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Redox-associated changes in healthy individuals at risk of Alzheimer's disease. A ten-year follow-up study

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ABSTRACT

Carrying an allele 4 of the apolipoprotein E (ApoE) is the best-established genetic risk factor to develop Alzheimer's disease (AD). Fifty percent of ApoE4/4 individuals develop the disease at 70 years of age. ApoE3/4 carriers have a lower risk of developing the disease, still 50% of them suffer AD at around 80 years. In a previous study we showed that healthy young individuals, who had a parent with AD and were carriers of at least one ApoE4 allele displayed reductive stress. This was evidenced as a decrease in oxidative markers, such as oxidized glutathione, p-p38, and $NADP^+$/NADPH ratio, and an increase of antioxidant enzymes, such as glutathione peroxidase (Gpx1) and both the catalytic and regulatory subunits of glutamyl-cysteinyl (GCLM and GCLC). Moreover, we found an increase in stress-related proteins involved in tau physiopathology.

Now, 10 years later, we have conducted a follow-up study measuring the same parameters in the same cohort. Our results show that reductive stress has reversed, as we could now observe an increase in lipid peroxidation and in the oxidation of glutathione along with a decrease in the expression of Gpx1 and SOD1 antioxidant enzymes in ApoE4 carriers. Furthermore, we found an increase in plasma levels of IL1β levels and in PKR (eukaryotic translation initiation factor 2 alpha kinase 2) gene expression in isolated lymphocytes.

Altogether, our results suggest that, in the continuum of Alzheimer's disease, people at risk of developing the disease go through different redox phases, from stablished reductive stress to oxidative stress.

1. Introduction

The use of biomarkers in clinical practice has shown that the molecular alterations of Alzheimer's disease (AD) may be evident even two decades before the onset of clinical symptoms [1]. Therefore, studies carried out in people at risk of suffering AD are of interest. In this regard, the apolipoprotein E ε4 allele (ApoE4) is considered a risk factor for many diseases, but it is specially related to AD [2]. ApoE is a multifunctional protein involved in many cellular processes. It acts in lipid homeostasis, in the response to intracellular calcium, in energy metabolism, in the modulation of intracellular pathways, in endothelial activation and repair, in the maintenance of the blood brain barrier integrity, in the elimination of cellular debris, and in the control of inflammatory response [3]. It is also involved in redox homeostasis [4]. However, the ApoE4 isoform presents altered conformation which affects its function; specifically, ApoE4 has been related to redox imbalance, causing both reductive stress [5] and oxidative stress [6,7].

AD causes a progressive neurological deterioration, affecting different cognitive domains. However, the most common phenotype is amnestic AD, which initially affects episodic memory and focused attention, and is strongly associated with ApoE4 and a positive family history of AD [8]. In a previous study we showed that healthy young people carrying of at least one ApoE4 allele who had a parent with AD presented reductive stress [5]. We found a decrease in blood oxidized glutathione (GSSG) levels and an increased expression in glutathione peroxidase (Gpx1) and in glutamyl-cysteinyl ligase (GCLM and GCLC subunits) in peripheral lymphocytes. We also showed that these individuals had increased levels of proteins related to AD physiopathology

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in lymphocytes, including p-tau, PKR, calcineurin and RCAN1. Moreover, this cohort also showed increased subjective cognitive complaints at that time [9]. Finally, we did not find any difference in inflammation parameters.

Now, after 10 years, we have recruited the same ApoE4 carriers that participated in the previous analysis and performed a follow-up study measuring their current cognitive status and stress and oxidative parameters. We wanted to assess the current redox state along with the cognitive performance of these subjects in late adulthood. We have observed a shift from reductive stress status to oxidative stress. An increase in the depression incidence was also observed. No cognitive changes have been observed in any group. This study highlights the importance of following subjects at risk of suffering neurodegenerative diseases over time, to encourage the development of preventive measures in the future.

2. Materials and methods

2.1. Subjects

The current study is a 10 year-follow-up of a previous study conducted by our research group in 2008 [5,9]. In 2019 we contacted the same group of ApoE3/4 and ApoE4/4 subjects and 26 of them (of an initial 33) agreed to participate in the current study. ApoE4 carriers were subdivided in heterozygous (n = 14) and homozygous (n = 12). All groups were comprised of both men and women, who were children of AD patients, were aged between 35 and 65 years and of all levels of education. We also recruited 15 ApoE3/3 subjects as a fresh control group.

In all subjects, cognitive tests were performed and 20 ml of blood were extracted from the antecubital vein of each person.

Participation in the study was voluntary and an informed consent was obtained from all individual participants. This study was conducted in accordance with the Helsinki declaration, UNESCO's Universal Declaration on Bioethics and Human Rights, the International Ethical Guidelines for Biomedical Research Involving Human Subjects from the Council for International Organizations of Medical Sciences', the Council of Europe's Convention on Human Rights and Biomedicine and the European Charter of Fundamental Rights. Furthermore, it was conducted in accordance with the Spanish legislation regarding, personal data protection, biomedical research, and bioethics. This study was approved by the human ethics committee of the University of Valencia (reference: H1542117584721).

2.2. Cognitive measures

We analyzed objective memory with the Rey Auditory Verbal Learning Test [RAVLT], in which a list of 15 words is read to the subject and, immediately after, the subject is asked to recall as many words as they can (trial I). The same procedure is done four more times (Trials II-V). After a 30-min interval, the subject is asked again to recall the words from the list (Trial VIII). The raw score for each trial is the number of words correctly recalled. In this work, we evaluated immediate recall (trial I), long-delay recall (trial VIII) and total learning (expressed by the sum of trials I–V). Raw scores were converted into psychometric T-scores (standard score with mean of 50 and standard deviation of 10).

We analyzed focused attention using a Spanish version of the Stroop Color and Word Test. Raw scores for the word (W), color (C) and color-word (CW) trials were converted into psychometric T-scores.

Lastly, we used the 17-item Spanish version of the Hamilton Depression Rating Scale to evaluate prevalence and severity of depression in our subjects. Subjects were questioned about depressive symptoms in the previous week and the interviewer would then answer the items of the scale. The scores for each item were then summed, generating a global severity score. Prevalence was expressed as percentage of individuals with a global score higher than 7.

2.3. Lymphocyte isolation

Blood samples were extracted by phlebotomy of the cubital vein and collected in BD Vacutainer® CPT™ Mononuclear Cell Preparation Tube with Sodium Citrate. After centrifuging the tubes at 1,800G for 35 min at 22 °C, mononuclear cells were collected. Plasma was also collected, aliquoted and stored at −80 °C. RPMI culture media (Sigma-Aldrich) was used to wash mononuclear cells. Then, they were incubated for 3 h at 37 °C in RPMI supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin. Floating lymphocytes were carefully collected and were counted in an automated cell counter (TC10 automated cell counter; Bio-Rad). After washing in RPMI medium cell pellet was stored at −80 °C for PCR analysis.

2.4. Glutathione and malondialdehyde determination

Blood sample was collected in BD Vacutainer tube with dipotassium ethylenediaminetetraacetic acid (K_2-EDTA). We performed reduced and oxidized glutathione analysis following the method described in a method-paper [10]. Briefly, samples were mixed with trichloroacetic acid (TCA) and *N*-ethyl-maleimide (NEM) to precipitate proteins. Supernatant was mixed with 5,5′-Dithio-Bis-2-nitrobenzoic acid (Ellman reagent), NADPH, and glutathione reductase in phosphate buffer and absorbance was recorded at 412 nm. To measure total glutathione (TG), samples were treated with TCA but without NEM and we followed the same described method. Reduced glutathione was calculated as following: GSH = TG-2*GSSG. The GSSG/GHS redox ratio is at equilibrium in the $NADP^+$/NADPH ratio [11] and it may be calculated using the formula:

$$K = \frac{[GSSG] * [NADPH] * H^+}{[GSH]^2 * [NADP^+]}$$

Where, $K = 1.98*10^{-2}\ M.^{-1}$

Malondialdehyde (MDA) was measured by HPLC in plasma samples. We added thiobarbituric acid (TBA) to plasma and incubated it at 100 °C to form an MDA-TBA adduct. HPLC was performed using a column of octadecyl silica gel, thus we separated the MDA-TBA adduct from interfering chromogens. The adduct was eluted from the column with 50 mM phosphate buffer pH 6.8 in 50% methanol and quantified spectrophotometrically at 532 nm.

2.5. ApoE genotype and IL1-β determination

We used Apolipoprotein E4 (human) ELISA Kit [K4699-100, Bio-Vision] to assess the ApoE genotype from plasma samples following the manufacturer's recommendations. We also used the Human IL-1 beta ELISA Kit [2ab214025, Abcam] to determine IL1-β plasma levels, following the manufacturer's recommendations.

2.6. PCR analysis

RNA was isolated from lymphocytes using the PARIS Protein and RNA isolation kit (Ambion) according to the manufacturer's instructions. For the reverse transcription (RT) reaction, 1 mg of purified RNA was transcribed using random hexamers with the cDNA Archive High-Capacity kit (Applied Biosystems). Reverse transcription conditions were an initial incubation at 25 °C for 10 min, followed by cDNA synthesis reaction at 37 °C for 120 min and a final inactivation step of 5 min at 95 °C. The measurement of mRNA levels was determined by quantitative PCR with the ABI Prism 7900 H T Fast Real-Time PCR System (Applied Biosystems). The specific primers used were obtained from Qiagen (Applied Biosystems). The PCR conditions were 10 min at 95 °C to activate the enzyme, followed by 40 cycles of 15 sat 95 °C and 1min at 60 °C. The expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured in all samples with the aim

of normalizing the expression of each gene, RNA quality, and efficiency of RT. The primers were from TaqMan Gene Expression Assays GCLC, Hs00155249_m1; GCLM, Hs00157694_m1; and GPX1, Hs00829989_gH; PKR (EIF2AK2; eukaryotic translation initiation factor 2 alpha kinase 2), Hs00169345_m1; SOD1 (superoxide dismutase 1), Hs00533490_m1; GAPDH, Hs02786624_g1. Each sample was tested in triplicate and the expression was calculated according to the method $2^{-\Delta\Delta Ct}$.

2.7. Statistical analysis

Statistical analysis was performed using the software SPSS 24 and graphs were constructed using GraphPad prism version 8. All variables were subjected to normality tests, specifically the Kolmogorov-Smirnov test with the Lilliefors correction, prior to statistical hypothesis testing. We used different statistical tests to analyze cross-sectional (subjects recruited in this study) and longitudinal data (subjects recruited in this study compared with themselves in the previous study).

For cross-sectional analysis, nominal and ordinal variables were analyzed with Pearson's Chi-square test. Parametrical variables were analyzed using the student's t-test and Analysis of variance (ANOVA), for 2 variables and 3 or more variables, respectively. Conversely, 2 non-parametric variables were analyzed with Mann–Whitney *U* test, while 3 or more non-parametric scale variables were analyzed with Kruskal–Wallis H test.

For longitudinal analysis, paired nominal data were analyzed with McNamar's test. Parametric related variables were analyzed with paired Student's t-test and non-parametric related variables were analyzed with Wilcoxon signed-rank test. In all tests, a result was considered statistically significant when the p-value was lower than 0.05.

Finally, relationship between plasma oxidative parameters, inflammation parameters and cellular stress with ApoE4 were assessed using binomial regression analysis (Kendall's tau).

3. Results

3.1. Sample characterization

Table 1 shows the characterization of our study sample. As we can see there are no differences in age, sex nor educational level.

Cognitive tests were performed in all recruited subjects. No alterations in attention and memory were found in these subjects, as reflected by the Stroop and RAVLT test results, neither among genotypes nor through time (Fig. 1a and b). This indicates that subjects do not show AD related cognitive alterations. Nevertheless, we found a significant increase in the prevalence of clinical depression after 10 years. As we can see in Fig. 1c, the prevalence of depression in 2019 is 43% in ApoE3/4 and 40% in ApoE4/4 carriers. Through time, the cases of depression have increased about 30% in each group.

3.2. Reductive stress shifted to oxidative stress in the ten-year follow-up study

We measured oxidation parameters and antioxidant enzyme expression to evaluate the evolution of redox status over time. We found that GSSG and GSSG/GSH levels were increased along time in ApoE 3/4 and 4/4 subjects (Fig. 2a, b, 2c). In a similar fashion, lipid peroxidation measured as MDA also showed an increment in 3/4 and 4/4 subject with time (Fig. 2d). We did not observe changes in 3/3 population in this ten year follow up study.

We then calculated $NADP^+$/NADH ratio in whole blood and found that current ratios were increased in ApoE3/4 and 4/4 subjects but not in 3/3 individuals compared to 10 years before (Fig. 3 and Table 2).

Regarding antioxidant enzyme expression, ten years ago ApoE4 carriers presented an increase in GCLC and GCLM expression, when compared to non-carriers. Nowadays, the differences were no longer observed (Fig. 4). Furthermore, Gpx1 expression, which was previously increased, now showed a significant decrease in both ApoE 3/4 and 4/4 carriers when compared to non-carriers and we also found that ApoE 3/4 subjects showed lower SOD1 expression levels than non-carriers (Fig. 4).

Fig. 5 provides a comparison between antioxidant enzymes from 2008 versus 2019 per group. As we can see, there is a marked decrease in GCLC and GCLM in ApoE3/4 and APoE4/4 subjects respectively over time. We also found a decrease in GPX and SOD1 expression over time in both genotypes.

Table 1
Sample characterization by ApoE genotype.

	ApoE 3/3	ApoE 3/4	ApoE 4/4	p
Participants (n)	15	14	12	–
Female n (%)	67	71	58	0,256
High studies (%) (higher education versus other study levels)	33	50	58	0,301
Age (mean± SD)	51,47 ± 7,43	51,86 ± 8,80	54,08 ± 7,89	0,667

3.3. PKR change its expression levels in ApoE4 carriers

Previously we found that PKR levels were overexpressed in peripheral lymphocytes from ApoE4/4 carrier subjects only [9]. Ten years later we found an increase not only in ApoE4/4 but also in the ApoE3/4 individuals (Fig. 6). Furthermore, PKR levels correlate with ApoE4 carriage (ρ 0.549, p = 0.003) (see Table 3 below).

3.4. IL-1β increases in ApoE4 carriers in an allele dose-dependent manner

Heterozygous ApoE4 carriers presented higher levels compared to non-carriers, while homozygous carriers presented increased levels compared to both non-carriers and heterozygous carriers (Fig. 7). Moreover, IL-1β levels correlate with ApoE4 genotype (ρ 0608, p < 0.001) (see Table 3 below). We did not find changes in the previous study regarding inflammation.

Finally, we performed Kendal's tau correlations between ApoE genotype and the levels of antioxidant enzymes obtained in this new study (Table 2). We found significant negative correlation between being an ApoE4 carrier and the expression levels of SOD1 and of Gpx1. MDA levels correlated positively with the ApoE genotype.

In the same table, we also include IL-1β levels determined by ELISA and PKR levels determined by PCR, and we report a significant correlation with ApoE4 genotype.

4. Discussion

4.1. ApoE4 and cognition

We did not find any difference between the ApoE 3/3, 3/4 and 4/4 genotypes, nor a difference through time, in RAVLT and Stroop scores. Studies analyzing the connection between ApoE4 and cognition in healthy individuals have found divergent results, and many studies, like ours, did not find any difference between carriers and non-carriers [12,13]. In our study, most individuals had a high educational attainment and were bilingual, speaking both Spanish and Valencian as their native language; and both factors are known to increase cognitive reserve and resilience to AD pathology [14,15]. Also, the influence of ApoE4 in healthy individuals may cause subtle cognitive differences in middle-aged subjects [16],

We also found that cases of depression were significative increased after 10 years. Depression is a frequent neuropsychiatric symptom related to AD that can be found in individuals even before they develop mild cognitive impairment [MCI] [17,18]. If depression is a risk factor

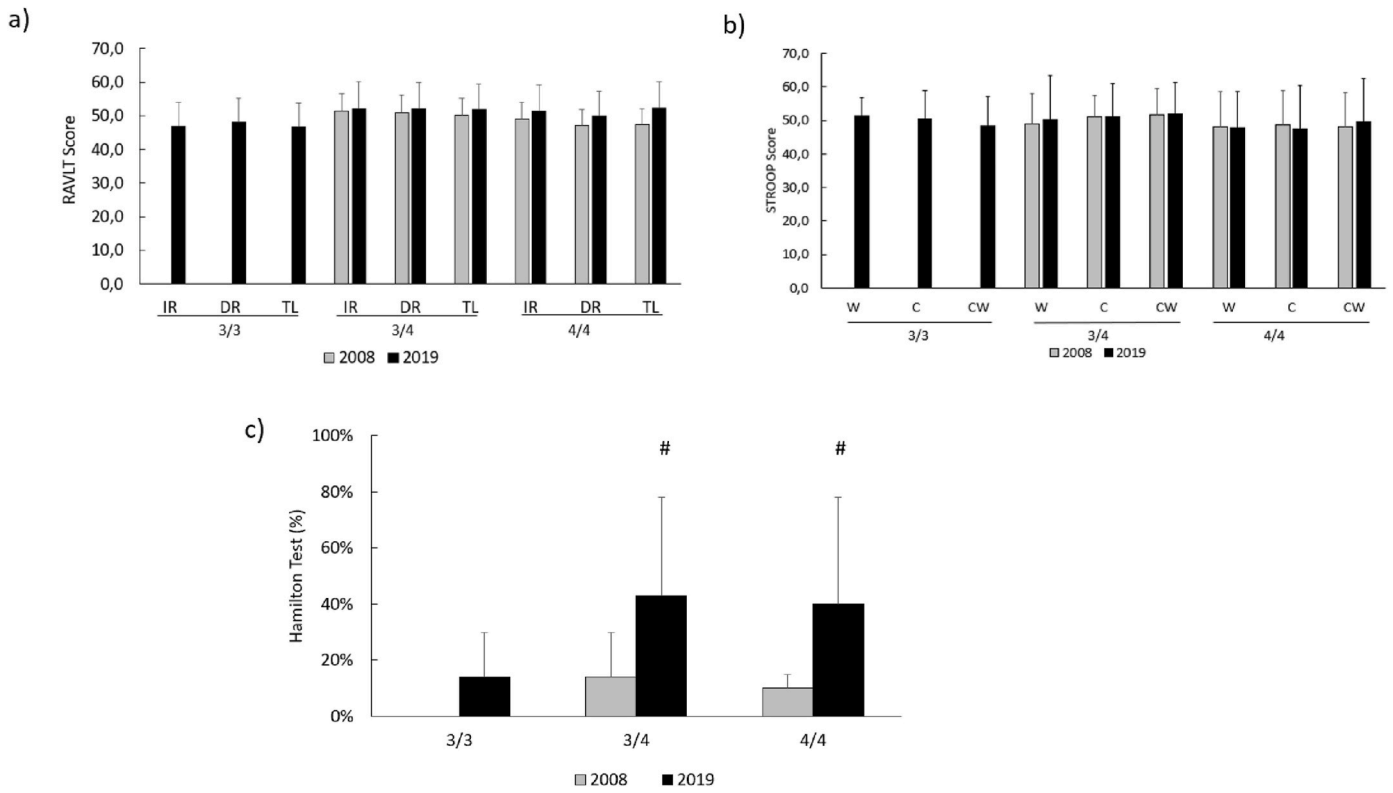

Fig. 1. Cognitive tests scores according to ApoE Genotype. 1a) Rey Auditory Verbal Learning Test (RAVLT) in ApoE3/3, 3/4 and 4/4 individuals. IR stands for Immediate Recall, DR stands for Delayed recall, TL stands for total learning. 1b) Stroop Color and Word Test (Stroop), W stands for word count, C stands for Color count, CW stands for Color-word count. RAVLT and Stroop scores are represented as psychometric T-scores (standard score with mean of 50 and standard deviation of 10). 1c) Prevalence of depression in the Hamilton Depression Rating Scale (HDRS) shown in percentage. #p≤0.05 vs. 2008.

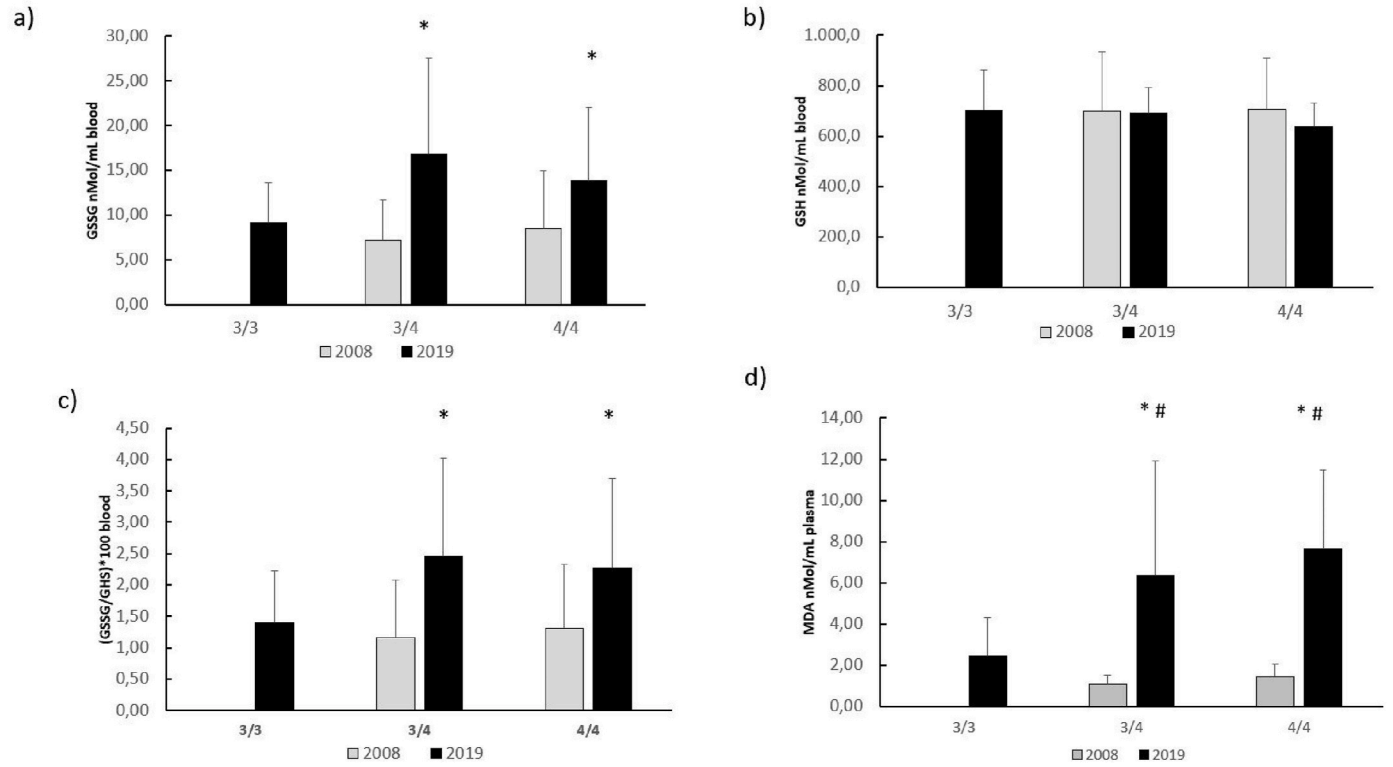

Fig. 2. Oxidative stress parameters according to ApoE Genotype. Oxidized glutathione (GSSG) (2a), reduced glutathione (GSH) (2b), and the GSSG/GSH ratio measured by spectrophotometric ssay method. 2d) Lipid peroxidation estimated as malondialdehyde (MDA) measured by HPLC. *p≤0.05 vs. 3/3 2019; #p≤0.05 vs. 2008.

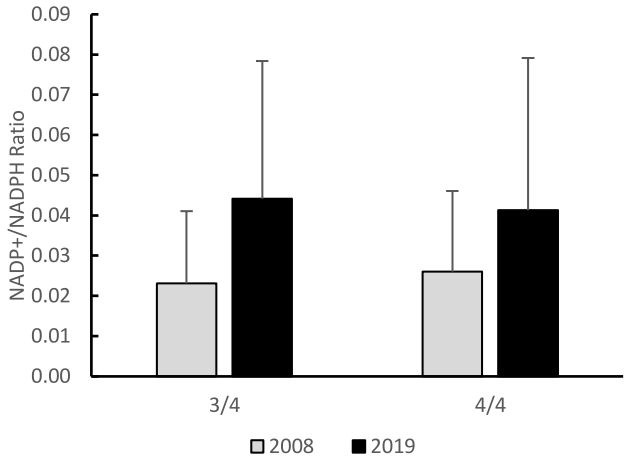

Fig. 3. $NADP^+$/NADPH ratio in healthy young individuals carrying ApoE 3/4 or 4/4 genotype compared over time. *The ratio $NADP^+$/NADPH has been calculated thanks to its relationship with the GSSG/GSH ratio.*

Table 2
$NADP^+$/NADPH ratio in healthy young individuals carrying ApoE3/3, 3/4 or 4/4 genotype compared over time.

Genotype	$NADP^+$/NADPH Ratio		
	2008	2019	Δ (%)
3/3	0,049 x 10^{-3}	0,032 x 10^{-3}	0,64
3/4	0,023x 10^{-3}	0,042 x 10^{-3}	1,81
4/4	0,026x 10^{-3}	0,046 x 10^{-3}	1,79

(with environmental components) or a prodromal symptom of Alzheimer's disease (probably due to anomalies in amygdala function) is still controversial. In this regard, we did not find a correlation between the incidence of depression and the age of onset of AD in the parent (data not shown). Furthermore, ApoE4 has been associated with increased risk of late-life depression in healthy elders [19]. However, the relationship between *ApoE4* and overall life-time depression is still equivocal; while some studies found increased overall prevalence in *ApoE4* carriers, others did not find this relationship [20,21]. However, it is possible that ApoE4 may increase susceptibility to depression, as has been described in female mice, where ApoE4 interacted with estrogen receptor β to decrease the expression of brain-derived neurotrophic factor and of serotonin receptor, increasing the risk for depression [22].

Finally, we had considered the influence of depression on our subjects, as studies have shown alterations in oxidative stress and in memory tests related to depression. However, we did not find any difference in the cognitive scores between subjects with or without depression (data not shown). Therefore, we considered that the increase in oxidative markers seen in our study was not depression-related.

4.2. ApoE4 and inflammation

Our results show that homozygous *ApoE4* carriers present increased IL-1β when compared to non-carriers and to heterozygous carriers. Furthermore, heterozygous carriers have higher IL-1β levels than non-carriers. As IL-1β is a pro-inflammatory cytokine, this suggests that ApoE4 is related to increased inflammation even in healthy middle-aged individuals, in an allele dose-dependent manner.

ApoE4 is related to increased inflammation and higher levels of pro-inflammatory cytokines due to a shift to an inflammatory immune phenotype [23,24]. IL-1β is a pro-inflammatory cytokine that depends on the transcription factor NFκβ, which, in turn is activated by ApoE4 [25]. Moreover, IL-1β is also increased by CCAAT/enhancer-binding protein [C/EBP] [26]. This transcription factor, which is activated by ApoE4, also increases ApoE4 transcription, thus causing positive feedback that leads to the onset of inflammation [27,28].

Our results agree with studies in AD patients, where ApoE4 is related to increased levels of pro-inflammatory cytokines in a dose-dependent manner [29]. Conversely, in healthy *ApoE4* carriers, a recent study by Wang and colleagues [30] did not find a correlation between inflammatory cytokines and *ApoE4*. This could be because, unlike us, they analyzed both homozygous and heterozygous *ApoE4* carriers in the same group; this could affect results as the increase is much higher for the homozygous genotype.

4.3. ApoE and oxidative stress

There is plenty of evidence that oxidative stress occurs in individuals suffering from AD, as well as in those individuals suffering from MCI [

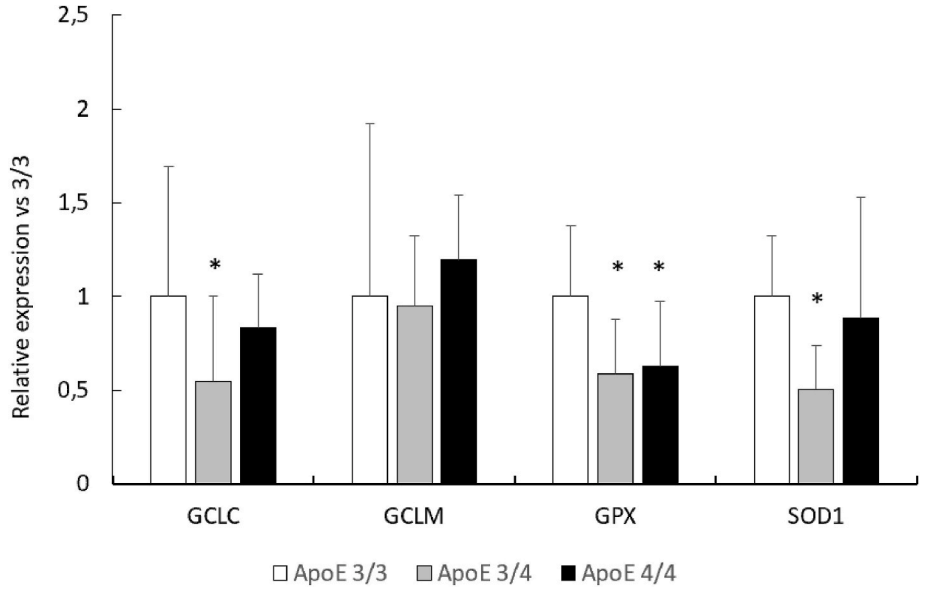

Fig. 4. Antioxidant enzymes expression in 2019 samples. E*xpression of glutamyl-cysteinyl ligase (GCLM and GCLC subunits), glutathione peroxidase 1 (Gpx1), and superoxide dismutase1 (SOD1) in isolated peripheral lymphocytes measured by PCR, relative to ApoE3/3 group expression. *p≤0.05 vs. 3/3 group.*

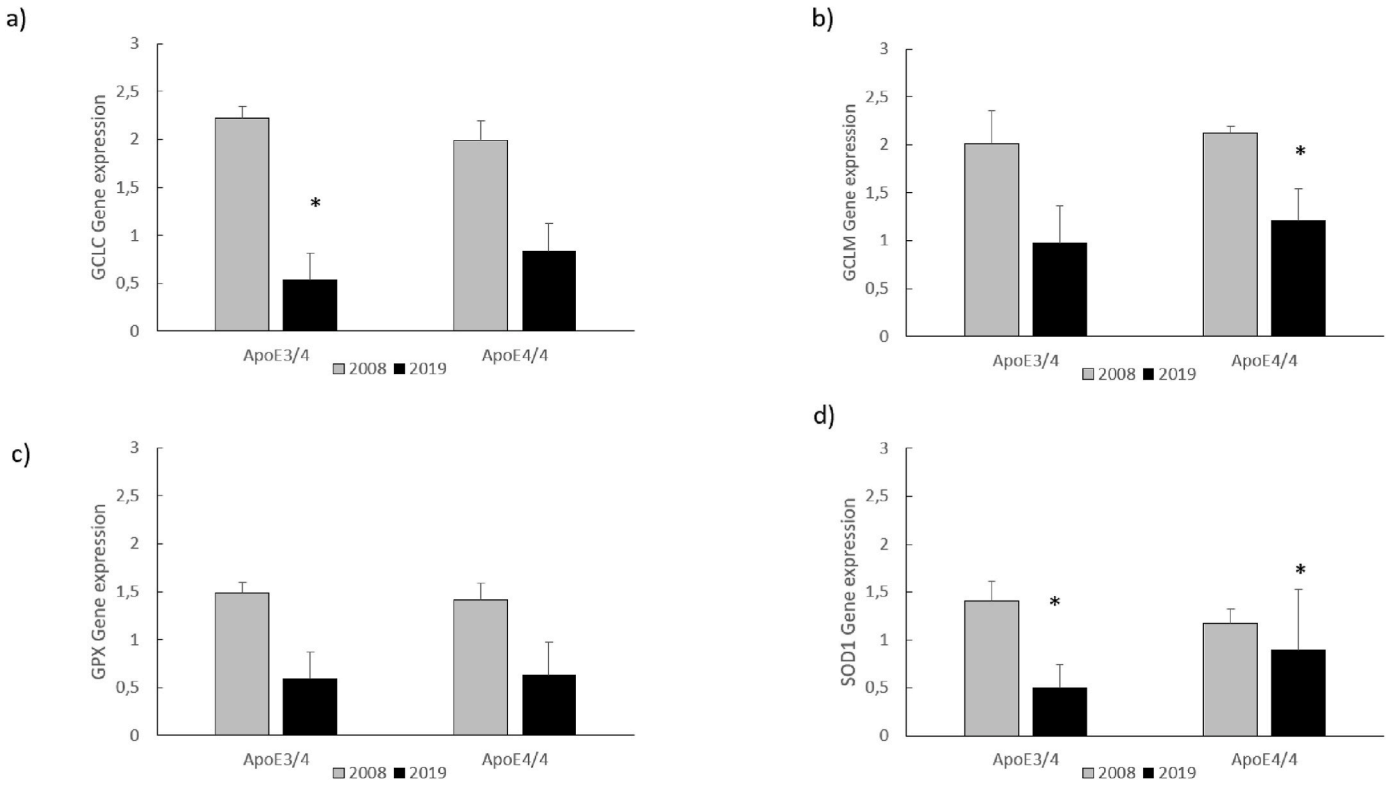

Fig. 5. Comparison of the expression of antioxidant enzymes over time. Relative e*xpression of glutamyl-cysteinyl ligase catalytic (a) and modifier subunits(b), glutathione peroxidase 1 (Gpx (c)), and superoxide dismutase1 (SOD1 (d)) in isolated peripheral lymphocytes measured by PCR, respect to ApoE3/3 group expression. *p≤0.05 vs 2008.*

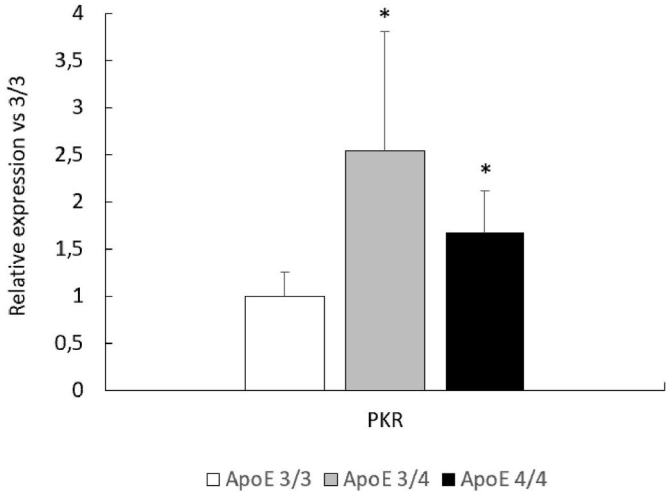

Fig. 6. The eukaryotic translation initiation factor 2 alpha kinase 2 (PKR) expression. *Expression of the eukaryotic translation initiation factor 2 alpha kinase 2 (PKR) in isolated peripheral lymphocytes by PCR. *p≤0.05 vs. 3/3 group.*

[6,31,32]]. Several studies report elevated oxidative stress and oxidative damage in both blood and brain of MCI and AD patients [33,34] which is more marked in *ApoE4* carriers [35]. Interestingly, oxidative stress is shown as an early phenomenon as it occurs even in cognitively healthy individuals [7,36].

Here, we show a shift overtime in the redox status of ApoE4 carriers, with healthy individuals at risk of AD no longer suffering from reductive stress, as seen in our previous study [5]. Furthermore, we have found that *ApoE4* carriers of both genotypes present increased MDA levels and decreased GPx1 levels when compared to non-carriers. Moreover,

Table 3
Kendal's tau correlation coefficients of antioxidant and stress proteins and Il-1β.

	ApoE4 Carrier	
	Coef.	p value
GCLC	−0,01	0,95
GCLM	0,25	0,21
Gpx	−0,47*	0,01
SOD1	−0,39*	0,05
MDA	0,52**	0,00
PKR	0,55**	0,00
IL-1B	0,61**	0,00

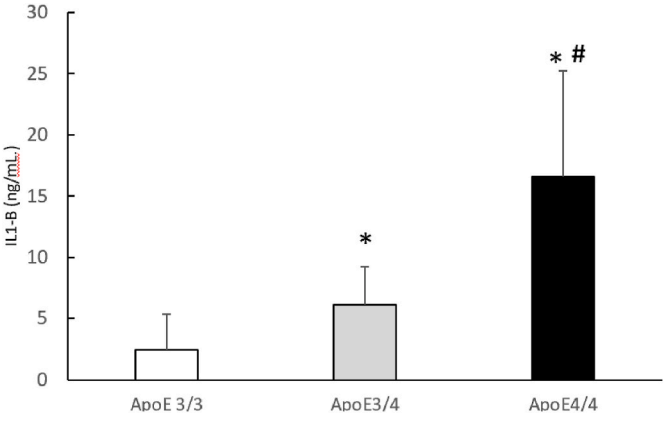

Fig. 7. Plasma levels of Interleukin1-beta (IL1β). *Levels of Interleukin-1-beta (IL1β) in plasma by ELISA. *p≤0.05 vs. 3/3; #p≤0.05 vs. 3/4.*

heterozygous carriers presented lower SOD1 levels. This altered oxidative status was further supported by the longitudinal increase in lipoperoxidation that was present only in ApoE4 carriers and not in non-carriers. Thus, in our study, middle-aged *ApoE4* carriers present oxidative damage, which was not present when they were younger. This agrees with other studies that found elevated oxidative damage [37,38], reduced plasma antioxidant capacity and altered Nrf2 signaling [39] in healthy *ApoE4* carriers. It also agrees with *in vitro* and with mouse studies which found an ApoE4-related decrease in the level and activity of antioxidant enzymes, including Gpx1 [40,41]. The lower levels of GPx1 might account for the absence of an important increase in current GSSG levels in carriers compared to non-carriers, as without this enzyme reactive species cannot be coupled with GSH [42].

To rule out the possible effect of age rather than the ApoE genotype, we divided our sample into those under and over 50 years of age. We did not observe any difference between groups in all parameters measured (data not shown). This agrees with previous results from our group that showed that oxidation depends more on functional status (frailty) than on chronological age [43].

4.4. The shift from reductive to oxidative stress

Reductive stress may be explained by an overcompensation to increased generation of oxidants, specially by mitochondria in the presence of β-Amyloid peptide, resulting in an increase of antioxidants [44–46]. When the cellular capacity to promote antioxidant defenses is overwhelmed, then oxidative stress takes place. This process could coincide with the onset of cognitive decline in the continuum of AD evolution. Butterfield and Halliwell reviewed that oxidative stress leads to altered brain glucose metabolism in AD and consequently, ATP-requiring processes for cognitive function could be impaired [47].

Reductive stress itself may cause serious metabolic alterations [48] and moreover, by chemical reactions, induce increased oxidant production [49]. In this line, Zhang and colleagues showed that glutathione-dependent reductive stress may trigger mitochondrial oxidation and cytotoxicity [50]. Our findings after ten years of follow up reveal that reductive stress in individuals at risk of AD shifts to oxidative stress. This shift could be explained by the fact that reductive stress can induce oxidation and reactive oxygen species (ROS) production overtime, as mentioned above [49]. It has been shown in hepatocyte hypoxia, that reductive stress increases the release of intracellular iron which enhances oxidation. Furthermore, the increase of iron converts xanthine dehydrogenase to xanthine oxidase leading to ROS generation. Besides, the increased levels of NADH is a source of oxidative stress, due to more NADH recycling by mitochondrial complex I, which leads to electron leakage. Finally, abnormally reduced electron chain components, in particularly ubisemiquinone, react directly with oxygen to form toxic radicals (for a review see 51).

ApoE4 carriers at middle-age may suffer oxidative stress, as a result of decreased antioxidant defenses leading to subsequent damage. This was further influenced by the reductive stress previously seen in our subjects, which is known to cause increased ROS production, oxidative damage [49] and accelerated senescence [52] even in the presence of increased reducing equivalents.

4.5. ApoE4 and cellular stress

Both *ApoE4* and AD are known to cause cellular stress and both activate multiple stress response pathways [5,53,54]. In the present study we have measured the same stress proteins as in 2008, in the same subjects, after 10 years of evolution [9]. We found no significant differences in RCAN1, calcineurin, and phospho-tau levels (data not shown). However, we found increased PKR gene expression in *ApoE4* carriers, especially heterozygous carriers. Furthermore, PKR levels increased in heterozygous carriers and remained high in homozygous carriers the last 10 years. This concurs with studies which found *ApoE4*-related upregulation of PKR activity and activation of stress responses [55–57]. PKR is a kinase that phosphorylates the eukaryotic translation initiation factor 2α (eIF2α) [58]. This occurs when the integrated stress response is activated by cellular stress signals, increasing PKR and causing transcriptional changes and protein synthesis attenuation [59]. Interestingly, PKR induces the up-regulation of IL-1β as studies using a specific PKR inhibitor show a decrease in IL-1β production [60].

To conclude, our results show that there is a shift from reductive stress to oxidative stress in healthy ApoE4 carriers over time. Chronic reductive stress in our subjects could have led to the exhaustion of the antioxidant system and oxidative damage [48,51] that we now see in these same individuals. This may be important as oxidative stress leads to increased p-tau and has a role in β-Amyloid excitotoxicity [61] paving the way for severe damage seen in full-blown AD dementia.

CRediT authorship contribution statement

Mariana Nepomuceno: Writing – review & editing, Methodology, Data curation. **Paloma Monllor:** Writing – review & editing, Writing – original draft, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation. **Maria Jose Cardells:** Methodology. **Artemis Ftara:** Methodology. **Maria Magallon:** Methodology. **Francisco Dasí:** Methodology, Investigation. **Mari Carmen Badia:** Methodology, Investigation, Conceptualization. **Jose Viña:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition. **Ana Lloret:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that no conflict of interest exists.

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