Apolipoprotein E polymorphism and oxidative stress in human peripheral blood cells: can physical activity reactivate the proteasome system through epigenetic mechanisms?

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Abstract

Background Alzheimer’s Disease (AD) is characterised by proteasome activity impairment and oxidative stress, resulting in β-amyloid (Aβ) production/degradation imbalance. Apolipoprotein E (ApoE) is implicated in Aβ clearance, and ApoE ε4 isoform predisposes to AD development. Regular physical activity is known to reduce AD progression. However, the impact of ApoE polymorphism and physical exercise on Aβ production and proteasome system activity has never been investigated in human peripheral blood cells.

Methods Healthy subjects were enrolled and classified based on the ApoE polymorphism (by the restriction fragment length polymorphism technique) and physical activity level (Borg Scale), dividing them in ApoE ε4/non-ε4 carriers and active/non-active subjects. The plasma antioxidant capability (AOC), the erythrocyte Aβ production/accumulation, and the nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated proteasome functionality were evaluated in all groups by chromatographic and immunoenzymatic assay, respectively. Moreover, epigenetic mechanisms were investigated considering the expression of the histone deacetylase 6, employing a competitive ELISA, and the modulation of two keys miRNAs (miR-153-3p and miR-195-5p), through miRNeasy Serum/Plasma Mini Kit.

Results ApoE ε4 subjects showed a reduction in plasma AOC and an increase of the Nrf2 blocker, miR-153-3p, contributing to an enhancement of the erythrocyte concentration of Aβ. Physical exercise increased plasma AOC and reduced the amount of Aβ and its precursor, involving a reduced miR-153-3p expression and a miR-195-5p enhancement.

Conclusions Our data highlight the impact of ApoE genotype on the amyloidogenic pathway and the proteasome system, and suggest the positive impact of physical exercise, also through epigenetic mechanisms.

1. Background

Apolipoprotein E (ApoE) belongs to the family of apolipoproteins, i.e. proteins involved in the lipoprotein assembly, lipid transport, and metabolism by mediating interactions with receptors, enzymes, and lipid transport proteins [1]. The ApoE is mainly associated with chylomicron, very-low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) [2], delivering lipids through blood, cerebrospinal fluid, and lymph [3]. It is chiefly synthesized in the peripheral system by the liver and macrophages, and in the central nervous system (CNS) by astrocytes, microglia or in pathological condition by neurons [4]. The ApoE is a 34 kDa glycoprotein of 299 amino acids, encoded by the ApoE gene located on chromosome 19q13.2, that exists in three different isoforms: ApoE ε2, ApoE ε3, and ApoE ε4. Each isoform differs from the others for the presence of a cysteine (C) or arginine (R) in positions 112 and 158 of the amino acid sequence of the protein. These dissimilarities in the primary sequence lead to alterations in the structure and in the function of the ApoE isoforms [5].
Beyond the function in the regulation of the transport and metabolism of lipids, ApoE is implicated in the maintenance of the normal brain functions, playing an important role in neurological disorders (NDs) [6]. In the CNS, ApoE regulates the clearance of amyloid beta (Aβ), which is a common hallmark of NDs, mainly associated with Alzheimer’s Disease (AD). Notably, ApoE ε4 is the main genetic risk factor that leads to AD, especially when the genotype is homozygous (ε4/ε4) [4]. Interestingly, the ε4 isoform of ApoE shows a reduced affinity to Aβ compared to the other isoforms, decreasing Aβ clearance and giving rise to the formation of Aβ toxic oligomers, which constitute the extracellular plaques, a typical pathogenic feature of AD [6].

Many studies have extensively demonstrated the association between the ApoE genotype and oxidative stress. Particularly, the ε4 is the lowest effective isoform of ApoE in protecting cells from oxidative stress, both in vitro and in vivo [7]. Oxidative stress is the main process that initiates and enhances the pathological processes that characterized NDs. Of note, a significant degree of oxidative damage is associated with accumulation of Aβ in the brain of AD patients [8, 9]. Indeed, besides the imbalance between the dangerous generation of reactive oxygen species (ROS) and the lacking ability of the biological system to remove them, AD is characterised by the incapacity to maintain a homeostatic balance between amyloid production and its degradation, resulting in the direct inhibition of proteasome activity and indirect elevation of oxidative stress, both of which contribute to protein dysfunction [10]. In this sense, accumulating evidence indicates that the dysfunction of the ubiquitin-proteasome system (UPS) is a key factor to initiate and aggravate the pathogenesis of NDs. On the other hand, Aβ accumulation has been proven to reduce proteasomal activity in cultured neurons [11]. From a molecular point of view, misfolded protein accumulation and aggregation induce an atypical production of ROS, that modify the ubiquitin E3-ligase Kelch-like ECH-associated protein 1 (Keap1), leading to the release, stabilization and nuclear localization of the nuclear factor erythroid 2-related factor 2 (Nrf2), finally increasing the transcription of antioxidant response element (ARE)-genes [12]. Physiologically, Nrf2 up-regulates the proteasome system subunits, protecting cells from accumulation of toxic proteins [12, 13]. However, altered levels of Nrf2 and of its ubiquitinated form have been found in NDs, including AD [12, 14]. Furthermore, decreased levels of Nrf2-dependent target gene expression has been found in ApoE ε4 compared to other isoforms [15], strengthening the link between ApoE polymorphism, proteasome activity, and neurodegenerative processes.

Many studies have demonstrated that regular and moderate physical activity can prevent or reduce the progression of NDs [16]. Physical exercise has demonstrated to upregulate the antioxidant capability, modulating oxidative stress, and increase degradation of amyloidogenic oligomers [16]. Furthermore, aerobic exercise has been shown to upregulate UPS in healthy mice [17].

In this regard, we have recently demonstrated that physical activity modulates the accumulation of NDs-related misfolded proteins in peripheral cells, both in healthy subjects and in NDs patients, and plays a pivotal role in maintaining the physiological erythrocytes well-being and plasma antioxidant capability in healthy volunteers [18–21]. Erythrocytes are emerging as a good model to study biochemical alterations
related to NDs because this kind of cells is particularly susceptible to oxidative stress and accumulation of misfolded proteins, as Aβ [22, 23].

Therefore, the present study aimed to investigate the influence of the ApoE polymorphism and the physical activity on the oxidative stress levels, the amyloidogenic pathway of Aβ production/accumulation, and the Keap1-Nrf2-mediated proteasome functionality. Finally, the epigenetic mechanisms around the amyloidogenic pathway and Keap1-Nrf2 axis were evaluated considering the expression of histone deacetylase 6 (HDAC6) and the modulation exerted by two key miRNAs (miR-153-3p and miR-195-5p). All these aspects were evaluated in peripheral cells, i.e. erythrocytes, and plasma of healthy subjects classified on the base of ApoE polymorphism and the level of physical activity.

2. Methods

2.1. Recruitment of healthy volunteers and genotyping of ApoE polymorphism

The healthy subjects (forty-two age- and sex-matched, Table 1) were engaged from the Sports Medicine Unit (Department of Clinical and Experimental Medicine, University of Pisa). This study was approved by the Ethics Committee of the Great North West Area of Tuscany (271/2014 to F.F.), and it was carried out in accordance with the Declaration of Helsinki. All subjects gave informed consent to participate in the study. Fully informed consent was obtained from each subject entering the study [21].

The blood was collected from each subject and, subsequently, genomic DNA was extracted from the whole blood. The restriction fragment length polymorphism (RFLP) technique has been employed to classify the subjects in ApoE ε4 carriers and non-ε4 carriers. Briefly, the polymerase chain reaction (PCR) was made with 1.5 pmol of each primer (forward 5′-TCG-GCCGCA-GGG-CGC-TGA-TGG-3′ and reverse 5′-CTCGCG-GGC-CCC-GGC-CTG-GTA-3′), 250 µmol/L dNTPs, GC-rich (10% of the final volume), 2 units of Taq DNA polymerase (Applied Biosystems Inc., Branchburg, NJ), 10 ng/µL of genomic DNA, 25 mM MgCl2, and buffer 10X. A thermal cycler (PerkinElmer) was employed for reactions: one cycle at 94 °C for 6 min, 30 cycles at 94 °C for 40 s, 67 °C for 30 s, 72 °C for 45 s, and a final extension at 72 °C for 5 min. The amplified fragments, resulting from digestion with 3 U of HhaI restriction enzyme, were divided through agarose (5%) gel electrophoresis. The restriction patterns were displayed using ethidium bromide staining and UV light.

The subjects’ genotypes were established by ABI PRISM310 Automated Sequencer (Applied Biosystems, Forster City, CA, USA). Thus, the subjects have been classified in ApoE ε4 carriers (sixteen, age- and sex-matched, Table 1), who included heterozygotes subjects ε4/ε3, and ApoE non-ε4 carriers (twenty-six, age- and sex-matched, Table 1), who included heterozygotes subjects (ε2/ε3) or homozygotes ones (ε3/ε3). The lowered number of ApoE ε4 carriers is due to a reduced extent of this genotype in the human race compared to other polymorphisms of the same protein (ApoE ε2 or ApoE ε3) [5, 18]. When the DNA
concentration was too low to allow a correct discrimination of ApoE alleles by RFLP, subjects’ genotypes were established by ABI PRISM310 Automated Sequencer.

| Groups                      | Number of subjects | Age (years) | Sex (M/F) | Physical activity level (Borg scale) |
|-----------------------------|--------------------|-------------|-----------|-------------------------------------|
| ApoE ε4 carriers            | 16                 | 39±14       | 7/9       | 9±3                                 |
| ApoE non-ε4 carriers        | 26                 | 40±13       | 12/14     | 10±4                                |
| NA ApoE ε4 carriers         | 8                  | 38±11       | 3/5       | 7±1                                 |
| A ApoE ε4 carriers          | 8                  | 41±21       | 4/4       | 13±2                                |
| NA ApoE non-ε4 carriers     | 13                 | 41±14       | 5/8       | 7±1                                 |
| A ApoE non-ε4 carriers      | 13                 | 39±12       | 7/6       | 13±2                                |

The subjects were grouped in ApoE ε4 carriers and ApoE non-ε4 carriers, on the base of ApoE polymorphism (Sect. 2.1.). In each group, the subjects are further classified in non-active (NA) and active (A), on the base of physical activity level (Sect. 2.3.). The number of recruited subjects (N), the age (years), and sex (M/F) are indicated. Values are expressed as mean ± SD.

2.2. Clinical parameters of the enrolled subjects

Italian healthy subjects with an upper-middle socio-economic status have been recruited for the current study. Each participant shows neither cardiovascular disease nor other major medical disorders, thus established by clinical history, physical examination, blood pressure, blood chemistry, haematology, urine analysis, basal and stress electrocardiography, with a maximal graded cycle ergometry test executed by a cardiologist blinded to the other data [18, 19, 24]. Familiar AD cases were excluded from subjects’ sampling.

Generally, the major inclusion criteria were as follows: diastolic arterial blood pressure lower than 90 mmHg, systolic arterial blood pressure lower than 140 mmHg, body mass index lower than 30 kg/m², plasma triglycerides from 30 to 150 mg/ml, total plasma cholesterol ranging from 120 to 220 mg/ml, and HDL cholesterol from 26 to 75 mg/ml. Smokers and subjects in treatment with drug/nutraceutical were excluded from the study [18, 19].

2.3. Levels of physical activity of the enrolled subjects

The participants were grouped into non-active (NA) and active (A) based on the habit’s questionnaire (Table 1). According to the World Health Organization (WHO) [25], a non-active subject performs less than 150 minutes per week of physical activity. Moreover, the Borg Rating and Perceived Exertion (RPE) Scale has been employed to evaluate the intensity level of physical activity [26]. The scale ranges from 6 to 20:
6 corresponds to no exertion at all, 7.5 to extremely light, 9 to very light, 11 to light, 13 to somewhat hard, 15 to hard, 17 to very hard, 19 to extremely hard, and 20 to maximal exertion [18].

2.4. Blood specimen collection

The whole blood was collected from each volunteer at least 48 h later the last exercise bout and it was conserved into an anticoagulant EDTA tube. The blood was centrifuged at 200 x g at 4 °C for 10 min to separate erythrocytes from plasma.

The plasma supernatant was isolated and conserved at -20 °C until use. The erythrocyte pellet was suspended in 3 mL of PBS, centrifuged at 1000 x g for 10 min, and washed with PBS. Following further centrifugation at 1500 x g for 10 min, the isolated erythrocytes were conserved at -20 °C until use [18].

2.5. Assessment of the total antioxidant capability (AOC) in plasma

The total antioxidant capability (AOC) in plasma was assessed using the total oxyradical scavenging capacity (TOSC) assay, a gas chromatographic assay able to define oxyradical scavenging capacity of biological fluids [18, 19, 27]. Hydroxyl radicals were generated at 35 °C by the iron plus ascorbate-driven Fenton reaction (1.8 mM Fe$^{3+}$, 3.6 mM EDTA, and 180 mM ascorbic acid in 100 mM PBS, pH 7.4). Reactions with 0.2 mM KMBA (alpha-keto gamma-methylthiobutyric acid) were performed in 10 mL vials sealed with gas-tight Mininert valves (Supelco, Bellefonte, PA) in a final volume of 1 mL. Ethylene production was quantified by gas chromatographic analysis of 200 µL aliquots taken from the headspace of vials at timed intervals during the reaction (Hewlett-Packard gas chromatograph, HP 7820A Series, Andoven, M, equipped with a Supelco DB-1 capillary column and a flame ionization detector, FID). Total ethylene formation was measured from the area under the curves that best define the experimental points obtained for control reactions and after the addition of plasma during the reaction [18, 27, 28]. The equation TOSC = 100 − (SA / CA x 100) was used to determine the TOSC values: SA is the area under the curve (AUC) for the sample and CA is the control reaction. A TOSC value of 100 is correlated with a sample able to suppresses the ethylene formation, while a negative TOSC value is attributed to a pro-oxidant sample. A TOSC value of 0 corresponds to a sample without scavenging capacity [29]. Each experiment was performed twice to consider the intrinsic variability of the method. The results were indicated in TOSC units/ml [18, 27, 30].

2.6. Quantification of Amyloid Beta (Aβ) in Erythrocytes

The concentration of Aβ in erythrocytes was measured by an enzyme-linked immunosorbent assay (ELISA), as described [18, 19]. The plate was pre-coated with a specific antibody to Aβ (sc-9129, Santa Cruz Biotechnology), diluted in poli-L-ornithine, and maintained overnight at 4 °C. Following washing with PBS-T (PBS, containing 0.01% Tween 20), to block non-specific sites, BSA 1% was added and incubated for 2 h at 37 °C. After washes with PBS-T, erythrocytes (0.05 mg/100 µL) were added to each well (100 µL/well) and incubated for 1 h at 25 °C. Then, a polyclonal antibody to Aβ (sc-5399, Santa Cruz Biotechnology) was employed and incubated for 1.5 h at 25 °C. Consequently, a HRP antibody (Santa
Cruz Biotechnology) was added to each well and incubated for 1 h at 37 °C. The 3,3′,5,5′-tetramethylbenzidine (TMB) (Thermo Scientific) and, consequently, the Stop Solution (H₂SO₄), were added and the absorbance was read at 450 nm (EnSight Multimode Plate Reade, PerkinElmer). All measurements were performed in duplicate. The standard curve for ELISA assay was constructed using recombinant human Aβ solution at different concentrations [18, 19, 21].

2.7. Evaluation of erythrocyte amyloid precursor protein (APP)

The amyloid precursor protein (APP) levels in erythrocytes were evaluated through a sandwich ELISA kit (Human Amyloid Precursor Protein, ELISA kit, MyBioSource, #MBS731247).

Erythrocytes (50 µL), isolated from whole blood as already described (Sect. 2.4.), were diluted 1:10 in PBS pH = 7.0-7.2 and incubated in the wells of the pre-coated plate, together with balance solution (5 µL) and conjugate (100 µL), for 1 h at 37 °C. Then, each well was thoroughly washed to remove all unbound components. Substrate solutions were added to each well. After a short incubation period necessary to the substrate to react with the enzyme (HRP), and following the addition of sulphuric acid to terminate the enzyme-substrate reaction, the absorbance was read at 450 nm (EnSight Multimode Plate Reade, PerkinElmer). A standard curve was designed relating the intensity of the colour (O.D.) to the concentration of standards. The APP concentration (ng/mg of total proteins) was interpolated from the standard curve.

2.8. Evaluation of erythrocyte expression of β-secretase 1 (BACE1)

The β-secretase 1 (BACE1) amount in erythrocytes was evaluated through a sandwich ELISA kit (Human Beta-secretase 1, ELISA kit, Thermo Scientific Pierce, #EHBACE1).

Erythrocytes (100 µL), isolated from whole blood as already described (Sect. 2.4.), were diluted 1:25 in 1X assay diluent and incubated for 2.5 h at room temperature with gentle shaking. Following washing, a 1X biotinylated antibody (100 µL) was added to each well and incubated for 1 h at room temperature with gentle shaking. After washing, Streptavidin-HRP solution (100 µL) was added to each well and incubated for 45 min at room temperature with gentle shaking. Then, the wells were washed and TMB substrate (100 µL) was added to each well. The colorimetric reaction was stopped by the adding of the stop solution (50 µL) to each well. The absorbance was read at 450 nm (EnSight Multimode Plate Reade, PerkinElmer). The standard curve was generated by plotting the absorbance obtained from each standard. The BACE1 concentration (ng/mL) was quantified according to the standard curve.

2.9. Quantification of the total amount of erythrocyte nuclear factor erythroid 2-related factor 2 (Nrf2)

The nuclear factor erythroid 2-related factor 2 (Nrf2) was quantified in erythrocytes by a high throughput assay, that combines a quick ELISA assay with a sensitive and specific non-radioactive one for
transcription factor activation (Nrf2 Transcription Factor Assay Kit, Colorimetric, abcam, #ab207223). Through this assay, only active Nrf2 that is present in the sample is detected by a primary antibody that recognizes an epitope of Nrf2 accessible only when the protein is activated.

Erythrocytes (10 µL, i.e. 5–20 µg), separated from the whole blood as above described (Sect. 2.4.), were diluted in the completed binding buffer and incubated for 1 h at room temperature with mild agitation (100 rpm). After extensive washes, a primary antibody (100 µL) was added and incubated for 1 h at room temperature without shaking. Following washing, a secondary antibody (100 µL) was added and incubated for 1 h at room temperature without shaking. Then, the wells were washed and the developing solution was added and incubated. After the addition of the stop solution, the absorbance was read at 450 nm (EnSight Multimode Plate Reade, PerkinElmer). The Nrf2 amount was calculated from Nrf2 activation absorbance and normalised to the absorbance of the total proteins in the sample (µg/µL).

2.10. Measurement of erythrocyte histone deacetylase 6 (HDAC6)

The histone deacetylase 6 (HDAC6) was detected in erythrocytes with a competitive ELISA kit (Human Histone Deacetylase 6, HDAC6, Elisa Kit, Competitive ELISA, MyBioSource, #MBS7254230).

Erythrocytes (100 µL), isolated from whole blood as already described (Sect. 2.4.), were diluted 1:10 in PBS pH = 7.0-7.2 and incubated in the wells of the pre-coated plate, together with balance solution (10 µL) and conjugate (50 µL), for 1 h at 37 °C. Afterward, the wells were carefully washed to remove all unbound components. Substrate solutions were added to each well and incubated for a few minutes. Following the addition of sulphuric acid to terminate the enzyme-substrate reaction, the absorbance was read at 450 nm (EnSight Multimode Plate Reade, PerkinElmer). A standard curve was designed relating the intensity of the colour (O.D.) to the concentration of standards. The HDAC6 concentration (pg/mg of total proteins) was interpolated from the standard curve.

2.11. Expression of plasma Kelch-like ECH-associated protein 1 (Keap1)

The expression of plasma Kelch-like ECH-associated protein 1 (Keap1) was detected by western-blot analysis.

Briefly, plasma (10 µg of total proteins, quantified through Lowry assay), opportunely isolated from the whole blood as above described (Sect. 2.4.), with additional Laemmli solution, was resolved by electrophoresis using a 4–20% Criterion TGX stain-free precast gel (Bio-Rad, #5678094). Afterward, the samples were transferred by the Trans-Blot Turbo transfer system (Bio-Rad) to Trans-Blot Turbo Midi 0.2 µM PVDF membrane (Bio-Rad, #1704157). Then, the membrane was incubated for at least 1 h with a buffer able to block non-specific sites (5% Milk). Primary antibody against Keap1 (rabbit, #AV38981, Sigma-Aldrich) was used and incubated overnight at 4 °C, under continuous agitation. Following the incubation with a secondary antibody HRP-conjugated, protein bands were detected with a chemiluminescent substrate (Clarity Western ECL Substrate, Bio-Rad, #1705061). Densitometry was
performed by ImageJ Software. Images were obtained in different western blots using a reference standard for each running gel, due to the impossibility to show all the samples at the same time.

2.12. Analysis of the expression of circulating miRNAs

Plasma, isolated from the whole blood as previously described (Sect. 2.4.), were processed by miRNeasy Serum/Plasma Mini Kit (Qiagen, Hilden, Germany) to isolate total RNA, including microRNAs (miRNAs). Retro-transcription was carried out using miRCURY LNA miRNA RT Kit (Qiagen, Hilden, Germany) and the obtained cDNA was diluted 1:30, immediately before use. Real-time PCR was run on the MiniOpticon CFX 48 real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) using miRCURY LNA miRNA SYBR Green PCR and specific miRCURY LNA miRNA PCR Assay (Qiagen, Hilden, Germany), as previously reported [31]. MiRCURY Primer Assay specific for hsa-miR-195-5p (MIMAT0000461), hsa-miR-153-3p (MIMAT0000439) and hsa-miR-93-5p (MIMAT0000093) were purchased by Qiagen (Hilden, Germany).

The relative miRNA expression was calculated using the Ct method and normalized on miR-93-5p. Several pieces of evidence reported high stability of miR-93-5p in biofluids [32–35] thus miR-93-5p was suggested as plasmatic reference gene in manufacturer's handbook. According to this, the expression levels of miR-93-5p in plasma samples of our cohort showed comparable expression levels without significant difference among groups (data unshown).

2.13. Statistical analysis

The data are shown as the mean value ± S.D. (Standard Deviation). The data relative to Aβ, APP, BACE1 were depicted as median values. Sample size calculator was performed to estimate the accuracy of the results. Accordingly, the study group required 40 patients to obtain the same difference with α = 0.05 and a statistical power of 85%.

Kolmogorov–Smirnov tests were used to data meeting the assumption of a normality distribution. The variance between groups was statistically significant using Bartlett's test. One-way analysis of variance (ANOVA) test was used to evaluate differences among groups for data meeting the assumption of homogeneity of variance. Pearson correlation analysis and t-tests were applied when only two groups were present for data with distributions that met parametric assumptions. Chi-square tests (Pearson's, Yates-adjusted or Fisher's exact test according to sample size), Mann–Whitney U tests, and Spearman correlation analysis were employed in situations where parametric assumptions were not met. Tukey's multiple comparison was applied for the densitometry analysis. Statistical analysis for miRNA expression was performed using the Kruskal-Wallis (non-parametric) followed by Dunn's multiple comparisons test.

Correlation between variables was determined by linear regression analysis, while interactions between variables were analysed by correlation and multiple regression analyses. P values < 0.05 were considered significantly different. All statistical analysis were carried out by commercial software (GraphPad Prism, version 7.0; GraphPad Software Inc., San Diego, CA) [18, 21, 28].
3. Results

3.1. Descriptive statistics

The whole cohort of healthy volunteers \((n = 42)\) was divided in ApoE \(\varepsilon4\) carriers (mean age 39±14) and ApoE non-\(\varepsilon4\) carriers (mean age 40±13), and, based on the results of the Borg score (Sect. 2.3.), the subjects were additionally classified in non-active (NA) and active (A).

The population did not show significant differences in age, sex distribution, and body mass index (BMI).

3.2. Plasma Antioxidant Capability (AOC)

The plasma antioxidant capability (AOC) was evaluated as an index of oxidative stress, using the TOSC assay: increased TOSC levels are linked to an improved AOC, the main tool in the assessment of the antioxidant status [36]. The AOC levels versus hydroxyl radicals (Fig. 1) were significantly decreased in ApoE \(\varepsilon4\) compared to non-\(\varepsilon4\) carriers \((P < 0.0001)\), underlining the impact of ApoE polymorphism on the AOC. A significant higher AOC was evidenced in active compared to non-active subjects, both in ApoE \(\varepsilon4\) \((P < 0.0001)\) and non-\(\varepsilon4\) carrier group \((P < 0.0001)\). Moreover, among non-active subjects, the non-\(\varepsilon4\) carriers presented increased plasma AOC levels compared to \(\varepsilon4\) ones \((P < 0.0001)\), while no differences have been identified among active subjects \((P = 0.3649)\). The data confirmed that both ApoE polymorphism and physical activity can influence plasma AOC.

3.3. Erythrocyte amyloid beta (A\(\beta\))

The oxidative stress initiates and enhances the accumulation and aggregation of A\(\beta\) in the brain of AD patients [8, 9]. To this regard, A\(\beta\) has been recently demonstrated to also accumulate in erythrocytes [18, 37–39]. On this basis, A\(\beta\) accumulation was measured in erythrocytes of the enrolled subjects.

The concentration of A\(\beta\) (Fig. 2a) showed a trend of increase in ApoE \(\varepsilon4\) carriers compared to non-\(\varepsilon4\) ones \((P = 0.4261)\). Moreover, in both \(\varepsilon4\) and non-\(\varepsilon4\) carrier groups, non-active showed higher A\(\beta\) levels than active subjects \((\varepsilon4: P = 0.0060; \text{non-}\varepsilon4: P < 0.0001)\).

Furthermore, among active subjects, \(\varepsilon4\) carriers displayed increased amount of A\(\beta\) in erythrocytes compared to non-\(\varepsilon4\) carriers \((P = 0.0004)\), while no differences have been identified in non-active groups \((P = 0.8003)\).

Overall, these results confirmed the influence of both ApoE polymorphism and physical activity on the accumulation of A\(\beta\) in erythrocytes.

3.4. Erythrocyte amyloid precursor protein (APP)

The physiologic generation of the neurotoxic A\(\beta\) peptide has been established from sequential APP proteolysis [40]. Since oxidative stress has revealed to promote APP amyloidogenic pathway,
consequently increasing the Aβ production and accumulation [41], the amount of APP was assessed in erythrocytes as the pivotal precursor of the production of the Aβ in peripherals cells [18, 19, 21].

The total amount of APP (Fig. 2b) was comparable in ApoE ε4 and non-ε4 carriers (P = 0.8370), underling that the APP levels in erythrocytes are independent of ApoE polymorphism. Generally, non-active subjects showed increased erythrocyte APP levels when compared to the active ones (ε4 carriers: P = 0.0119; non-ε4: P = 0.0018). Overall, these results demonstrated that the APP concentration in erythrocytes is influenced by the levels of physical activity but it is independent of ApoE genotype.

### 3.5. Erythrocyte expression of β-secretase 1 (BACE1)

Oxidative stress has shown to increase the BACE1 activity, a key enzyme involved in Aβ production [42]. On this basis, the total BACE1 (Fig. 2c) was detected in erythrocytes of the enrolled cohort. A trend of increase of BACE1 levels in erythrocytes have been identified in ApoE non-ε4 carriers compared with ε4-carriers group (P = 0.1681). Moreover, among non-ε4 carriers, non-active subjects showed an increased BACE1 amount compared to the active ones (P = 0.0067), while no differences have been identified among ε4 carriers (P = 0.2467).

Furthermore, among non-active subjects, non-ε4 carriers presented increased BACE1 levels compared to ε4 ones (P = 0.0015).

Globally, the results underlined the influence of the ApoE polymorphism and physical activity on the peripheral levels of BACE1.

### 3.6. Erythrocyte nuclear factor erythroid 2-related factor 2 (Nrf2)

The lessened antioxidant enzymes activity and the increased oxidative stress have been shown to be a consequence of the reduction of Nrf2 activity in the cells, a condition that characterized several diseases, including AD [43]. Herein, the levels of Nrf2 (Fig. 3a) were detected in erythrocytes, also considering that the transcription factor is crucial for the redox homeostasis of erythrocytes, under pro-oxidant stress conditions [44].

The detection of Nrf2 in the erythrocytes of ApoE ε4 carriers versus non-ε4 carriers did not reveal significant differences (P = 0.2128), even if among non-active subjects, non-ε4 carriers displayed increased amount of Nrf2 compared to ε4 carriers (P = 0.0011). Of note, in the non-ε4 subgroup, the data showed a higher Nrf2 amount in non-active than active subjects (P < 0.0001).

Thus, the results revealed that the erythrocyte quantity of Nrf2 depends neither on ApoE polymorphism nor physical activity, even if physical activity could influence the expression levels in peripheral cells.

### 3.7. Plasma Kelch-like ECH-associated protein 1 (Keap1)
The role of Nrf2 in the regulation of antioxidant response, through the induction of AREs transcription, has been demonstrated to involve Keap1, which also contributes to the Nrf2-mediated autophagy and proteasome activity [12].

Keap1 concentrations (Fig. 3b) were significantly lower in non-ε4 carriers compared to ε4-carriers, in the whole group (P < 0.0001), and among both non-active (P < 0.0001) and active (P < 0.0001) subjects. Moreover, among ApoE ε4 carriers and non-ε4 carriers, Keap1 was significantly lower in active subjects compared to non-active ones (ε4: P < 0.0001; non-ε4: P = 0.0138).

These data highlight that ApoE polymorphism and physical exercise modulate Keap1 expression.

3.8. Erythrocyte histone deacytalyase 6 (HDAC6)

In order to investigate the epigenetic mechanisms around the Keap1-Nrf2 axis [45], the expression of the histone deacytalyase HDAC6 was investigated. Indeed, HDAC6 has been proven to modulate the Nrf2-Keap1 association and thus Nrf2 activity [46].

Concerning this evidence, the total erythrocyte amount of HDAC6 (Fig. 4a) was measured. ApoE non-ε4 carriers showed lower levels of HDAC6 in erythrocytes than ApoE ε4 carriers in the whole cohort (P = 0.0062) and in non-active subgroup (P = 0.0083), while among active subjects it was comparable (P = 0.1750). Beyond the emerged differences on the base of ApoE polymorphism in the whole cohort, the concentration of HDAC6 in erythrocytes were comparable between active and non-active subjects (ε4 carriers, P = 0.3918; non-ε4 carriers, P = 0.6574).

The data demonstrated that HDAC6 concentration depends on ApoE polymorphism and it is independent of the physical activity.

3.9. Expression levels of circulating miRNAs

Increased oxidative stress influences the levels of miRNAs, that have been identified as oxidative stress response actors, inducing premature cell senescence [43, 47] and emerging as candidate biomarkers for numberless diseases [48, 49], including AD [50].

Among neurodegenerative disorders-related miRNAs, miR-195-5p and miR-153-3p caught our attention [51, 52]. Indeed, the expression of miR-195-5p is related to physical activity [53] and Aβ accumulation, and it directly targets BACE1 and APP [54], while miR-153-3p, targeting Nrf2, is involved in the control of oxidative stress response [51, 53]. In detail, the circulating expression of miR-195-5p and miR-153-3p were evaluated. For both miRNAs, all the comparisons performed were not modified by considering each sex separately (data not shown).

The levels of miR-195-5p (Fig. 4b) were almost comparable among ApoE ε4 and non-ε4 carriers. Nevertheless, a significant difference inside these groups appeared when active and non-active subjects were separately analysed. In particular, the circulating miR-195-5p levels were significantly higher in active than in non-active subjects, with a greater difference observed between the groups when the ε4
polymorphism was present (8.2-fold change, \( P = 0.0013 \) for ApoE \( \varepsilon4 \) and 2.3-fold, \( P = 0.0489 \) for ApoE non-\( \varepsilon4 \)). Overall, these results demonstrate that circulating levels of miR-195-5p are influenced by the levels of physical activity and this influence is more relevant in ApoE \( \varepsilon4 \).

As regards the expression of miR-153-3p (Fig. 4c), it was significantly lower in ApoE non-\( \varepsilon4 \) carriers than \( \varepsilon4 \) ones (\( P = 0.0002 \)). Furthermore, in \( \varepsilon4 \) carriers the expression of miR-153-3p was significantly higher (6.1-fold change, \( P = 0.045 \)) in plasma of non-active compared to active subjects. Increased levels (12-fold, \( P = 0.0003 \)) appeared also from the comparison between non active subjects belonging to the different polymorphic state. Instead, no significant differences were detected among active subjects from the \( \varepsilon4 \) and non-\( \varepsilon4 \) groups, respectively.

Taken together, these data suggest that physical exercise may play a relevant role in the control of changes linked to the \( \varepsilon4 \) polymorphism. Moreover, between the two miRNAs investigated, miR153-3p may represent a good biomarker of oxidative stress linked to the ApoE \( \varepsilon4 \) polymorphism.

3.10. **Correlation of plasma and erythrocytes parameters with the plasma antioxidant capability (AOC)**

No significant correlation with age was evidenced for all the analysed parameters.

The plasma AOC showed an inverse correlation with A\( \beta \) (Fig. 5a, \( P = 0.0048, R^2 = 0.183 \)) or APP (Fig. 5b, \( P = 0.0255, R^2 = 0.142 \)) accumulation in erythrocytes.

Interestingly, a significant positive correlation was observed between plasma AOC and miR-195-5p (Fig. 5c, \( P = 0.0456, R^2 = 0.158 \)). In contrast, a negative correlation was evidenced for miR-153-3p (Fig. 5d, \( P = 0.0378, R^2 = 0.111 \)).

The plasma AOC level was not significantly related to the other examined parameters (BACE1: \( P = 0.8702 \); Nrf2: \( P = 0.8655 \); HDAC6: \( P = 0.8660 \)).

3.11. **Correlation of plasma and erythrocytes parameters with the level of physical activity**

The physical activity level showed a direct correlation with the plasma AOC (Fig. 6a, \( P = 0.0408, R^2 = 0.109 \)), as previously obtained in a similar cohort of subjects [18, 21].

An inverse correlation was observed between the level of physical exercise and erythrocytes accumulation of A\( \beta \) (Fig. 6b, \( P < 0.0001, R^2 = 0.412 \)) or its precursor APP (Fig. 6c, \( P = 0.04, R^2 = 0.316 \)). These data confirm that the protein levels in these blood cells may be influenced by exercise.

Interestingly, the amount of physical exercise was inversely related to the levels of miR-153-3p (Fig. 6d, \( P = 0.0464, R^2 = 0.103 \)). In contrast, a strong positive correlation was observed with miR-195-5p (Fig. 6e, \( P = 0.0078, R^2 = 0.647 \)).
The Borg’s score was not significantly related to the other examined parameters (BACE1: P = 0.1289; HDAC6: P = 0.5731).

3.12. Correlation with Aβ synthesis and accumulation in erythrocytes

As expected, Aβ accumulation in erythrocytes showed a positive correlation with APP (Fig. 7a, P = 0.0166, $R^2 = 0.162$). In contrast, a significant inverse correlation was observed between the erythrocyte's levels of Aβ and plasma miR-195-5p (Fig. 7b, P = 0.0011, $R^2 = 0.260$). The latter's negative correlation was also observed between erythrocyte APP concentration and plasma miR-195-5p (Fig. 7c, P = 0.0279, $R^2 = 0.146$), consistent with the fact that Ab and APP represent two consolidated miR-195-5p targets [55].

Aβ accumulation was not significantly related to the other examined parameters (BACE1: P = 0.0957; HDAC6: P = 0.3744; miR-153-3p: P = 0.1787).

Of note, erythrocyte BACE1 levels showed an inverse correlation with plasma miR-153-3p (Fig. 7d, P = 0.0415, $R^2 = 0.211$).

4. Discussion

The current study evaluated the impact of ApoE polymorphism and oxidative stress on the amyloidogenic pathway in peripheral blood cells; moreover, the protective effects of physical activity in reactivating the proteasome system was explored. The main results of the paper are as follows (Fig. 8): i) plasma AOC was significantly reduced in ApoE ε4 subjects; ii) the concentration of Aβ, the proteasome-related Keap1, HDAC6, and the Nrf2 blocker, miR-153-3p, augmented in ε4 carriers compared to non-ε4 carriers; iii) physical exercise was associated to increased plasma AOC and to a reduced amount of Aβ and of its precursor APP; iv) physical exercise-induced effects involved a reduced expression of Keap1, HDAC6 and the Nrf2 blocker, miR-153-3p, together with an enhancement of miR-195-5p. Taken together, our study highlights the impact of ApoE genotype on the amyloidogenic pathway and on the proteasome system, and suggest the positive impact of physical exercise on the protective mechanisms against Aβ accumulation and proteasome inhibition, also through the modulation exerted by epigenetic mechanisms.

ApoE polymorphism plays a pivotal role in the transport and metabolism of lipids. Moreover, it has been widely implicated in the modulation of the oxidative status and misfolded proteins’ accumulation, even before the onset of neurological diseases [6]. On the other hand, regular physical activity has been proven to reduce the accumulation of toxic oligomers, modulate the levels of oxidative stress, finally enhancing neurogenesis and counteracting neurodegeneration processes [18, 56].

Herein, erythrocytes and plasma were elected as a good peripheral models to investigate neurodegeneration-related proteins, because they are particularly susceptible to oxidative stress and capable of accumulating misfolded proteins [22, 23, 57]. By taking advantage of this peripheral models, the present study aimed to investigate the influence of the ApoE polymorphism and physical activity on
the oxidative stress levels, the amyloidogenic pathway of A\(\beta\) production, and the Nrf2-mediated proteasome functionality.

Oxidative stress has been proven to increase in ApoE \(\varepsilon4\) carriers and to rise with aging [18, 58]. Accordingly, plasma AOC, an indirect measure of oxidative stress, was significantly lower in the presence of \(\varepsilon4\) polymorphism. Nevertheless, plasma AOC was independent from age, probably because of the poor age interval of the enrolled subjects. Furthermore, physical exercise enhanced the antioxidant capability, independently from ApoE polymorphism, thus confirming previous reports [18, 21]. In particular, plasma AOC showed a significant positive correlation with the level of physical activity, as previously described in human subjects [18, 19, 21, 59].

As noticed for plasma oxidative status, A\(\beta\) accumulation in erythrocytes occurred particularly in ApoE \(\varepsilon4\) carriers, confirming that this polymorphism plays a pivotal role in A\(\beta\) deposition [5, 60], even in peripheral fluids [61]. Despite A\(\beta\) origins form its precursor APP, no significant differences were evidenced between ApoE \(\varepsilon4\) carriers and non-carriers. These results suggest that the polymorphism does not influence the initial amount of APP but rather its processing through the amyloidogenic pathway. Nevertheless, our results showed an inverse correlation between APP and A\(\beta\) with plasma AOC towards hydroxyl radicals, confirming the link between oxidative stress and the amyloidogenic pathway.

A\(\beta\) generates from APP through the rate limiting enzyme BACE1. Increased BACE1 levels and activity have been reported in the brain of patients with sporadic AD, and well correlated to its end product A\(\beta\) [62]. Surprisingly, in our hands BACE1 concentrations were significantly higher in non-\(\varepsilon4\) carriers than in \(\varepsilon4\) carriers. Additional experiments will be required to measure the enzyme activity, rather than its concentration, to verify putative differences among the different ApoE polymorphisms. In this respect, BACE1 activity in platelets has been found to be increased in AD patients [63]. Of note, the use of different blood cells (i.e., platelets versus erythrocytes) may be another cause explaining our data. Finally, an additional explanation may come from putative BACE1 polymorphisms carried by the subjects [64].

As concern the impact of physical exercise on the amyloidogenic pathway, APP, and A\(\beta\) levels in erythrocytes were significantly lower in active subjects compared to non-active ones, thus confirming that physical exercise modulates A\(\beta\) production and accumulation. Consistent with the data discussed above, BACE1 decreased with physical activity in the absence of ApoE polymorphism. In particular, APP and A\(\beta\) concentrations strictly and inversely depended from the level of physical exercise in the whole group. These data are consistent with those reporting that physical exercise can reduce A\(\beta\) synthesis and accumulation in plasma [65], and erythrocytes [18, 19, 21].

Accumulating evidence indicates that neuroinflammatory and neurodegenerative processes are triggered by the dysfunction of the UPS [11, 66]. In particular, decreased levels and transcriptional activity of the nuclear factor Nrf2 contributes to propagate oxidative stress and neurodegeneration-related proteins [12]. Considering the strict link between the proteasome system and misfolded proteins accumulation, we investigated the impact of ApoE polymorphism and physical activity on the modulation of the UPS pivotal actors.
Herein, the erythrocytes concentrations of Nrf2 did not change between ApoE non-ε-4 carriers and ε-4 carriers. In contrast, Keap1 levels decreased in non-ε-4 carriers compared to ε-4 carriers. Accordingly, Keap1 has been proven to inhibit Nrf2, and direct Keap1 inhibitors have been suggested as an efficient target for the re-activation of Nrf2 in neurodegeneration [67]. Consistent with Keap1 inhibitory role on Nrf2, Keap1 concentration was significantly lower in active subjects in the ε-4 carriers subgroup. According to our data, exercise trials have been proven to significant increase nuclear Nrf2 levels in different peripheral tissues [68, 69].

In order to investigate the epigenetic mechanisms around the Keap1-Nrf2 axis [45], the expression of the HDAC6 and two specific miRNAs was investigated. Indeed, HDAC6 has been proven to inhibit the transcription factor Nrf2 and HDAC inhibition can reduce Keap1-mediated Nrf2 suppression, Nrf2 nuclear translocation, and Nrf2 binding to antioxidant response elements [46]. In our hands, HDAC6 concentration in erythrocytes was lower in non-ε-4 carriers than in ε-4 carriers, consistent with the trend obtained for Keap1 in the same subjects.

Finally, the expression of miR-153-3p and miR-195-5p was explored. Among the different miRNAs nowadays implicated in neurodegeneration, miR-153-3p was chosen because of its link with Keap1-Nrf2 axis and the downstream genes [43, 70]; in contrast, miR-195-5p exerts neuroprotective and neurogenic effects, also down regulated the transcriptional expression levels of BACE1 and APP [54, 55]. Herein, the expression of miR-153-3p significantly decreased in subjects not carrying the ε-4 carrier.

A negative correlation was observed between both Aβ and APP concentrations and miR-195-5p, consistent with the fact that Aβ and APP are strongly modulated by miR-195-5p [55]. Furthermore, miR-153-3p showed an inverse correlation with BACE1 levels. In this sense, miR-153-3p has been proven, in animal models and AD patients, to target directly APP by binding to its 3’ UTR, finally downregulating Aβ [71, 72]: in this sense, Ab downregulation may involve a decrease in BACE1 too.

Interestingly, miR-153-3p showed a negative correlation with plasma AOC towards hydroxyl radicals. In contrast, miR-195-5p positively related to plasma AOC. The same miRNA-related correlations were observed with the level of physical exercise. These data suggest that the two miRNAs positively and negatively regulate the redox response to physical exercise.

### 5. Conclusions

In conclusion, in the present paper we showed that ApoE ε-4 polymorphism was associated with an elevated concentration of Aβ, the proteasome-related Keap1, and miR-153-3p, as well as to a minor plasma AOC. Independently of ApoE polymorphism, physical exercise was associated with increased plasma AOC and reduced amount of Aβ and of its precursor APP. Moreover, physical exercise-induced effects involved a reduced expression of Keap1, HDAC6, and miR-153-3p, together with an enhancement of miR-195-5p.
Overall, our study highlights the impact of ApoE genotype on the amyloidogenic pathway and the proteasome system and evidenced the positive effects of physical activity against Aβ accumulation and proteasome inhibition, also through epigenetic mechanisms that involved miR-153 and miR-195-5p (Fig. 8).

List Of Abbreviations

β-secretase 1 (BACE1)
Alzheimer's Disease (AD)
Amyloid β (Aβ)
Amyloid precursor protein (APP)
Antioxidant response element (ARE)
Apolipoprotein E (ApoE)
Central nervous system (CNS)
Histone deacetylase 6 (HDAC6)
Kelch-like ECH-associated protein 1 (Keap1)
Neurological Disorders (NDs)
Nuclear factor erythroid 2-related factor 2 (Nrf2)
Reactive oxygen species (ROS)
Total antioxidant capability (AOC)
Total oxyradical scavenging capacity (TOSC)
Ubiquitin-proteasome system (UPS)

Declarations

Ethics approval and consent to participate: The current study was accepted by the Ethics Committee of the Great North West Area of Tuscany (271/2014 to F.F.) and it was carried out by the Declaration of Helsinki. All participants have received the informed consent and they have agreed to participate in the study.

Consent for publication: not applicable.
Availability of data and materials: the datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: RP, SD, BP, SC and JF conducted all the experiments. SD, JF, FB and FF recruited subjects. RP, SD, BP, SC, FB and FF analysed the data. RP, SD and CM wrote the manuscript. SD, GS, UB, PN, FF and CM designed the study and provided whole supervision of the project. All authors have given contribution to the drafting and critical revision of the manuscript and contributed to approval of the version to be published.

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References

1. Ramasamy I. Recent advances in physiological lipoprotein metabolism. Clin Chem Lab Med. 2014;52:1695–727.
2. Mahley RW, Weisgraber KH, Huang Y. Apolipoprotein E. structure determines function, from atherosclerosis to Alzheimer’s disease to AIDS. J Lipid Res. 2009;50(Suppl):183–8.
3. Baars HF, Doevendans PAFM, Smagt JJ van der, editors. Clinical Cardiogenetics. London: Springer-Verlag; 2011. doi:10.1007/978-1-84996-471-5.
4. Giau VV, Bagyinszky E, An SSA, Kim SY. Role of apolipoprotein E in neurodegenerative diseases. Neuropsychiatr Dis Treat. 2015;11:1723–37.
5. Liu C-C, Liu C-C, Kanekiyo T, Xu H, Bu G. Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. Nat Rev Neurol. 2013;9:106–18.
6. Mahley RW, Rall SC. Apolipoprotein E: far more than a lipid transport protein. Annu Rev Genomics Hum Genet. 2000;1:507–37.
7. Jofre-Monseny L, Minihane A-M, Rimbach G. Impact of apoE genotype on oxidative stress, inflammation and disease risk. Mol Nutr Food Res. 2008;52:131–45.
8. Huang W-J, Zhang X, Chen W-W. Role of oxidative stress in Alzheimer’s disease (Review). Biomedical Reports. 2016;4:519–22.
9. Prasad KN, Bondy SC. Inhibition of early upstream events in prodromal Alzheimer’s disease by use of targeted antioxidants. Curr Aging Sci. 2014;7:77–90.
10. Bonet-Costa V, Pomatto LC-D, Davies KJA. The Proteasome and Oxidative Stress in Alzheimer’s Disease. Antioxid Redox Signal. 2016;25:886–901.
11. Oh S, Hong HS, Hwang E, Sim HJ, Lee W, Shin SJ, et al. Amyloid peptide attenuates the proteasome activity in neuronal cells. Mech Ageing Dev. 2005;126:1292–9.
12. Pajares M, Cuadrado A, Rojo AI. Modulation of proteostasis by transcription factor NRF2 and impact in neurodegenerative diseases. Redox Biol. 2017;11:543–53.
13. Kwak M-K, Wakabayashi N, Greenlaw JL, Yamamoto M, Kensler TW. Antioxidants Enhance Mammalian Proteasome Expression through the Keap1-Nrf2 Signaling Pathway. Mol Cell Biol. 2003;23:8786–94.
14. Kuusisto E, Kauppinen T, Alafuzoff I. Use of p62/SQSTM1 antibodies for neuropathological diagnosis. Neuropathol Appl Neurobiol. 2008;34:169–80.
15. Graeser A-C, Boesch-Saadatmandi C, Lippmann J, Wagner AE, Huebbe P, Storm N, et al. Nrf2-dependent gene expression is affected by the proatherogenic apoE4 genotype-studies in targeted gene replacement mice. J Mol Med. 2011;89:1027–35.
16. Radak Z, Suzuki K, Higuchi M, Balogh L, Boldogh I, Koltai E. Physical exercise, reactive oxygen species and neuroprotection. Free Radic Biol Med. 2016;98:187–96.
17. Cunha TF, Moreira JBN, Paixão NA, Campos JC, Monteiro AWA, Bacurau AVN, et al. Aerobic exercise training upregulates skeletal muscle calpain and ubiquitin-proteasome systems in healthy mice. J Appl Physiol. 2012;112:1839–46.
18. Piccarducci R, Daniele S, Fusi J, Chico L, Baldacci F, Siciliano G, et al. Impact of ApoE Polymorphism and Physical Activity on Plasma Antioxidant Capability and Erythrocyte Membranes. Antioxidants (Basel). 2019;8.
19. Daniele S, Frosini D, Pietrobono D, Petrozzi L, Lo Gerfo A, Baldacci F, et al. α-Synuclein Heterocomplexes with β-Amyloid Are Increased in Red Blood Cells of Parkinson’s Disease Patients and Correlate with Disease Severity. Front Mol Neurosci. 2018;11:53.
20. Daniele S, Pietrobono D, Fusi J, Lo Gerfo A, Cerri E, Chico L, et al. α-Synuclein Aggregated with Tau and β-Amyloid in Human Platelets from Healthy Subjects: Correlation with Physical Exercise. Front Aging Neurosci. 2018;10:17.
21. Daniele S, Pietrobono D, Fusi J, Iofrida C, Chico L, Petrozzi L, et al. α-Synuclein Aggregates with β-Amyloid or Tau in Human Red Blood Cells: Correlation with Antioxidant Capability and Physical Exercise in Human Healthy Subjects. Mol Neurobiol. 2018;55:2653–75.
22. Singh S. Antioxidants as a preventive therapeutic option for age related neurodegenerative diseases. 2015.
23. Kiko T, Nakagawa K, Satoh A, Tsuduki T, Furukawa K, Arai H, et al. Amyloid β Levels in Human Red Blood Cells. PLOS ONE. 2012;7:e49620.
24. Whaley MH, Brubaker PH, Otto RM, Armstrong LE. Medicine ACoS Guidelines for exercise testing and prescription. 7th edition. Philadelphia, USA: Lippincott Williams & Wilkins; 2006.
25. Wicker P, Frick B. Intensity of physical activity and subjective well-being: an empirical analysis of the WHO recommendations. J Public Health (Oxf). 2017;39:e19–26.
26. Borg GA. Psychophysical bases of perceived exertion. Med Sci Sports Exerc. 1982;14:377–81.
27. Regoli F, Winston GW. Quantification of Total Oxidant Scavenging Capacity of Antioxidants for Peroxynitrite, Peroxyl Radicals, and Hydroxyl Radicals. Toxicol Appl Pharmacol. 1999;156:96–105.

28. Franzoni F, Ghiadoni L, Galetta F, Plantinga Y, Lubrano V, Huang Y, et al. Physical activity, plasma antioxidant capacity, and endothelium-dependent vasodilation in young and older men. Am J Hypertens. 2005;18(4 Pt 1):510–6.

29. Bianchi S, Fusi J, Franzoni F, Giovannini L, Galetta F, Mannari C, et al. Effects of recombinant human erythropoietin high mimicking abuse doses on oxidative stress processes in rats. Biomed Pharmacother. 2016;82:355–63.

30. Franzoni F, Colognato R, Galetta F, Laurenza I, Barsotti M, Di Stefano R, et al. An in vitro study on the free radical scavenging capacity of ergothioneine: comparison with reduced glutathione, uric acid and trolox. Biomed Pharmacother. 2006;60:453–7.

31. Fogli S, Polini B, Carpi S, Pardini B, Naccarati A, Dubbini N, et al. Identification of plasma microRNAs as new potential biomarkers with high diagnostic power in human cutaneous melanoma. Tumour Biol. 2017;39:1010428317701646.

32. Niu Y, Wu Y, Huang J, Li Q, Kang K, Qu J, et al. Identification of reference genes for circulating microRNA analysis in colorectal cancer. Sci Rep. 2016;6:35611.

33. Bae I-S, Chung KY, Yi J, Kim TI, Choi H-S, Cho Y-M, et al. Identification of Reference Genes for Relative Quantification of Circulating MicroRNAs in Bovine Serum. PLOS ONE. 2015;10:e0122554.

34. Song J, Bai Z, Han W, Zhang J, Meng H, Bi J, et al. Identification of Suitable Reference Genes for qPCR Analysis of Serum microRNA in Gastric Cancer Patients. Dig Dis Sci. 2012;57:897–904.

35. Liu X, Zhang L, Cheng K, Wang X, Ren G, Xie P. Identification of suitable plasma-based reference genes for miRNAome analysis of major depressive disorder. J Affect Disord. 2014;163:133–9.

36. Prior RL. Plasma Antioxidant Measurements. J Nutr. 2004;134:3184S–3185S.

37. Baldacci F, Daniele S, Piccarducci R, Giampietri L, Pietrobono D, Giorgi FS, et al. Potential Diagnostic Value of Red Blood Cells α-Synuclein Heteroaggregates in Alzheimer’s Disease. Mol Neurobiol. 2019;56:6451–9.

38. Piccarducci R, Pietrobono D, Pellegrini C, Daniele S, Fornai M, Antonioli L, et al. High Levels of β-Amyloid, Tau, and Phospho-Tau in Red Blood Cells as Biomarkers of Neuropathology in Senescence-Accelerated Mouse. Oxid Med Cell Longev. 2019;2019:5030475.

39. Hooper C, De Souto Barreto P, Payoux P, Salabet AS, Guyonnet S, Andrieu S, et al. Association of Cortical β-Amyloid with Erythrocyte Membrane Monounsaturated and Saturated Fatty Acids in Older Adults at Risk of Dementia. J Nutr Health Aging. 2017;21:1170–5.

40. O’Brien RJ, Wong PC. Amyloid precursor protein processing and Alzheimer’s disease. Annu Rev Neurosci. 2011;34:185–204.

41. Muche A, Arendt T, Schliebs R. Oxidative stress affects processing of amyloid precursor protein in vascular endothelial cells. PLoS One. 2017;12. doi:10.1371/journal.pone.0178127.
42. Tamagno E, Guglielmotto M, Aragno M, Borghi R, Autelli R, Giliberto L, et al. Oxidative stress activates a positive feedback between the γ- and β-secretase cleavages of the β-amyloid precursor protein. J Neurochem. 2008;104:683–95.

43. Prasad KN. Oxidative stress and pro-inflammatory cytokines may act as one of the signals for regulating microRNAs expression in Alzheimer's disease. Mech Ageing Dev. 2017;162:63–71.

44. Kawatani Y, Suzuki T, Shimizu R, Kelly VP, Yamamoto M. Nrf2 and selenoproteins are essential for maintaining oxidative homeostasis in erythrocytes and protecting against hemolytic anemia. Blood. 2011;117:986–96.

45. Cheng D, Wu R, Guo Y, Kong A-NT. Regulation of Keap1-Nrf2 signaling: The role of epigenetics. Curr Opin Toxicol. 2016;1:134–8.

46. Wang B, Zhu X, Kim Y, Li J, Huang S, Saleem S, et al. Histone deacetylase inhibition activates transcription factor Nrf2 and protects against cerebral ischemic damage. Free Radic Biol Med. 2012;52:928–36.

47. Li G, Luna C, Qiu J, Epstein DL, Gonzalez P. Alterations in microRNA Expression in Stress-induced Cellular Senescence. Mech Ageing Dev. 2009;130:731–41.

48. Carpi S, Polini B, Fogli S, Podestà A, Ylösmäki E, Cerullo V, et al. Circulating microRNAs as biomarkers for early diagnosis of cutaneous melanoma. Expert Rev Mol Diagn. 2020;20:19–30.

49. Polini B, Carpi S, Romanini A, Breschi MC, Nieri P, Podestà A. Circulating cell-free microRNAs in cutaneous melanoma staging and recurrence or survival prognosis. Pigment Cell Melanoma Research. 2019;32:486–99.

50. Wiedrick JT, Phillips JI, Lusardi TA, McFarland TJ, Lind B, Sandau US, et al. Validation of MicroRNA Biomarkers for Alzheimer's Disease in Human Cerebrospinal Fluid. J Alzheimers Dis. 2019;67:875–91.

51. Gong G-H, An F-M, Wang Y, Bian M, Wang D, Wei C-X. MiR-153 regulates expression of hypoxia-inducible factor-1α in refractory epilepsy. Oncotarget. 2018;9:8542–7.

52. van den Berg MMJ, Krauskopf J, Ramaekers JG, Kleinjans JCS, Prickaerts J, Briedé JJ. Circulating microRNAs as potential biomarkers for psychiatric and neurodegenerative disorders. Prog Neurobiol. 2020;185:101732.

53. Konovalova J, Gerasymchuk D, Parkkinen I, Chmielarz P, Domanskyi A. Interplay between MicroRNAs and Oxidative Stress in Neurodegenerative Diseases. Int J Mol Sci. 2019;20.

54. Zhu H-C, Wang L-M, Wang M, Song B, Tan S, Teng J-F, et al. MicroRNA-195 downregulates Alzheimer’s disease amyloid-β production by targeting BACE1. Brain Res Bull. 2012;88:596–601.

55. Ai J, Sun L-H, Che H, Zhang R, Zhang T-Z, Wu W-C, et al. MicroRNA-195 Protects Against Dementia Induced by Chronic Brain Hypoperfusion via Its Anti-Amyloidogenic Effect in Rats. J Neurosci. 2013;33:3989–4001.

56. Liu PZ, Nusslock R. Exercise-Mediated Neurogenesis in the Hippocampus via BDNF. Front Neurosci. 2018;12. doi:10.3389/fnins.2018.00052.
57. Jayakumar R, Kusiak JW, Chrest FJ, Demehin AA, Murali J, Wersto RP, et al. Red cell perturbations by amyloid beta-protein. Biochim Biophys Acta. 2003;1622:20–8.

58. Muralidharan N, Bhat T, Kumari SN. A study on effect of ageing on the levels of total antioxidant and lipid peroxidation. IJCMR. 2017;4:8–10.

59. Gawron-Skarbek A, Chrzczanowicz J, Kostka J, Nowak D, Drygas W, Jegier A, et al. Physical Activity, Aerobic Capacity, and Total Antioxidant Capacity in Healthy Men and in Men with Coronary Heart Disease. Oxid Med Cell Longev. 2015;2015:197307.

60. Head D, Bugg JM, Goate AM, Fagan AM, Mintun MA, Benzinger T, et al. Exercise Engagement as a Moderator of the Effects of APOE Genotype on Amyloid Deposition. Arch Neurol. 2012;69:636–43.

61. Nakamura T, Kawarabayashi T, Seino Y, Hirohata M, Nakahata N, Narita S, et al. Aging and APOE-ε4 are determinative factors of plasma Aβ42 levels. Ann Clin Transl Neurol. 2018;5:1184–91.

62. Timmers M, Barão S, Van Broeck B, Tesseur I, Slemmon J, De Waepenaert K, et al. BACE1 Dynamics Upon Inhibition with a BACE Inhibitor and Correlation to Downstream Alzheimer’s Disease Markers in Elderly Healthy Participants. J Alzheimers Dis. 2017;56:1437–49.

63. Johnston JA, Liu WW, Coulson DTR, Todd S, Murphy S, Brennan S, et al. Platelet beta-secretase activity is increased in Alzheimer’s disease. Neurobiol Aging. 2008;29:661–8.

64. Clarimón J, Bertranpetit J, Calafell F, Boada M, Tàrraga L, Comas D. Association study between Alzheimer’s disease and genes involved in Abeta biosynthesis, aggregation and degradation: suggestive results with BACE1. J Neurol. 2003;250:956–61.

65. Stillman CM, Lopez OL, Becker JT, Kuller LH, Mehta PD, Tracy RP, et al. Physical activity predicts reduced plasma β amyloid in the Cardiovascular Health Study. Ann Clin Transl Neurol. 2017;4:284–91.

66. Daniele S, Giacomelli C, Martini C. Brain ageing and neurodegenerative disease: The role of cellular waste management. Biochem Pharmacol. 2018;158:207–16.

67. Kerr F, Sofola-Adesakin O, Ivanov DK, Gatillif J, Gomez Perez-Nievas B, Bertrand HC, et al. Direct Keap1-Nrf2 disruption as a potential therapeutic target for Alzheimer’s disease. PLoS Genet. 2017;13:e1006593.

68. Wang P, Li CG, Qi Z, Cui D, Ding S. Acute exercise stress promotes Ref1/Nrf2 signalling and increases mitochondrial antioxidant activity in skeletal muscle. Exp Physiol. 2016;101:410–20.

69. Done AJ, Newell MJ, Traustadóttir T. Effect of exercise intensity on Nrf2 signalling in young men. Free Radic Res. 2017;51:646–55.

70. Zhu J, Wang S, Qi W, Xu X, Liang Y. Overexpression of miR-153 promotes oxidative stress in MPP+-induced PD model by negatively regulating the Nrf2/HO-1 signaling pathway. Int J Clin Exp Pathol. 2018;11:4179–87.

71. Liang C, Zhu H, Xu Y, Huang L, Ma C, Deng W, et al. MicroRNA-153 negatively regulates the expression of amyloid precursor protein and amyloid precursor-like protein 2. Brain Res. 2012;1455:103–13.
72. Delay C, Calon F, Mathews P, Hébert SS. Alzheimer-specific variants in the 3’UTR of Amyloid precursor protein affect microRNA function. Mol Neurodegener. 2011;6:70.

Figures

Figure 1
Plasma AOC levels detected by TOSC assay. The plasma AOC toward hydroxyl radicals was detected by TOSC assay, as described in Material and Methods: high TOSC values are associated with elevated antioxidant capacity. The data are shown as the mean value ± S.D. and are representative of three independent experiments (n = 3). P-values were adjusted with Unpaired t test: **** P < 0.0001 between the indicated subgroups. GraphPad Prism 7 was used to create the figure.

Figure 2
Erythrocyte Aβ, APP and BACE1 accumulation measured by a sandwich ELISA kit. The whole blood was collected by volunteers and erythrocytes were isolated through sequential centrifugations, as described in Material and Methods. A sandwich ELISA kit was employed to measure the erythrocyte concentration of Aβ (a), APP (b) and BACE1 (c). The data are shown as the median value and are representative of three independent experiments (n = 3). P-values were adjusted with Unpaired t test: * P < 0.05, ** P < 0.01, *** P < 0.001 and **** P < 0.0001 between the indicated subgroups. GraphPad Prism 7 was used to create the figure.
Figure 3

Erythrocyte Nrf2 and plasma Keap1 quantified by transcription factor assay and Western blot analysis, respectively. (a) Nrf2 was measured in erythrocytes, isolated from the whole blood of healthy volunteers, through a high throughput assay, that combines a quick ELISA assay with a sensitive and specific non-radioactive one for transcription factor activation. By this kind of assay, only the activated Nrf2 is detected (Material and Methods). (b) Plasma collected from healthy volunteers was considered to identify the presence of the Keap1 through Western blot analysis, that is shown as densitometric unit of the bands (Material and Methods). The data are shown as the mean value ± S.D. and are representative of three independent experiments (n = 3). P-values were adjusted with Unpaired t test: * P < 0.05, **P < 0.01, and ****P < 0.0001 between the indicated subgroups. GraphPad Prism 7 was used to create the figure.
Erythrocyte HDAC6 concentration quantified by competitive ELISA kit and circulating expression of miR-195-5p and miR-153-3p. (a) HDAC6 was quantified in erythrocytes, isolated from the whole blood of the total cohort, employing a competitive ELISA kit (Material and Methods). The data are shown as the mean value ± S.D. and are representative of three independent experiments (n = 3). P-values were adjusted with Unpaired t test: **P < 0.01 between the indicated subgroups. The expression of miR-195-5p (b) and miR-
153-3p (c) were analysed in plasma samples of non-active and active subjects, classified based on ApoE ε4 polymorphism. The analysis was performed through miRNeasy Serum/Plasma Mini Kit, as described in Material and Methods. The relative expression was calculated by Ct method and normalized on miR-93-5p. Statistical analysis was performed by Kruskal-Wallis (non-parametric) followed by Dunn's multiple comparisons test. * P < 0.05, **P < 0.01, and ***P < 0.001 between the indicated subgroups. GraphPad Prism 7 was used to create the figure.

**Figure 5**
Correlation of erythrocyte and plasma parameters with AOC expressed as TOSC values versus hydroxyl radicals. Correlation analysis between A\(\mu\) (a), APP (b), miR-195-5p (c), and miR-153-3p (d) with plasma AOC. Correlation between variables was determined by simple linear regression analysis, using the StatView program (Abacus Concepts, Inc., SAS Institute, Cary, NC). P and R2 values obtained for each correlation are reported in the respective panel.

Figure 6
Correlation of plasma and erythrocytes parameters with the physical activity level expressed as Borg scale. Correlation analysis between AOC (a), A(β) (b), APP (c), miR-153-3p (d), and miR-195-5p (e), with physical activity. Correlation between variables was determined by simple linear regression analysis, using the StatView program (Abacus Concepts, Inc., SAS Institute, Cary, NC). P and R2 values obtained for each correlation are reported in the respective panel.

Figure 7
Correlation of erythrocytes and plasma parameters with erythrocytes Aβ accumulation. Correlation analysis between erythrocyte Aβ and APP (a), or plasma miR-195-5p (b). Correlation analysis between erythrocyte APP and plasma miR-195-5p (c). Correlation analysis between erythrocyte BACE1 concentrations and plasma miR-153-3p (d). Correlation between variables was determined by simple linear regression analysis, using the StatView program (Abacus Concepts, Inc., SAS Institute, Cary, NC). P and R2 values obtained for each correlation are reported in the respective panel.
Influence of ApoE €4 polymorphism and physical activity in the pathways of the proteasome system. The presence of ApoE €4 polymorphism was associated to an elevated concentration of the proteasome-related Keap1, which inhibited Nfr2 and impaired the proteasome system. These events lead to an enhancement of A\textsubscript{\textalpha}, and of miR-153-3p, as well as to a minor plasma AOC. Aside from ApoE polymorphism, regular physical exercise reduced oxidative stress, by increasing the plasma antioxidant capability, and blocked the pathway of A\textalpha production/accumulation. Physical exercise can also regulate the epigenetic mechanisms involved in the Nrf2-Keap1 axis and in A\textalpha production by reducing miR-153-3p and HDAC6 and enhancing miR-195-5p expression.