consisted of voluntary and NMES 30-repetition MVIC protocols (40 subjects). The voluntary fatigue protocol consisted of a series of MVICs, where each contraction was 5 seconds in duration with 5 seconds rest between contractions for 5 minutes (30 total contractions). CAR testing was administered and calculated during the 1st and 30th contraction of the voluntary fatigue test. A standard clinical electrical stimulator (Rich-Mar, Chattanooga, TN) was utilized for the NMES-induced fatigue protocol (50 Hz, 450 μsec biphasic pulses) and also consisted of contractions that were 5 seconds on and 5 seconds off for 5 minutes (30 total contractions).

2.3.6. Body Composition. Total fat, lean tissue, and leg lean tissue were measured using dual-energy X-ray absorptiometry (Prodigy; Lunar Radiation, Madison, WI). The scans were analyzed with the use of the GE Lunar enCORE software standard analysis module. Total body and right and left leg analyses were performed by adjusting *Total Body Cut* lines using the software's *ROI* (Region Of Interest) tool. Leg ROI's required adjusting the following Total Body Cuts: the *Pelvis Top* cut was placed immediately superior to the iliac crests; the *Left and Right Pelvis* cuts were adjusted tangential to the ischium and passing through the femoral necks without touching the pelvis; the *Left and Right Leg* cuts were adjusted to separate the hands and forearms from the legs; the *Center Leg* cut was adjusted to separate the right and left leg.

2.3.7. Arterial Elasticity. Noninvasive radial artery pulse wave analysis was used for measurement of arterial elasticity (which in turn is related to endothelial function) [6]. Pulse wave analysis was performed in duplicate, and average values were reported. The radial artery waveform was obtained with a sensor positioned over the brachial artery and calibrated using an oscillometric method on the opposite arm. Thirty seconds of analog waveforms were digitized at 200 samples/sec, and a beat marking algorithm determined the beginning of systole, peak systole, onset of diastole, and end of diastole for all beats in the 30 sec measurement period. An average beat determination was constructed, and a parameter estimating algorithm (Hypertension Diagnostics, Eagan, MN) was applied to define a third-order equation that replicated the diastolic decay in the waveform. Small artery and large artery elasticity were then determined based on the modified Windkessel model [18]. The method used was the predecessor of the CR 2000 and DO 2020 instruments (Hypertension Diagnostics), which incorporates a new transducer and similar software with a revised analytical algorithm.

2.3.8. Self-Reported Fatigue. Perceptions of fatigue were assessed with relevant items from the Beck Depression Inventory-ll (BDI) [19]. Participants completed the BDI at home, during the week prior to strength and physical function testing. Items 15 (loss of energy) and 20 (tiredness or fatigue) were the only two that were included in these analyses, with higher scores indicating greater loss of energy or fatigue. Self-reported fatigue was measured on 74 subjects due to incomplete/incorrect questionnaires.

2.4. Statistical Analyses. Means and standard deviations were calculated for all variables of interest. Pearson product correlations were determined for all contrasts of interest. Reported hormone replacement therapy was not related to any fatigue, endurance, or function measure so it was not included in the results. In order to determine the independent relationship between arterial elasticity variables and performance measures stepwise linear multiple regression was used to model central fatigue, treadmill endurance, fatigue/tiredness, and knee extension strength after adjusting for potential confounders. Potential confounders considered were VO_2 max, knee extension strength, age, and percent body fat for central fatigue, treadmill endurance, and fatigue/tiredness. Potential confounders for strength were lean tissue and age. Only those potential confounders that had significant Pearson product correlations with the performance measures of interest were included in the multiple regression models. Multicollinearity was within acceptable limits for all models with variance inflation factor $(1/1 - R_i^2)$ less than 1.5 for all variables in all models. The Central Activation Ratio (CAR) was not added to the study until the final two years, hence the reduced sample size for analyses including this variable ($n = 40$). Because of missing data for several variables, due to equipment malfunction and sickness, correlations and regression models have varying observations as indicated in the tables. IBM SPSS Statistics version 20 was used in the analyses. An alpha level of $P < 0.05$ was the criteria for significance.

3. Results

Descriptive characteristics are contained in Table 1. Table 2 is a correlation table showing simple unadjusted associations

Table 1: Subject characteristics and physiologic, Fatigue, and performance measures. Sample size is 91 (15 AA and 76 EA) unless otherwise indicated. (mean ± SD).

Age (years)	65.0 ± 3.9
Height (cm)	165.0 ± 5.8
Weight (kg)	73.9 ± 11.4
% Fat	42.8 ± 6.0
Leg lean tissue (kg)	12.9 ± 1.9
VO_2 max (mL/kg/min, $N = 77$)	23.7 ± 4.1
Treadmill endurance (min, $N = 77$)	13.1 ± 3.0
Loss of energy[a] ($N = 74$)	0.68 ± 0.68
Fatigue/tiredness[b] ($N = 74$)	0.53 ± 0.53
Maximum voluntary contraction (N.M, $N = 40$)	116.9 ± 26.7
Maximum voluntary contraction + electrical stimulation (N.M, $N = 40$)	123.6 ± 26.5
VOL ($N = 40$)	22.2 ± 18.6
ESTIM ($N = 40$)	40.2 ± 14.5
CAR1 ($N = 40$)	90.9 ± 6.4
CAR30 ($N = 40$)	89.5 ± 9.1
Large artery elasticity	13.1 ± 4.7
Small artery elasticity	4.0 ± 1.8
SBP (mm Hg, $N = 90$)	126.7 ± 14.9
DBP (mm Hg, $N = 90$)	70.7 ± 10.2

[a]Scored as 4, very low energy, to 0, full of energy; [b]scored as 4, exceedingly fatigued or tired, to 0, no feelings of fatigue or tiredness.
VOL = % drop in force for 30 voluntary maximal contractions.
ESTIM = % drop in force for voluntary contraction + electrical stimulation during 30 voluntary maximal contractions. CAR1 = Central Activation Ratio for the first repetition in the fatigue test.
CAR30 = Central Activation Ratio for the 30th repetition in the fatigue test.

between potential fatigue mediators and the various measures of performance/fatigue. Age was negatively related to treadmill endurance and both large and small artery elasticity. Percent fat was negatively related to treadmill endurance, loss of energy, and large artery elasticity. Leg lean tissue was related to maximum voluntary contraction, maximum voluntary contraction plus electrical stimulation, and small artery elasticity. Maximum oxygen uptake was positively related to treadmill endurance and large artery elasticity. Maximum voluntary contraction and maximum voluntary contraction plus electrical stimulation were highly related to each other, as well as both large and small artery elasticity. Loss of energy was significantly related to fatigue/tiredness and negatively related to large artery elasticity, while fatigue/tiredness was negatively related to both large and small artery elasticity. Systolic blood pressure was related only to small artery elasticity.

Based upon intercorrelations with fatigue measures and known potential confounding relationships between performance/fatigue and arterial elasticity, regression equations were developed that were designed to observe the independent relationship between arterial elasticity and the various fatigue measures. Table 3 shows that large artery elasticity remains significantly related to central fatigue after adjusting for knee extension strength (maximal voluntary contraction) and VO_2 max (partial $R = -0.46$, $P < 0.01$). Model 1 in Table 4 shows that large artery elasticity is independently related to treadmill endurance independent of VO_2 max, knee extension strength, and age (partial $R = 0.42$, $P < 0.02$). Because of limitations in sample size a potential confounder % fat was not included in Model 1. Therefore a second model was developed for treadmill endurance. After adjusting for VO_2 max, knee extension strength, and % fat, large artery elasticity remained independently related to treadmill endurance. Subjective feelings of fatigue/tiredness are modeled in Table 5. Large artery elasticity is negatively related to fatigue/tiredness independent of small artery elasticity (partial $= -0.29$, $P < 0.03$) while small artery elasticity approached a significant negative relationship with fatigue/tiredness after adjusting for large artery elasticity (partial $R = -0.22$, $P < 0.09$). Finally small artery elasticity was related to knee extension strength (maximum voluntary contraction) after adjusting for leg lean tissue and age (Table 6, partial = 0.52, $P < 0.01$).

4. Discussion

As we hypothesized, arterial elasticity was independently related to fatigue. This is particularly apparent when observing variables that are controlled by the central nervous system. Not only there was a relationship between large artery elasticity and fatigue/tiredness and lack of energy, but also central activation both during one maximal contraction and during a fatiguing maximal contraction protocol of 30 repetitions was related to arterial elasticity. In addition, similar to Fahs et al. [7] where arterial stiffness was inversely related to strength, we found that strength was positively related to arterial elasticity. This relationship occurred during activation of muscle voluntarily and voluntarily with electrical stimulation superimposed. It is tempting to hypothesize that arterial elasticity may positively affect both central (brain) and peripheral (muscle) function; however relationships do not prove cause and effect and could result from confounding of some other variable. However, we considered known potential confounders, such as age, strength (for fatigue measures), aerobic fitness, and leg lean tissue (surrogate of muscle), and still observed similar relationships. Thus the results of this study are supportive of the concept that arterial elasticity may influence central nervous system drive as well as muscle function.

Elasticity of both large and small arteries was related to leg strength (Table 2). This was the case whether strength was evaluated using just voluntary contractions or voluntary contractions plus electrical stimulation suggesting that the relationship was largely due to function of skeletal muscle. Small artery elasticity seemed to be more strongly related to strength than large artery elasticity. In addition, small artery elasticity continued to be related after adjusting for leg lean tissue (a surrogate of leg muscle [20]) and age. We know of no studies that have shown that arterial elasticity is related to muscular strength adjusted for volume of muscle, indicating

Table 2: Pearson product correlation table.

	Age	% Fat	Leg lean tissue	VO_2 max	Maximum voluntary contraction	Maximum voluntary contraction + electrical stimulation	Loss of energy	Feelings of tiredness	Large artery elasticity	Small artery elasticity	SBP
Treadmill endurance	−0.38 (<0.01) ($N = 77$)	−0.28 (<0.02) ($N = 77$)	0.01 (0.98) ($N = 77$)	0.54 (<0.01) ($N = 77$)	0.09 (0.64) ($N = 39$)	0.11 (0.58) ($N = 39$)	−0.11 (0.45) ($N = 71$)	0.03 (0.82) ($N = 71$)	0.27 (<0.03) ($N = 68$)	0.03 (0.98) ($N = 68$)	−0.20 (0.06) (67)
Maximum voluntary contraction	−0.24 (0.14) ($N = 40$)	−0.01 (0.97) ($N = 40$)	0.57 (<0.01) ($N = 40$)	0.17 (0.37) ($N = 39$)		0.99 (0.01) ($N = 40$)	−0.11 (0.61) ($N = 37$)	−0.23 (0.28) ($N = 37$)	0.32 (<0.05) ($N = 40$)	0.63 (<0.01) ($N = 40$)	−0.23 (0.16) ($N = 39$)
Maximum voluntary contraction + electrical stimulation	−0.26 (0.12) ($N = 40$)	0.01 (0.95) ($N = 40$)	0.57 (<0.01) ($N = 40$)	0.16 (0.41) ($N = 39$)	0.99 (<0.01) ($N = 40$)		−0.11 (0.60) ($N = 26$)	−0.19 (0.37) ($N = 26$)	0.36 (<0.03) ($N = 40$)	0.65 (<0.01) ($N = 40$)	−0.04 (0.84) ($N = 39$)
CAR1	0.09 (0.60) ($N = 40$)	−0.21 (0.22) ($N = 40$)	0.11 (0.51) ($N = 40$)	0.01 (0.99) ($N = 39$)	0.25 (0.14) ($N = 40$)	0.15 (0.39) ($N = 40$)	−0.06 (0.78) ($N = 26$)	−0.12 (0.57) ($N = 26$)	−0.37 (<0.03) ($N = 40$)	0.06 (0.73) ($N = 40$)	−0.08 (0.83) ($N = 39$)
CAR30	0.02 (0.93) ($N = 40$)	0.07 (0.68) ($N = 40$)	0.10 (0.10) ($N = 40$)	0.10 (0.59) ($N = 39$)	0.12 (0.46) ($N = 40$)	0.04 (0.83) ($N = 40$)	−0.09 (0.68) ($N = 26$)	−0.29 (0.15) ($N = 26$)	−0.34 (<0.04) ($N = 40$)	−0.08 (0.63) ($N = 40$)	−0.05 (0.78) ($N = 39$)
Loss of energy	0.20 (0.09) ($N = 74$)	0.26 (<0.03) ($N = 74$)	−0.02 (0.85) ($N = 74$)	−0.21 (0.12) ($N = 49$)	−0.11 (0.61) ($N = 26$)	−0.11 (0.60) ($N = 26$)		0.49 (<0.01) ($N = 74$)	−0.27 (<0.04) ($N = 74$)	−0.16 (0.20) ($N = 74$)	0.04 (0.74) ($N = 73$)
Feelings of tiredness	0.06 (0.60) ($N = 74$)	0.10 (0.42) ($N = 74$)	−0.07 (0.59) ($N = 74$)	−0.04 (0.76) ($N = 49$)	−0.23 (0.28) ($N = 26$)	−0.19 (0.37) ($N = 26$)	0.49 (<0.01) ($N = 74$)		−0.32 (<0.01) ($N = 74$)	−0.26 (<0.04) ($N = 74$)	0.03 (0.81) ($N = 73$)
Large artery elasticity	−0.27 (<0.01) ($N = 91$)	−0.22 (<0.04) ($N = 91$)	0.02 (0.82) ($N = 91$)	0.34 (<0.01) ($N = 77$)	0.32 (<0.05) ($N = 40$)	0.36 (<0.03) ($N = 40$)	−0.27 (<0.04) ($N = 74$)	−0.32 (<0.01) ($N = 74$)		−0.37 (<0.03) ($N = 91$)	−0.017 (0.89) ($N = 90$)
Small artery elasticity	−0.30 (<0.01) ($N = 91$)	0.17 (0.10) ($N = 91$)	0.38 (<0.01) ($N = 91$)	0.01 (0.98) ($N = 77$)	0.63 (<0.01) ($N = 40$)	0.65 (<0.01) ($N = 40$)	−0.16 (0.20) ($N = 74$)	−0.26 (<0.04) ($N = 74$)	0.25 (<0.02) ($N = 91$)		−0.28 (0.02) ($N = 90$)

[a] Scored as 4, very low energy, to 0, full of energy; [b] scored as 4, exceedingly fatigued or tired, to 0, no feelings of fatigue or tiredness.
CAR1 = Central Activation Ratio for the first repetition in the fatigue test.
CAR30 = Central Activation Ratio for the 30th repetition in the fatigue test.
SBP = systolic blood pressure.

Table 3: Linear regression model for estimation of central fatigue (CAR30) from aerobic fitness, knee extension strength, and large artery elasticity (38 subjects).

Central fatigue	Beta	$R = 0.49$ Partial R	$P < 0.02$ P
Constant	0.861		<0.01
VO_2 max	0.006	0.35	<0.04
Knee extension strength	<0.001	0.08	0.64
Large artery elasticity	−0.008	−0.42	<0.01

Table 4: Linear regression models for estimation of treadmill endurance. Model 1 from aerobic fitness, knee extension strength, large artery elasticity, and age. Model 2 from aerobic fitness, knee extension strength, large artery elasticity, and % fat (both models, 39 subjects).

	Beta	$R = 0.66$ Partial R	<0.01 P
	Treadmill model 1 endurance		
Constant	7.184		0.31
VO_2 max	0.262	0.47	<0.01
Knee extension strength	0.007	0.09	0.62
Large artery elasticity	0.201	0.37	<0.03
age	−0.128	−0.10	0.46
	Treadmill model 2 endurance		
VO_2 max	0.30	0.53	<0.01
Knee extension strength	0.01	0.10	0.58
Large artery elasticity	0.22	0.40	<0.02
% fat	0.06	0.15	0.39

Table 5: Linear regression model for estimation of subjective feelings of fatigue from large and small artery elasticity (64 subjects).

Feelings of tiredness	Beta	$R = 0.38$ Partial R	$P < 0.01$ P
Constant	1.361		<0.01
Small artery elasticity	−0.086	−0.22	<0.09
Large artery elasticity	−0.043	−0.29	<0.02

Table 6: Linear regression model for estimation of knee extension strength from leg lean tissue, small artery elasticity, and age ($n = 39$).

Knee extension strength	Beta	$R = 0.65$ Partial R	$P \leq 0.01$ P
Constant	118.7		0.08
Leg lean tissue (kg)	0.97	0.08	0.65
Small artery elasticity	6.42	0.52	<0.01
age	−1.02	−0.21	0.22

that muscle quality/specific strength was enhanced in the individuals who possessed more arterial elasticity.

On the other hand large artery elasticity but not small artery elasticity was related to both reduced perceived fatigue/tiredness while at rest as well as central fatigue during maximal isometric contractions suggesting that brain function may be influenced by arterial elasticity. It is possible that arterial elasticity may be just a marker of overall health and the relationship may be mediated by poor health for the individuals with less elastic arteries. However, these relationships were independent of blood pressure (either not related to variable in question or continued to be related to arterial elasticity even after adjusting for blood pressure) or blood lipids (none of the blood lipids even approached a significant relationship with any performance measure). Another explanation for the aforementioned findings may be that increased arterial elasticity improves the steady state flow to the capillaries. Consistent with this premise lowering of oxygen tension in the brain can amplify central fatigue [21].

We found differences between the voluntary and electrically-elicited muscle fatigue. During voluntary contractions the neuromuscular system utilizes multiple strategies to offset fatigue. For example, recruiting motor units in an orderly manner such that fatigue resistant muscle is recruited first or altering activated motor units to maintain force production is a common way in which force production is preserved. However, during electrical stimulation, these strategies are not available because motor units are recruited in a nonselective, spatially fixed, and temporally synchronous pattern [17]. We utilized maximum voluntary contractions during our voluntary fatigue protocol to reduce the ability of the neuromuscular system to offset fatigue and be similar to that which is seen during electrically elicited contractions. However, subjects may not have always provided a true "maximum" contraction of the quadriceps, as evidenced from their Central Activation Ratios which were less than 0.95. Because of this, they may have had motor units in reserve that could be called upon when others were fatiguing.

Tiredness was not correlated with measures of voluntary fatigue but was related to the fatigue as a result of the electrical stimulation. The electrical stimulation induced contractions provide information that is specific to the periphery and removes most of the input from the higher central systems. This may provide evidence of a link between peripheral muscle function and central tiredness. The lack of a relationship between the tiredness and voluntary fatigue is probably related to variability in each person's effort at the beginning of the fatigue protocol.

5. Conclusions

In conclusion, arterial elasticity is related to strength independent of leg lean tissue (surrogate of leg muscle) and to fatigue independent of ethnic origin, strength, and aerobic fitness. This is especially apparent in the central nervous system where arterial elasticity is independently related to perceptions of fatigue at rest and central fatigue during 30 maximal contractions. These results suggest that arterial health may be involved with the ability for the central nervous system to activate muscle in older women.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Differences in Trunk Kinematic between Frail and Nonfrail Elderly Persons during Turn Transition Based on a Smartphone Inertial Sensor

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Objective. Firstly, to, through instrumentation with the *iPhone4* smartphone, measure and describe variability of tridimensional acceleration, angular velocity, and displacement of the trunk in the turn transition during the ten-meter Extended Timed-Get-up-and-Go test in two groups of frail and physically active elderly persons. Secondly, to analyse the differences and performance of the variance between the study groups during turn transition (frail and healthy). *Design*. This is a cross-sectional study of 30 subjects over 65 years, 14 frail subjects, and 16 healthy subjects. *Results*. Significant differences were found between the groups of elderly persons in the accelerometry ($P < 0.01$) and angular displacement variables ($P < 0.05$), obtained in the kinematic readings of the trunk during the turning transitions. The results obtained in this study show a series of deficits in the frail elderly population group. *Conclusions*. The inertial sensor found in the *iPhone4* is able to study and analyse the kinematics of the turning transitions in frail and physically active elderly persons. The accelerometry values for the frail elderly are lower than the physically active elderly, whilst variability in the readings for the frail elderly is also lower than the control group.

1. Background

Clinical frailty syndrome is a common geriatric syndrome which is characterized by physiological reserve decreases and increased vulnerability and which may, in the event of unexpected intercurrent processes, result in falls, hospitalization, institutionalization, or even death [1]. The changes associated with ageing and frailty are associated with changes in gait characteristics and the basic functional capacities of the individual [2]. This variability in different movement patterns has been interpreted as a more conservative gait pattern in order to increase gait stability and reduce the risk of falls [3]. This new, more conservative gait pattern has greater cognitive involvement and produces a result focused entirely on movement, whilst the perception of unexpected trigger factors may be overlooked [4]. Dual tasks have been shown to affect normal gait development even in healthy persons [5].

Turning while walking is a common occurrence in everyday life [6]. Turning requires transfer and rotation of the body towards the new walking direction while maintaining dynamic stability [7]. The Timed Get Up and Go (TGUG) test is a widely used tool to evaluate balance and some functional tasks through clinical evaluation of mobility and the risk of falls [2, 8–10]. The clinical potential of the TGUG test comes from the possibility of sequencing several basic functional abilities, such as standing up and sitting down transitions, and transitions which require balance, such as turning [11]. The TGUG test, despite being widely used in clinical practice, has limitations. As a consequence, the TGUG test is currently carried out in an instrumented manner by attaching inertial sensors to the body [2, 9, 12–16].

The latest generation of smartphones often includes inertial sensors with subunits such as accelerometers and gyroscopes which can detect acceleration and inclination

Table 1: Characteristics of sample ($n = 30$).

	Mean		SD	
	Frail ($n = 14$)	Healthy ($n = 16$)	Frail ($n = 14$)	Healthy ($n = 16$)
Age (years)	83.71	70.25	6.37	3.32
Weight (kg)	56.21	71.03	9.64	13.11
Height (cm)	155.79	159.44	7.81	10.61
Body mass index (kg/m^2)	23.36	27.87	3.48	3.79
Total score ETGUG (s)	53.64	15.52	24.12	2.91

Kg: kilograms; cm: centimeters; m: meters; s: seconds.

[17]. The apps developed for these smartphones mean the data from the accelerometer and the gyroscope these can be read, stored, transferred and displayed [18, 19]. These apps evaluate and assess kinematic variables related to gait [20], measures in the Cobb angles in X-rays, or provide an objective method to classify levels of physical activity and as indicator of the degree of functional capacity and quality of life [17, 21].

The goals of the present study are as follows. Firstly, to, through instrumentation with the *iPhone4* smartphone, measure and describe variability of tridimensional accelerations, angular velocity, and displacement of the trunk in the turn transition during the ten-meter Extended Timed-Get-up-and-Go test in two groups of frail and physically active elderly persons. Secondly, to analyse the differences and performance of the variance between the study groups during turn transition (frail and healthy).

2. Methods

2.1. Design and Participants. A cross-sectional study that involved 30 subjects over 65 years and 14 frail and 16 healthy elderly persons. The participants were classified with frailty syndrome by the Fried criteria (unintentional weight loss, self-reported exhaustion, weakness, slow walking speed, and low level of physical activity) [1]. Exclusion criteria were-history of pain in the last twelve months, previous surgery, presence of a tumour, and musculoskeletal disorders in the upper or lower extremity. Patients with impaired cognition, musculoskeletal back comorbidities, and problems associated with exercise intolerance were also excluded. All participants were clinically examined by a physiotherapist, and none of them were found to have any exclusion criteria. Table 1 shows the characteristics of the sample and stopwatch values in the ETGUG test.

Healthy elderly participants were recruited through notices at the Sport and Health Centre in Torremolinos, Spain. Frail elderly participants were recruited through notices at Geriatric Centres in Torremolinos and Benalmadena, Spain. Written informed consent was obtained from each individual. The study was approved by the Ethics Committee of the Faculty of Medicine at the University of Malaga, Spain.

2.2. Data Collection and Procedures. Linear acceleration was measured along three orthogonal axes using the *iPhone4* accelerometer snugly secured to the test subjects by a neoprene fixation belt over the sternum. Previous studies show that the essential spatiotemporal characteristics of overground walking can be obtained by trunk accelerometry; individual step or stride cycles can be identified, and fair estimations of step length and walking speed can be obtained using a single triaxial accelerometer [22].

The orientation and movement of the sensors are presented as roll, pitch, and yaw Euler angles (RPY). If the sensor's RPY axes are aligned with the anatomical axes of the trunk, the roll angle of a movement is around the anteroposterior (AP) axis, the pitch angle is around the left-right axis, and the yaw angle is around the vertical (V) axis.

This smartphone is equipped, as is the IC3, with three triaxial elements for the detection of kinematic variables: a gyroscope, a magnetometer, and an accelerometer. Apple uses an LIS302DL accelerometer in the *iPhone4* [23]. The application used to obtain kinematic data was *xSensor Pro, Crossbow Technology, Inc.*, available at the Apple *AppStore*. The *iPhone4* has storage capacity of 20 MB, and the data for each trial was transmitted as email for analysis and postprocessing. The data-sampling rate was set to 32 Hz. An *iPhone4* is required in order to obtain accelerometer, gyroscope, and magnetometer data together; earlier versions do not allow this possibility. A previous study showed an interobserver error (standard deviation of the difference between measurements by two different observers) of 4.0° for the iPhone and 3.4° for the protractor [17].

2.3. Extended Timed-Get-Up-and-Go Test. All subjects performed the Extended Timed-Get-up-and-Go test (ETGUG) three times, and the best trial was selected based on the total score for the full test. Devices were not removed between trials. Subjects had five minutes of rest between trials. All subjects used an armless chair and were instructed not to use their arms to stand up. Although in traditional ETGUG an armchair is used [24], we used an armless chair. The beginning and end of the walkway were marked with 2.5 cm green tape on the floor. The tape markings were shown to the subjects before the trials. Subjects were instructed to sit straight with their backs touching the back of the chair. Once the go signal was given by the tester, they stood up from the chair, walked as fast as possible but without running, turned left or right after passing the green tape at the end of the walkway, then returned to the chair, turned around, and sat down. The tester timed the performance with a stopwatch.

2.4. Turning Transitions of the Extended Timed-Get-Up-and-Go Test. The most important problem in analyzing turns is identifying the onset and offset of the turns. Offline data processing was used to identify the turning transition of the ETGUG test. The turn transition used in the study was the first one, the transition between the gait—go from the chair and the gait—come to the chair. The turning transition of the ETGUG test was detected with gyroscope data of the *iPhone4* accelerometer and was detected and analysed using a separate method [9].

2.5. Data Processing. Computerized automatic analysis was carried out to filter the inertial sensor data. This analysis, which was designed to systematically obtain kinematic data for further statistical analysis, was performed using basic software package R. Automatic analysis was guided in order to obtain kinematic information from the accelerometer and gyroscope independently for each subject in the turning transitions of the ETGUG test. The following was obtained from accelerometer: maximum peak, minimum peak, mean, and SDs of accelerations in the three axes of movements (x, y, and z). Also obtained were the maximum peak, minimum peak, mean, and SDs of the resultant vector (RV) accelerations (RV = $\sqrt{x2 + y2 + z2}$). The following was obtained from the gyroscope: maximum peak, minimum peak, mean and SDs of rotation motions in the three axis of movements (x, y and z). Finally, the following was obtained: maximum peak, minimum peak, mean, and SDs of the angular velocity in the three axes of movements (x, y, and z). The sign in the value measurements in accelerometer velocity along the x, y, and z axes is shown in Figure 1. The sign in the value measurements in the gyroscope rotation around the x, y, and z axes is shown in Figure 2. According to the information from Figure 2, if a subject performs a rotation to left during the test, the gyroscope records negative values in the y axes. In this study, all subjects performed the shift to the left (see Figure 2).

2.6. Statistical Analysis. Analysis was performed with SPSS version 15 for Windows, while data collection used inferential analysis between variables by type and normal. Mann-Whitney nonparametric tests were used, as determined by the normality of distribution variables. The statistical significance level was set at $P < 0.05$.

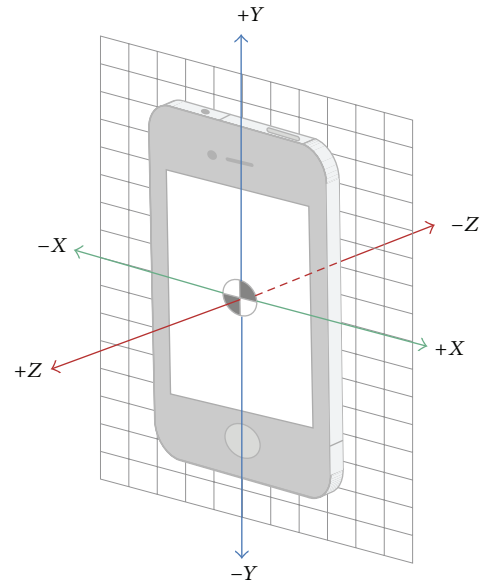

Figure 1: The accelerometer measures velocity along the x, y, and z axes.

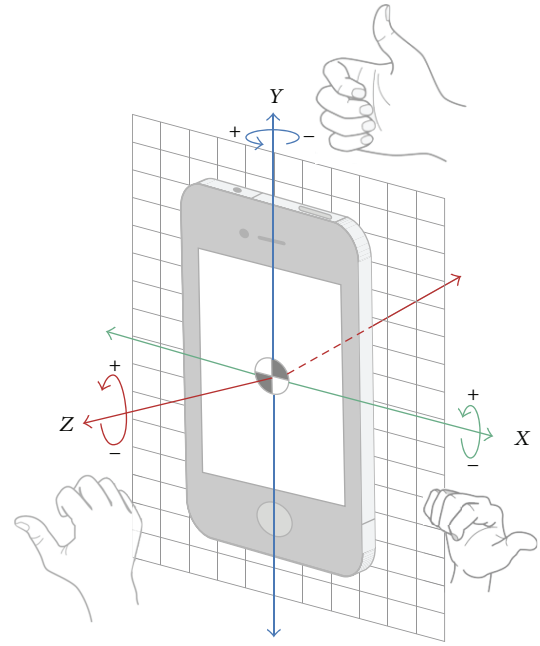

Figure 2: The gyroscope measures rotation around the x, y, and z axes.

3. Results

With regard to the mean accelerometry values, Table 2 summarizes the acceleration-based measurements of the turning transitions in the ETGUG test in the two groups. Stopwatch-based ETGUG duration showed higher duration for the frail patients compared to the fit control group, as expected. The best finding in the x-axis was the following: the difference between groups for the minimum acceleration was 3.72 m/s^2 ($P < 0.01$). The y-axis shows differences ($P < 0.001$) for maximum acceleration, 5.48 m/s^2 between groups; the minimum acceleration was 7.44 m/s^2 between groups. For the z-axis, the differences found ($P < 0.001$) were in minimum acceleration, 5.39 m/s^2 between groups. Finally, the differences found between groups for the resultant vector values for the three accelerations show ($P < 0.01$) in the maximum, acceleration was 8.13 m/s^2; in the minimum, acceleration was 0.78 m/s^2; and in the resultant vector mean, acceleration was 3.08 m/s.

With regard to the mean gyroscope values, Table 3 summarizes the gyroscope-based measurements of the turning transitions in the ETGUG test in the two groups. The difference between groups for the mean maximum peak value

Table 2: Acceleration-based values from the turning transition ($n = 30$).

	Mean		SD			
	Frail ($n = 14$)	Healthy ($n = 16$)	Frail ($n = 14$)	Healthy ($n = 16$)	U	P value
t.stopwatch (s)	5.329	2.815	1.344	2.069	2.000	<**0.001**
x.acc.min (m/s^2)	−2.053	−5.779	0.962	2.433	41.00	0.003
y.acc.max (m/s^2)	2.060	7.543	0.700	2.865	26.50	<**0.001**
y.acc.min (m/s^2)	−2.004	−9.448	0.945	6.937	14.00	<**0.001**
z.acc.min (m/s^2)	−1.815	−7.204	1.619	2.438	35.00	<**0.001**
z.acc.mean (m/s^2)	−0.264	−2.903	1.553	1.331	36.00	0.002
rv.acc.max (m/s^2)	3.634	11.985	1.165	6.523	41.00	0.003
rv.acc.min (m/s^2)	0.621	1.403	0.672	0.980	38.00	0.002
rv.acc.mean (m/s^2)	1.916	4.995	0.717	1.046	45.00	0.005

x: x-axis; y: y-axis; z: z-axis; acc: acceleration; t: time; max: maximum; min: minimum; rv: resultant vector; U: U-Mann-Whitney.

Table 3: Gyroscope-based values from the turning transition ($n = 30$).

	Mean		SD			
	Frail ($n = 14$)	Healthy ($n = 16$)	Frail ($n = 14$)	Healthy ($n = 16$)	U	P value
t.stopwatch (s)	5.329	2.815	1.344	2.069	2.000	<**0.001**
roll.rotation.min (deg)	−172.845	−53.578	12.758	64.308	60.00	0.031
roll.rotation.max (deg)	−5.770	63.360	35.422	97.818	62.00	0.038
rate.yaw.max (deg/s)	26.332	112.810	9.271	147.913	57.00	0.022
rate.yaw.min (deg/s)	−24.486	−52.809	8.867	34.733	49.00	0.009
rate.roll.max (deg/s)	25.508	134.558	14.217	135.523	13.00	<**0.001**
rate.roll.min (deg/s)	−20.396	−39.884	8.716	27.357	58.00	0.025

Max: maximum; min: minimum; t: time; s: second; deg: degrees; rate: angular velocity; U: U-Mann-Whitney.

for Yaw movement angular velocity was 86.48°/s ($P < 0.05$) (see Table 3). The difference between groups for minimum peak in angular velocity in this axis was 28.33°/s ($P < 0.01$) (see Table 3). Finally, with regard to the roll movement, the difference between groups was in the maximum angular velocity and peak was 109.04°/s ($P < 0.01$). In the minimum rotation, mean was 119.27° ($P < 0.05$). In the minimum angular velocity, peak was 19.49°/s ($P < 0.05$).

4. Discussion

The present study has described and examined the identification, analysis, and differentiation in the performance of kinematic variables using the inertial sensor in the *iPhone4* during the turning transitions of the ETGUG test in healthy and frail elderly persons. Significant differences were found between the groups of elderly persons in the accelerometry and angular displacement variables obtained in the kinematic readings of the trunk during the turning transitions of the ETGUG test.

The results obtained in this study show a series of deficits in the frail elderly population group. The statistically significant differences found between the groups were in the data obtained from the gyroscope and the accelerometer. From the results obtained, significant differences were obtained in the y-axis (Yaw movement), the z-axis (Pitch movement), and the x-axis (Roll movement).

As far as we are aware, this is the first study which has used *iPhone4* technology to analyse and study the kinematics of healthy and frail persons aged over 65 years during the turning transitions of the ETGUG test. Three recent studies [14, 25, 26] have instrumented the Timed Get Up and Go test, differentiating and analysing the kinematic data in each of the five subphases of the test between two groups of elderly persons. However, unlike the present study, they did not use *iPhone4* technology to collect kinematic variables. Their goal was to differentiate movement patterns for elderly persons with Parkinson's disease, carrying out the tests over a distance of seven meters.

It should be noted that frailty is defined as a clinical syndrome in which three or more of the following criteria should be present: unintentional weight loss, self-referred exhaustion, muscular weakness, low walking speed, and low physical activity levels [1]. Generically, the gyroscope and accelerometry data obtained for the turning transitions were similar to other studies with other types of study group. In this study, the frail elderly showed low magnitudes in the kinematic values with low variability (very small standard deviations) compared to the controls, the same as the subjects affected by Parkinson's disease [16, 25, 26], the elderly with a high risk of falls [2] and the frail elderly in a previous study [13].

Another recent study which has worked on the instrumentalization of the Timed Get Up and Go [2] test systematically evaluated the accelerometry values in elderly

persons with a high risk of falls during the traditional three-meter test, focusing solely on transitions in Sit to Stand and Stand to Sit. Like the present study, this study found numerous variables from acceleration which showed differences between groups. However, in this study both the variables and the methodology, amongst other aspects, were different. Moreover, the measurement units were not coincident, and this study was based on the acceleration increased amplitude and the acceleration slope.

From a clinical perspective, the present study demonstrates that these new accelerometry parameters play an important role in differentiating between subjects with different functional states. These results provide new knowledge, extending existing knowledge of the isolated study of other transitions in frail and physically active elderly persons [12, 13, 27].

With regard to analysis of the data obtained in the present study, the differences between the frail and the physically active elderly show a series of deficits in the group of frail persons in the turning transitions. It is notable that the most significant differences in the phase were described in the results section. Moreover, as can be seen, the standard deviation in values for the frail subjects was always lower than for the physically active subjects. A previous study which analysed the behaviour of kinematic variables during turning in persons suffering from Parkinson's disease [9] did not find statistically significant differences between the groups, except in the duration of the transition. However, the present study found statistically significant differences between groups in the aforementioned variables.

Finally, it is notable that in accelerometry, three variables (minimum acceleration in the x, y, and z axes) showed significant differences between the groups during the turning transitions in the ETGUG test. Other studies will be required in the future in order to analyse the predictive capability of the kinematic variables which showed statistically significant differences in the different phases of the ETGUG test between healthy and frail elderly persons. This not only will help to understand which variables are of interest and are associated with the identification of the frail elderly, but also will allow early differentiation of possible pre-frail elderly which may be of use in the sphere of prevention in clinical practice.

The results obtained open up the way for further research in the future, although this study presents a series of limitations. Firstly, men and women have different characteristics, and it would be interesting to analyse differences in the kinematic data by gender following turning exercises. A new study would be required in order to compare differences by gender. Moreover, it would be interesting to consider prospective studies to determine whether the measurements obtained from trunk acceleration can predict frailty syndrome in the elderly, possibly in combination with other measurements (risk of falls). Additional work is also needed to explore other properties of accelerometer-derived measures of the turning, including comparison with gold standard. In the meantime, the present results demonstrate the potential of using an accelerometer to measure turn performance in humans, while maintaining simplicity and requiring no additional time to acquire the data.

5. Conclusions

The *iPhone4* inertial sensor is able to study and analyse the kinematics of the turning transitions of the ETGUG test in frail and physically active elderly persons. The accelerometry values for the frail elderly are lower than the physically active elderly, whilst variability in the readings for the frail elderly is also lower than the control group. This suggests that the frail elderly carry out the transition in a more careful, restricted way during the turning, possibly showing their reduced ability to regulate movement when performing these transitions. Compensation mechanisms also play an important role. These results indicate that the additional, relevant information for future discriminant analysis comes mainly from the acceleration signal during the different transitions of the ETGUG test.

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