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The pleiotropic beneficial intervention of olive oil intake on the Alzheimer's disease onset via fibrinolytic system

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ABSTRACT

The daily consumption of Extra Virgin Olive Oil (EVOO) in Mediterranean nutrition is tightly associated with lower frequency of many diseases' appearance, including Alzheimer's disease (AD). Fibrinolytic system is already assumed to be involved in AD pathophysiology through various factors, especially plasminogen activator inhibitor-1 (PAI-1), a2-antiplasmin (α2AP) and tissue plasminogen activator (tPA). We, here, present a biochemical study, as a continuation of a clinical trial of a cohort of 84 participants, focusing on the pleiotropic effect of the annual EVOO consumption on the fibrinolytic factors of Mild Cognitive Impairment (MCI) patients. The levels of all these fibrinolytic factors, measured by Enzyme-Linked Immunosorbent Assay (ELISA) method, were reduced in the serum of MCI patients annually administered with EVOO, versus not treated MCI patients, as well as AD patients. The well-established AD hallmarks (Aβ1–40 and Aβ1–42 species, tau, and p-tau) of MCI patients' group, annually administered with EVOO, were restored to levels equal to those of the cognitively-healthy group; in contrast to those patients not being administered, and their AD hallmarks levels increased at the end of the year. Moreover, one of the EVOO annual consumption multimodal effects on the MCI patients focused on the levels of an oxidative stress trademark, malondialdehyde (MDA), which displayed also a visible quenching; On the other hand, an increase exhibited in the MCI patients not consuming EVOO one year after, was attributed to the lack of the EVOO anti-oxidative properties. These outcomes are exploitable towards the establishment of natural products like EVOO, as a preventive remedy fighting this neurodegenerative disorder, AD.

Clinical trial registration: https://clinicaltrials.gov/ct2/show/NCT03362996 MICOIL gov Identifier: NCT03362996

1. Introduction

Alzheimer's disease (AD) is a devastating disorder that affects more than 50 million people at a global level today. The dominant pathogenic features of the disease are the extracellular plaques composed primarily of amyloid-β (Aβ) (mixed peptides of 40 and 42 residues generated from the amyloid-β precursor protein), and the intracellular neurofibrillary tangles composed of the cytoskeletal protein tau in the hyper-phosphorylated form (Deture and Dickson, 2019; dos Santos Picanco et al., 2016; Masters et al., 2015; Zhang et al., 2011).

For the early detection and diagnosis of AD was reported plasminogen activator inhibitor-1 (PAI-1) as biomarker (Oh et al., 2014). PAI-1

Abbreviations: MCI, Mild Cognitive Impairment; AD, Alzheimer's Disease; EVOO, Extra Virgin Olive Oil; APP, amyloid precursor protein; Aβ, β-amyloid; Aβ1–40, amyloid beta 40; Aβ1–42, amyloid beta 42; PAI-1, plasminogen activator inhibitor 1; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; α2AP, α2-antiplasmin; p-tau, phosphorylated tau; MDA, malondialdehyde; TBA, thiobarbituric acid; TCA, thrichloroacetic acid; PBST, PBS-Tween 20.

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known as endothelial plasminogen activator inhibitor or serpin E1, is a serine protease's inhibitor that is expressed in the majority of cells and operates as a principal inhibitor of two proteases; the tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) resulting in canceling out the plasminogen activation and in maintaining balance between thrombosis and fibrinolysis. Elevated PAI-1 levels are a risk factor for thrombosis and atherosclerosis (Liu et al., 2011). PAI-1 implicates amyloid deposition through the inhibition of both tPA and plasmin (Reed et al., 2017). A malfunction of the tPA-plasmin system causes defective proteolytic degradation of Aβ plaques in advanced stages of AD (Diaz et al., 2020).

Plasmin is a significant enzyme degrades many blood plasma proteins, including fibrin clots, and is considered crucial for the regulation of Aβ accumulation in the cerebrum (Fekih-Mrissa et al., 2017). Reduced levels of brain plasmin in AD patients imply its down-regulation may lead to amyloid plaque deposition that accompanied sporadic AD (Ledesma et al., 2000). Thus, this protease clears both Aβ soluble and aggregated forms (Baranello et al., 2015; Ledesma et al., 2000; Liu et al., 2011; Tucker et al., 2000).

Plasmin activity is also regulated by another inhibitor, a member of the serine proteases family, which is considered as the dominant and the fastest inhibitor of plasmin, alpha 2-antiplasmin (alpha 2-AP), or α2-antiplasmin inhibitor (serpinf2) (Baranello et al., 2015; Reed et al., 2017). The α2AP suppresses plasmin activity by forming the plasmin-α2AP complex. The blood levels of this molecule act as a marker of plasmin nullification.

EVOO seems to have promising results in the fight against AD as it is recently reviewed (Tsolaki et al., 2017). Evidence has shown that Mediterranean populations emerge a smaller prevalence of dementia in the elderly thanks to the consumption of olive oil (Ntanasi et al., 2018; Tsolaki et al., 1999, 2020). Prevalent ingredients of EVOO are the monounsaturated fatty acids, mainly oleic acid and phenolic compounds-oleuropein, hydroxytyrosol (HT) etc. in which are attributed the pleotropic beneficial properties. Phenols exhibit significant biological effects in many diseases, participating in various cellular and biochemical processes (Papanikolaou et al., 2019). According to a health claim approved by the European Food Safety Authority, EVOO containing at least 5 mg of HT and its derivatives per 20 g rendered it to protect against oxidative stress-induced lipid peroxidation in human blood (Kouka et al., 2020). Olive oil is proved that reinforces the activation of signaling pathways in liver cells implicated in inflammatory cascade, oxidative stress, endoplasmic reticulum stress, mitochondrial dysfunction, and insulin resistance yielding in prevention or/and rescue of liver havoc (Soto-Alarcon et al., 2017). It is of high importance to mention that consuming HT even at high concentrations is totally safe and non-toxic or mutagenic showed in vitro studies. HT had proved to possess among others anti-cancer potential, endothelial and vascular function, anti-steatosis and mitochondrial function. The healthy effect of HT have been investigated in humans and in cellular level and animal models (Echeverría et al., 2017). Notably the composition of the EVOO used in this study is published in the Supplementary material (Tzekaki et al., 2021a).

The aim of this biochemical study, performed in the frame of the clinical trial MICOIL concerning AD, was to investigate the effect of the EVOO administration in MCI patients, focusing on the changes in the levels of fibrinolytic system factors For this purpose, using the ELISA method, we determined the levels of PAI-1, a2-antiplasmin, and tPA, the levels of AD hallmarks-related, of amyloid Aβ species (Aβ1–42, Aβ1–40), and p-tau protein, and finally, the levels of one major oxidative stress marker, MDA. Extensive comparisons of the measured parameters were also carried out thoroughly for the participated groups of MCI and AD patients, as well as the cognitively healthy group.

Table 1
Demographic and clinical characteristics of the tested individuals. The depicted values represent mean values, with SEM.

	Normal	MCI baseline	MCI without EVOO	MCI with EVOO	AD
Subject number	21	42	21	21	21
Age (years)	72.67 ± 0.7632	72.19 ± 0.9018	72.48 ± 1.281	71.76 ± 1.293	73.19 ± 0.9725
Gender (M/F)	9/12	20/22	10/11	11/10	9/12
Education	8.810 ± 0.7388	9.190 ± 0.4737	8.810 ± 0.5504	9.095 ± 0.6932	8.524 ± 0.7516
MMSE	28.81 ± 0.1636	27.21 ± 0.2374	26.90 ± 0.3645	28.67 ± 0.2323	14.62 ± 0.6813

2. Materials and methods

2.1. Participants in the clinical trial

This biochemical study was carried out by Prof. Pantazaki's group, and included participants recruited in the framework of the MICOIL clinical trial (NCT03362996, MICOIL: Management of Mild Cognitive Impairment Patients With Extra Virgin Olive Oil) performed by Prof. Tsolaki's group. The study, which is still ongoing, initiated in December 2017 and is financed by Alzheimer Hellas, Thessaloniki, Greece and Yanni's Olive Grove Company, providing the EVOO from Potidea Chalkidiki, Greece.

The design, realization, the ongoing and the outcomes of this clinical trial focused on the neuropsychological part of the study described in detail (Tsolaki et al., 2020). A number of 84 participants, in total, (ages 65–80), were recruited in the current study by the Greek Alzheimer's Association and out-patient memory and dementia clinic of the 1st University Neurology Department of AHEPA Hospital, living in a Mediterranean location (Northern Greece, Thessaloniki region). All participants had diagnosed according to international criteria (Petersen, 2004) using neurological, neuropsychological, neuroimaging tests, and blood analysis as described in detail in the MICOIL clinical trial (Tsolaki et al., 2020).

All participants underwent neuropsychological assessments, such as the Mini-Mental State Examination (MMSE) and the Alzheimer's Disease Assessment Scale-Cognitive sub-scale (ADAS-cog) (Folstein et al., 1975; Fountoulakis et al., 2000; Mohs et al., 1997; Tsolaki et al., 1997). The inclusion and exclusion criteria are described also in the study Tsolaki et al., 2020.

Cognitively healthy individuals were evaluated also through clinical and neuropsychological tests. Global cognitive performance was examined by Mini-Mental State Examination (MMSE) and other cognitive and behavioral tests before and after one year of the EVOO intervention in MCI patients on MeDi as well as in MCI patients who followed only MeDi (Tsolaki et al., 2020).

Participants in each experiment of this study were classified into the following groups as indicated: A. MCI patients' group-baseline ($n = 42$); The MCI patients of this group were randomly separated into two groups: B. MCI patients ($n = 21$) consuming 50 ml EVOO daily for one year (MCI with EVOO; $n = 21$) and in the group C. MCI patients ($n = 21$) not consuming EVOO for the respective period (MCI without EVOO; $n = 21$); D. AD patients (AD; $n = 21$) and E. normal cognitively individuals (Normal; $n = 21$) used as a reference.

The daily amount of raw EVOO (50 ml) administered to patients was divided equally over three meals throughout the day (main dish at lunchtime, a side dish (salad) at lunchtime, and salad at dinner). Compliance with the EVOO therapy in the context of the MICOIL clinical trial was assessed via patient/patient caregiver questionnaires and patient self-reports rates of prescription refills, as well as patient diaries. The characteristics of the demographic data of the participants are

Table 2
P-values obtained by comparison of the demographic characteristics between the participating groups.

P-values	Normal vs MCI baseline	Normal vs MCI without EVOO	Normal vs MCI with EVOO	Normal vs AD	MCI without EVOO vs MCI with EVOO	MCI baseline vs MCI without EVOO	MCI baseline vs MCI with EVOO
Education	0.6552 (ns)	1 (ns)	0.7793 (ns)	0.7877 (ns)	0.7485 (ns)	0.6250 (ns)	0.9090 (ns)
MMSE	<0.0001 (****)	<0.0001 (****)	0.3469 (ns)	<0.0001 (****)	0.0002 (***)	0.4672 (ns)	0.0002 (***)

P-value <0.05 is considered significant (*); <0.01 stands for **; <0.001 stands for ***; <0.0001 stands for ****.

shown in Tables 1 and 2. A full description of the EVOO content used in the MICOIL study is also provided (Tsolaki et al., 2020).

2.2. Serum samples

Whole blood specimens were collected from the participating individuals and allowed to clot by leaving them undisturbed at room temperature for 15–30 min. The clot was removed by centrifuging at 4.000 rpm for 5 min in a refrigerated centrifuge. The resulting supernatant designated as a serum, removed and apportioned into 0.5 ml aliquots, which are stored at −80 °C until their use in laboratory analyses.

2.3. Chemicals

Polystyrene 96 well-plates were provided by Greiner BioOne (Greiner BioOne, Germany). Nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and *p*-nitrophenyl phosphate, PBS, Tween-20, malondialdehyde (MDA), diethanolamine, thiobarbituric acid (TBA), trichloroacetic acid (TCA), were purchased by Sigma-Aldrich (St. Louis, MO, USA).

2.4. Serum PAI-1, α2-antiplasmin, tPA, Aβ 1–42, Aβ 1–40, p-tau protein-level detection

Serum PAI-1, α2-antiplasmin, tPA, Aβ 1–42, Aβ 1–40, p-tau protein levels were measured in individuals of all groups using the ELISA method. ELISA experiments were performed using the following kits, according to the manufacturers' instructions: Human PAI-1 Standard ABTS sandwich ELISA Development Kit was provided by PEPROTECH (PEPROTECH, France, and Catalog #900-K383), Human alpha2-antiplasmin and human phospho-tau protein ELISA Kits provided by MyBioSource (MyBioSource, San Diego, California, USA Catalog#MBS765756 and Catalog#MBS164899 respectively. Human t-Plasminogen Activator/tPA Quantikine ELISA Kit and Human Amyloid β (Aβ1–40) Immunoassay Quantikine® ELISA were provided by R&D Systems (R&D Systems, USA, Catalog Number DTPA00, DAB140B), Amyloid Beta 42 (Human) ELISA Kit was obtained from BioVision Inc. (San Francisco Bay Area, Catalog# E4288–1000). Human recombinant PAI-1 protein was purchased from Sigma-Aldrich (A8111-25UG). Primary mouse monoclonal antibodies were purchased from Santa-Cruz Biotechnology (Dallas, TX, USA) and were as follows: PAI-1 antibody (C-9: sc-5297), anti-β actin (#sc-130,300). A goat anti-mouse IgG conjugated with the enzyme alkaline phosphatase (ALP) (#A2429) was employed as secondary antibody (Sigma-Aldrich, St. Louis, MO, USA). All solvents used were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of analytical.

2.5. Serum proteins electrophoretic separation and immuno-detection of PAI-1 by Western blotting

An amount of a total protein (40 μg) from pools of serum samples, derived from each of the participant groups, determined by Bradford method (Bradford, 1976), and using bovine serum albumin as a protein standard, was separated onto a polyacrylamide gel electrophoresis (PAGE), in a 10% (*w/v*) acrylamide gel under sodium dodecyl sulfate (SDS)-denaturing conditions (Laemmli, 1970). Samples were suspended in 1× Laemmli loading buffer (0.1 M Tris-HCl pH 6.8, 4% w/v SDS, 12% *v*/v β-mercaptoethanol, 20% v/v glycerol, 0.1 mg/ml bromophenol blue) and boiled for 5 min.

After electrophoretic separation, proteins have been transferred onto a nitrocellulose membrane, at 4 °C, 200 mA for 45 min, using Towbin transfer buffer with 20% (*v*/v) methanol and 0.03% (w/v) SDS (Towbin et al., 1979) using a Labnet ENDURO VE10 apparatus. Then, the membrane was treated with a solution of primary antibody against PAI-1 (1000-times diluted), and then with a solution of secondary anti-mouse IgG antibody conjugated to the enzyme alkaline phosphatase (diluted 2000-times) (A-2429) purchased by Sigma-Aldrich (St. Louis, MO, USA). The membrane was washed after each step at least three times with PBS-T and finally, the membrane was immersed in an ALP buffer at final concentrations 0.5 mM NBT and 0.5 mM BCIP. PAI-1 bands have been visualized after membrane staying for 30 min in RT in the coloring reaction.

2.6. Lipid peroxidation via malondialdehyde (MDA) determination as an oxidative stress biomarker before and after EVOO consumption

Lipid peroxidation was determined via MDA measurement as an oxidative stress biomarker before and after EVOO consumption in all previously described groups. The concentration of serum MDA was quantified as previously described (Chowdhury et al., 2017), using thiobarbituric acid (TBA) reagent, and the absorbance of the supernatant was measured spectrophotometrically at 530 nm. The concentration of MDA was expressed as μM.

2.7. Statistical analysis

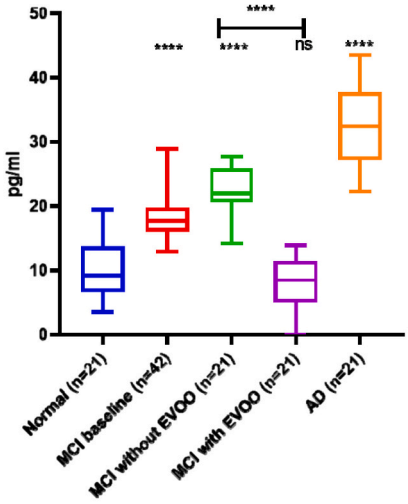
The mean and the standard error of the mean (Mean ± SEM) values were determined for each of the outcome measures described. Significance was determined using Student *t*-test or 1-way ANOVA; statistical significance between groups was determined using GraphPad Software, San Diego, CA. Significance was set at $p < 0.05$. Regression analysis with Pearson's test has been used for determining the relationships among analyzed variables and multilinear regression with stepwise elimination for evaluating the best model for the determinants of PAI-1 levels. To minimize selection bias, randomization and allocation concealment were applied for sample selection. The RAND function in Microsoft Excel 2010 was applied, which employs the Mersenne Twister algorithm (MT19937) to generate random numbers. RAND returns an evenly distributed random real number greater than or equal to 0 and less than 1. A new random real number is returned every time the worksheet is calculated (https://support.office.com/en-us/article/rand-function-4cbfa695-8869-4788-8d90-021ea9f5be73).

3. Results

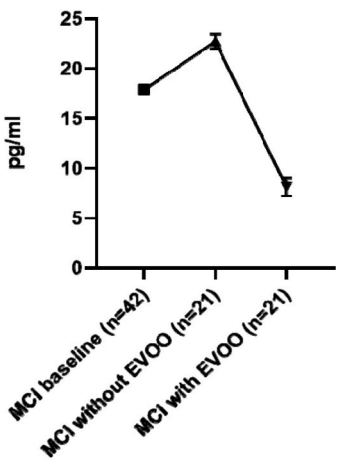
3.1. The individuals' demographic data

The individuals' demographic data participating in the clinical trial are summarized in Table 1 and in Table 2. Table 1 represents the mean

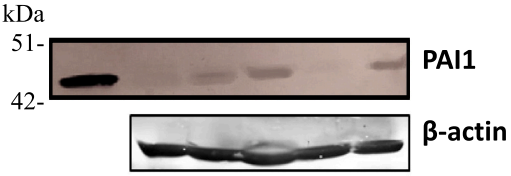
a.



b.



c.



PAI1	+	-	-	-	-	-
Normal	-	+	-	-	-	-
MCI baseline	-	-	+	-	-	-
MCI without EVOO	-	-	-	+	-	-
MCI with EVOO	-	-	-	-	+	-
AD	-	-	-	-	-	+

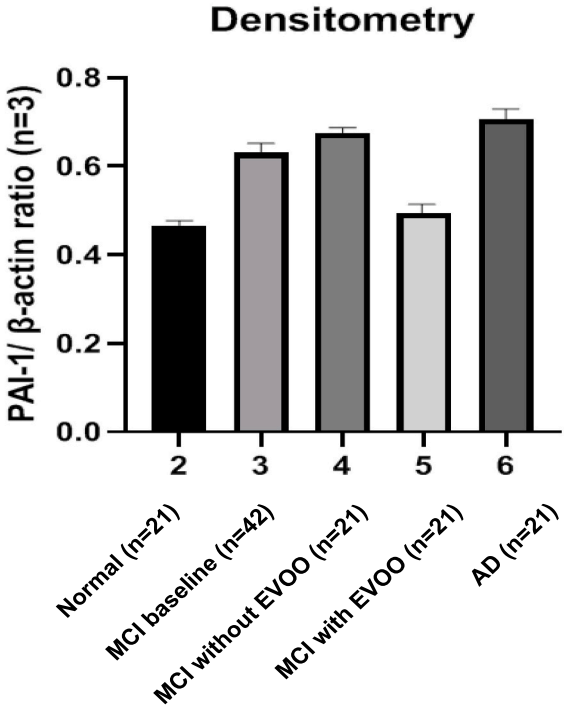


Fig. 1. a) Plasminogen activator inhibitor 1 (PAI-1) levels in serum of normal cognitively individuals, of MCI patient's baseline group, of MCI patients group supplemented and non-supplemented with EVOO and of AD patients' group. The results are expressed as the mean value ± SEM in a box-and-whisker plot from min to max value. Data are compared with cognitively individuals. Moreover, bracket depicts the comparison between group of MCI patients administered or not with EVOO (*$p = 0.05$, **$p = 0.001$, ***$p < 0.001$, ****$p < 0.0001$, ns, not significant). b) Comparison only of the PAI-1 levels in MCI patients' baseline group ($n = 42$), in MCI patients' group ($n = 21$) without EVOO treatment, and in MCI patients' group (n = 21) with EVOO treatment expressed as a mean value ± SEM. c) Assessment of PAI1 protein levels in participants' serum of the indicated samples through western blotting. EVOO treatment for 12 months retained PAI1 at levels comparable to those of the cognitively healthy individuals. Densitometer providing quantification for western blot presented in Fig. 1c.

and the standard error of the mean (Mean ± SEM) values of the parameters. It is noteworthy that MMSE value decreases according to the following order from the cognitively healthy individuals group (28.81 ± 0.1636) towards the MCI patient's group (baseline) (27.21 ± 0.2374), the MCI patient's group not subjected to EVOO consumption (26.90 ± 0.3645) and the AD patient's group (14.62 ± 0.6813) as it was expected. However, it should be highlighted also that MMSE value for the MCI patients' group subjected to EVOO consumption was as high as 28.67 ± 0.2323, and hence it was not very different from that of the cognitively healthy individuals' group (Table 1). Comparison of the demographic characteristics between the participating groups gave *P*-values for the age and education with no statistically significant difference. Whereas for MMSE, P-values exhibited a statistically significant difference in all cases, except the case of the comparison between the MCI patients group (baseline) against the MCI patients group not subjected to EVOO consumption, found with no statistically significant difference, as it was expected (Table 2).

3.2. Annual EVOO consumption restores the levels of MCI patients' serum fibrinolytic factors

Taken into consideration that PAI-1 implicates in amyloid deposition, this study aimed to assess the effect of EVOO consumption, before and after a systematic annual therapy, of diagnosed already MCI patients through the determination in their serum and evaluation of both biochemical key parameters, which operate in a certain metabolic pathway, like the fibrinolysis as well as in well-established biomarkers involved in AD pathology. Hence, we first measured the levels, of the fibrinolytic system factors before and after EVOO treatment, in the serum of MCI and AD patients compared to healthy age-matched cognitively subjects.

Initially, we recorded that before the EVOO treatment the levels of PAI-1 were increasing progressively from healthy individuals (9.994 ± 0.9658) towards the MCI (17.89 ± 0.4615) and AD (32.48 ± 1.324) pg/ml patients, respectively (Fig. 1a). However, at the end of the clinical trial the PAI-1 levels were found reduced in the serum of the MCI patients group (8.117 ± 0.8743 pg/ml) which consumed annually EVOO and surprisingly, approximately approaching those of the cognitively healthy individuals (9.994 ± 0.9658 pg/ml), in contrast to the respective group of MCI patients, which had not consumed EH EVOO, the sampling and measurement took place also after one year from the beginning of the trial, and the levels of PAI-1 exhibited increased fluctuation (22.70 ± 0.7289 pg/ml) compared to those of the healthy individuals and to the MCI patient's baseline (17.89 ± 0.4615 pg/ml) and AD (32.48 ± 1.324 pg/ml) (Fig. 1b). It was also performed a multi-range Tukey test and the results are depicted in Table S1.

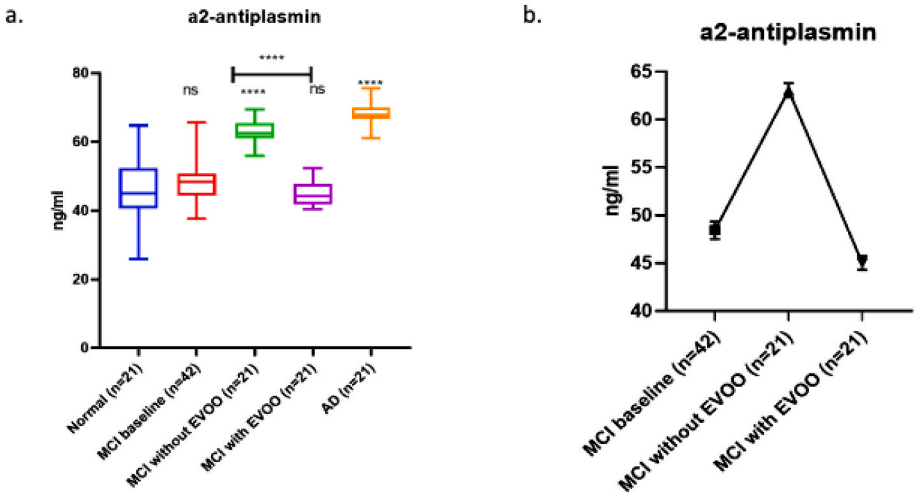


Fig. 2. a) a2-antiplasmin (a2-AP) levels in in serum of normal cognitively individuals, of MCI patient's baseline group, of MCI patients' group administered or not with EVOO and of AD patients' group. The results are expressed as the mean value ± SEM in a box-and-whisker plot from min to max value. Data are compared with cognitively individuals. Moreover, bracket depicts the comparison between group of MCI patients administered or not with EVOO (*p = 0.05, **p = 0.001, ***p < 0.001, ****p < 0.0001, ns, not significant). b) Comparison only of the a2-AP levels in MCI patients' baseline group (n = 42), in MCI patients' group (n = 21) without EVOO treatment, and in MCI patients' group (n = 21) with EVOO treatment expressed as a mean value± SEM.

Table 3
Clinical data of all measured biomarkers. The values are depicted as mean ± SEM.

	Normal (n = 21)	MCI baseline (n = 42)	MCI without EVOO (n = 21)	MCI with EVOO (n = 21)	AD (n = 21)
PAI-1 (pg/ml)	9.994 ± 0.9658	17.89 ± 0.4615	22.70 ± 0.7289	8.117 ± 0.8743	32.48 ± 1.324
a2-antiplasmin (ng/ml)	46.54 ± 1.9860	48.44 ± 0.9081	63.05 ± 0.7307	45.02 ± 0.7163	68.34 ± 0.7807
tPA (pg/ml)	919.1 ± 27.84	506.5 ± 14.62	466.9 ± 17.00	976.8 ± 40.52	455.4 ± 12.45
p-tau (pg/ml)	1.105 ± 0.0462	1.472 ± 0.0501	1.963 ± 0.0856	1.146 ± 0.0645	3.061 ± 0.1592
Aβ1–42 (pg/ml)	17.31 ± 2.202	16.06 ± 1.160	14.88 ± 1.248	17.13 ± 1.027	11.66 ± 1.743
Aβ1–40 (pg/ml)	286.1 ± 12.70	200.8 ± 6.961	168.4 ± 5.793	209.4 ± 6.840	168.6 ± 8.544
MDA (μM)	0.3244 ± 0.0750	1.060 ± 0.1009	2.655 ± 0.2419	0.2244 ± 0.03776	5.634 ± 0.5563
MMSE	28.81 ± 0.1636	27.21 ± 0.2374	26.90 ± 0.3645	28.67 ± 0.2323	14.62 ± 0.6813

In Western Blot analysis of serum samples which originated from the different under-investigation groups, despite that all contained equal protein loading as it was determined by the Bradford method and was proved by the equal amount of actin identification, PAI-1 is visualized as a single band around 45 kDa, (in accordance with standard PAI-1 run at lane 1), differing in quantity in all samples varying dependent of the cognitive stage and the annual EVOO dedication or not (Fig. 1c). In detail, we observed that the visualized PAI-1 protein bands, after immunostaining, from samples originated from the MCI patients group which had not been subjected to annual EVOO administration as well as from the patients group suffered from AD are more intense revealing the abundance of this protein in the respective samples; in contrast to the PAI-1 protein band derived from the MCI patients' group samples which had been subjected to annual EVOO administration being of less intense, and surprisingly almost equal to the normal cognitively individuals. Moreover, a confirmation of these outcomes originated from ELISA assays was supported from densitometry of the Western blot membranes immunostaining (Fig. 1c, down panel), representing the PAI-1/β-actin ratio (in triplicate) for all the under-investigation groups. In brief, the PAI-1 protein amount of the MCI patients' group samples, dedicated to annual EVOO administration, was almost identical to the cognitively normal group.

Secondly, since plasmin is inhibited by the α2AP we detected also the a2-antiplasmin levels in the serum of all groups. We hypothesized that if EVOO reflects in the restoration of PAI-1 levels, it would emerge the possibility that a2-antiplasmin could be activated as there is no hurdle to hamper it. For this reason, we measured the α2AP protein levels in all participated groups. At the beginning of the trial before the EVOO intervention, the levels of α2AP were found elevated with the progression of the disease from healthy individuals (46.54 ± 1.9860) towards the MCI patients (48.44 ± 0.9081), while the α2AP concentration was significantly higher in patients with AD (68.34 ± 0.7807) ng/ml. This result may imply that the augmented levels of α2AP during the progression of the disease inhibit the plasmin activity as it acts as an inhibitor of it (Fig. 2a).

Next, we examined whether the annual EVOO consumption by the MCI diagnosed patients displayed the efficacy to influence the α2AP levels. In the group of MCI patients, who had been annually subjected to EVOO therapy, the α2AP levels (45.02 ± 0.7163 ng/ml) are reduced significantly approaching those of the healthy individuals' levels (46.54 ± 1.9860 ng/ml), contrary to those levels of the MCI patients' group that had not consumed annually EH-EVOO (63.05 ± 0.7307 ng/ml), and they approached those of the AD patients (68.34 ± 0.7807 ng/ml) (Fig. 2b, Tables 3 and 4). It was also performed a multi-range Tukey test and the results are depicted in Table S2.

Taken into consideration reports that the depletion of endogenous tPA in Tg2576 mice has been reflected in an increase of cerebral Aβ accumulation, and as a consequence, worsening of AD pathophysiology (Oh et al., 2014), and that the restoration of tPA activity could be of therapeutic significance in diseases related to the amyloid accumulation (Park et al., 2020), these findings prompt us to determine the levels of the tPA enzyme - directly inactivated by PAI-1- in the serum of all participants.

In the cognitively normal individuals, the levels of the tPA protease were 919.1 ± 27.84 pg/ml and were found reduced in the MCI patients group (baseline) and equal to 506.5 ± 14.62 pg/ml and in the AD patients group equal to 455.4 ± 12.45 pg/ml respectively. On the other part, the levels of the tPA protease, in the MCI patients group subjected to annual EVOO supplementation, it is noteworthy that were measured to be as high as 976.8 ± 40.52 pg/ml, (a value a little higher even than this of the cognitively normal individuals, but with a non-significant statistical difference between them). Notably, in the respective MCI patients' group non-subjected to annual EVOO supplementation, the levels of the tPA protease measured at the end of one year were reduced at 466.9 ± 17.00, thus became similar to those measured for the AD patients' group. It was also performed a multi-range Tukey test and the results are depicted in Table S3. All descriptive values are summarized in Fig. 3a, b, and in Tables 3 and 4.

Table 4
P-values of groups' comparison for each measured marker.

P-value	Normal vs MCI baseline	Normal vs MCI without EVOO	Normal vs MCI with EVOO	Normal vs AD	MCI without EVOO vs MCI with EVOO	MCI baseline vs MCI without EVOO	MCI baseline vs MCI with EVOO
PAI-1 (pg/ml)	<0.0001 (****)	<0.0001 (****)	0.1574 (ns)	<0.0001 (****)	<0.0001 (****)	<0.0001 (****)	<0.0001 (****)
a2-antiplasmin (ng/ml)	0.1446 (ns)	<0.0001 (****)	0.7699 (ns)	<0.0001 (****)	<0.0001 (****)	<0.0001 (****)	<0.0001 (****)
tPA (pg/ml)	<0.0001 (****)	<0.0001 (****)	0.2474 (ns)	<0.0001 (****)	<0.001 (****)	<0.0001 (****)	<0.0001 (****)
p-tau (pg/ml)	<0.0001 (****)	<0.0001 (****)	0.6074 (ns)	<0.0001 (****)	<0.0001 (****)	<0.0001 (****)	<0.0001 (****)
Aβ1–42 (pg/ml)	0.5795 (ns)	0.3421 (ns)	0.9394 (ns)	0.051 (ns)	0.1719 (ns)	0.5308 (ns)	0.5531 (ns)
Aβ1–40 (pg/ml)	<0.0001 (****)	<0.0001 (****)	<0.0001 (****)	<0.0001 (****)	<0.0001 (****)	<0.01 (**)	0.4375 (ns)
MDA (μM)	<0.0001 (****)	<0.0001 (****)	0.2440 (ns)	<0.0001 (****)	<0.0001 (****)	<0.0001 (****)	<0.0001 (****)

P-value <0.05 is considered significant (*); <0.01 stands for **; <0.001 stands for ***; <0.0001 stands for ****.

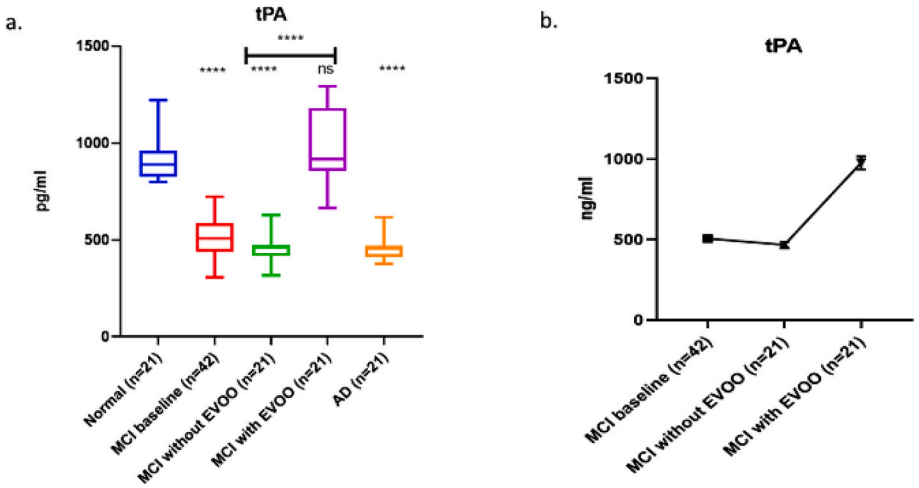


Fig. 3. a) Tissue plasminogen activator (tPA) levels in serum of normal cognitively individuals, MCI patients' baseline group, MCI patients' group administered or not with EVOO, and AD patients' group. The results are expressed as the mean value ± SEM in a box-and-whisker plot from min to max value. Data are compared with cognitively individuals. Moreover, bracket depicts the comparison between group of MCI patients administered or not with EVOO (*p = 0.05, **p = 0.001, ***p < 0.001, ****p < 0.0001, ns, not significant). b) Comparison only of the tPA levels in MCI baseline group patients (n = 42), in MCI patients' group (n = 21) without EVOO treatment, and in MCI patients' group (n = 21) with EVOO treatment expressed as a mean value± SEM.

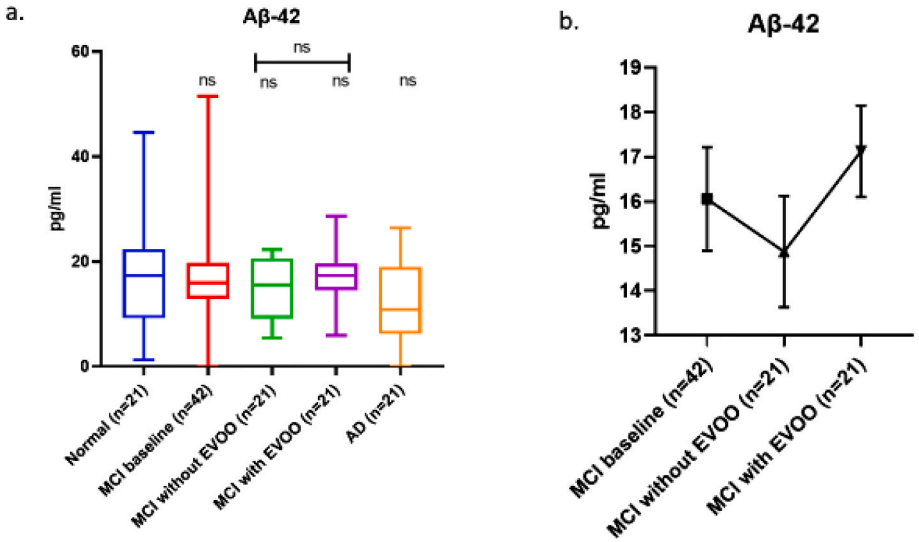


Fig. 4. a) Amyloid β 42 isoform (Aβ1–42) levels in serum of normal cognitively individuals, MCI patients' baseline group, MCI patients' group administered or not with EVOO, and AD patients' group. The results are expressed as the mean value ± SEM in a box-and-whisker plot from min to max value. Data are compared with cognitively individuals. Moreover, bracket depicts the comparison between group of MCI patients administered or not with EVOO (*p = 0.05, **p = 0.001, ***p < 0.001, ****p < 0.0001, ns, not significant). b) Comparison only of the Aβ1–42 levels in MCI patients' baseline group (n = 42), in MCI patients' group (n = 21) without EVOO treatment, and in MCI patients' group (n = 21) with EVOO treatment expressed as a mean value± SEM.

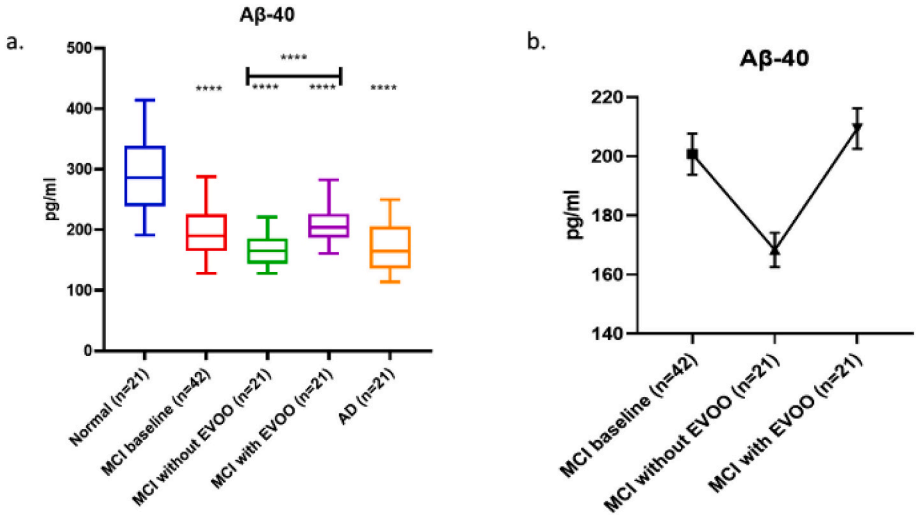


Fig. 5. a) Amyloid β1–40 isoform (Aβ1–40) levels in serum of normal cognitively individuals, MCI patients' baseline group, MCI patients' group administered or not with EVOO, and AD patients' group. The results are expressed as the mean value ± SEM in a box-and-whisker plot from min to max value. Data are compared with cognitively individuals. Moreover, bracket depicts the comparison between group of MCI patients administered or not with EVOO (*p = 0.05, **p = 0.001, ***p < 0.001, ****p < 0.0001, ns, not significant). b) Comparison only of the Aβ1–40 levels in MCI patients' baseline group (n = 42), in MCI patients' group (n = 21) without EVOO treatment, and in MCI patients' group (n = 21) with EVOO treatment expressed as a mean value± SEM.

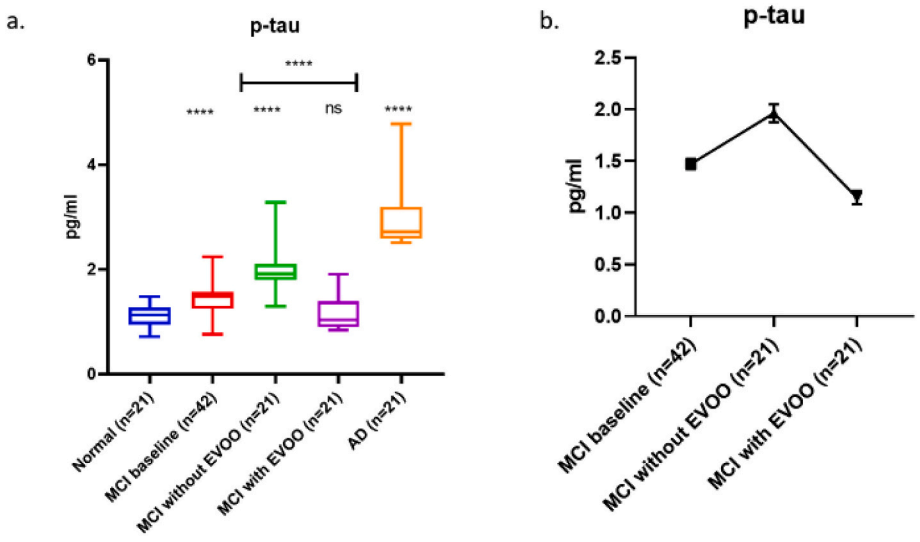


Fig. 6. a) Phosphorylated tau (p-tau) levels in serum of normal cognitively individuals, MCI patients' baseline group, MCI patients' group administered or not with EVOO, and AD patients' group. The results are expressed as the mean value ± SEM in a box-and-whisker plot from min to max value. Data are compared with cognitively individuals. Moreover, bracket depicts the comparison between group of MCI patients administered or not with EVOO (*p = 0.05, **p = 0.001, ***p < 0.001, ****p < 0.0001, ns, not significant). b) Comparison only of the p-tau levels in MCI patients' baseline group (n = 42), in MCI patients' group (n = 21) without EVOO treatment, and in MCI patients' group (n = 21) with EH EVOO treatment expressed as a Mean value± SEM.

3.3. The effect of annual EVOO consumption by MCI patients on Aβ levels

To estimate the effect of annual EVOO consumption by the MCI patients' group on the Aβ1–42 and Aβ1–40 levels we determined the levels of these Aβ species in the sera of all the cohort groups before and after EVOO treatment. We first found that Aβ1–42 levels are more elevated in cognitively normal individuals (17.31 ± 2.202 pg/ml) compared to any other group tested; In detail for the values obtained, the Aβ1–42 levels in the MCI patients group subjected to annual EVOO administration were augmented and equal to 17.13 ± 1.027 pg/ml, close to this of the cognitively normal group. Moreover, these values were found even a little more increased compared to the value obtained for the MCI patients group (baseline), equal to 16.01 ± 1.160 pg/ml, to the MCI patients group non-subjected that were 14.88 ± 1.248 pg/ml, as well as to the AD patients group, in which this value was obviously the lowest obtained equal to 11.66 ± 1.743 pg/ml (Fig. 4a, b, and Table 3). *P*-values obtained from groups' comparison for the Aβ1–42 levels in serum are summarized in Table 4.

We further detected that Aβ1–40 levels were higher a priori in the cognitively normal individuals (286.1 ± 12.70 pg/ml) compared to any other group, disease-affected. For the other groups, analytically, the Aβ1–40 levels of the MCI (baseline) patients group were lower than those of the cognitively normal individuals' group (200.8 ± 6.961 pg/ml), while Aβ1–40 levels measured in MCI patients' group subjected to annual EVOO administration were increased (209.4 ± 6.840 pg/ml) compared to the MCI (baseline) patients' group, to the MCI patients' group non-subjected (168.4 ± 5.793 pg/ml), and to the AD patients' group (168.6 ± 8.544 pg/ml), with the lowest levels of Aβ1–40 fragment of all groups (Fig. 5a, b, and Table 3). It is noteworthy that in MCI patients' group, who had consumed daily for one year EVOO we found an increased concentration of Aβ1–40 and closer to the cognitively normal individuals compared to those of the counterparts MCI groups. P-values obtained from groups' comparison for the Aβ1–40 levels in serum are summarized in Table 4. It was also performed a multi-range Tukey test and the results are depicted in Tables S4 and S5.

3.4. The effect of annual EVOO consumption by MCI patients on p-tau protein levels

We also investigated in all examined groups the p-tau levels, as a diagnosed biomarker of AD pathology (Lauretti et al., 2020). P-tau protein levels were as low as 1.105 ± 0.0462 pg/ml in the cognitively normal subjects and lower than those of all the examined groups, disease-affected: in the MCI patients' group (baseline) was 1.472 ± 0.0501 pg/ml, in the MCI patients' group non-subjected to EVOO supplementation was 1.963 ± 0.0856 pg/ml and in the AD patients' group was 3.061 ± 0.1592 pg/ml.

On the other hand, we should comment that the p-tau protein levels of the MCI patients' group who were dedicated to the annual consumption of EVOO, they were equal to 1.146 ± 0.0645 pg/ml, approaching those of the cognitively normal individuals and lower than those of all the other AD disease-affected groups. It was also performed a multi-range Tukey test and the results are depicted in Table S6.

These outcomes demonstrated the beneficial effect of the EVOO on the progression of the AD disease that may probably be occurred through reducing the hyperphosphorylation levels of tau protein, a well-established AD "trademark" intervening in the balance of the phosphatases-kinases balance activities on tau protein. All measures obtained are summarized in Fig. 6a, b, and Tables 3 and 4.

3.5. The effect of annual EVOO consumption by MCI patients on oxidative stress

Oxidative stress is considered as a key factor of AD's onset (Balez and Ooi, 2016; Barbagallo et al., 2015; Chauhan and Chauhan, 2006; Markesbery and Carney, 2006). Changes in oxidative metabolism and overproduction of ROS metabolites, followed by a decreased antioxidant activity and the incapacity of repairing the defective molecules, are recorded in AD.

For this reason, oxidative stress was assessed in the serum of all participated groups, through measurement of lipid peroxidation product levels, MDA as a characteristic marker. Due to the antioxidant properties of EVOO, we have also tested the capacity of the annual EVOO consumption by MCI patients to influence the levels of MDA compared to those of the MCI patients non-consuming, to explore their role in prevention of disease progression. The levels of MDA presented a gradual tendency of increase from stage of cognitively normal individuals (0.3244 ± 0.07500 μM) towards the MCI patients (baseline) (1.060 ± 0.1009 μM), to the MCI patients group non-consuming EVOO (2.655 ± 0.2419 μM) that measured after one year, and finally to the AD patients (5.634 ± 0.5563 μM), exhibiting the highest level of lipid peroxidation ending in abundant levels of MDA. On the other side, the MCI patients dedicated to the EVOO annual consumption displayed surprisingly very low levels of MDA (0.2244 ± 0.03776 μM) and comparable to those of the healthy individuals. It is concluded, by comparison, that starting from the MCI patients' group (baseline) the oxidative stress was duplicated in the MCI patients group non-consumed EVOO compared to this of the MCI patients' group annually consumed EVOO. Considered the

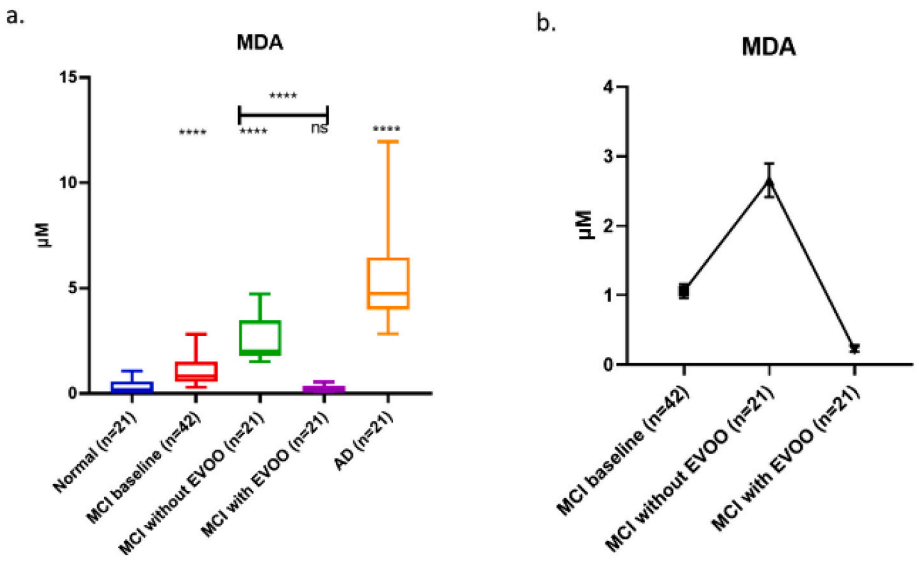


Fig. 7. a) Malondialdehyde (MDA) levels in serum of normal cognitively individuals, MCI patients' baseline group, MCI patients' group administered or not with EVOO, and AD patients' group. The results are expressed as the mean value ± SEM in a box-and-whisker plot from min to max value. Data are compared with cognitively individuals. Moreover, bracket depicts the comparison between group of MCI patients administered or not with EVOO (*p = 0.05, **p = 0.001, ***p < 0.001, ****p < 0.0001, ns, not significant). b) Comparison only of the MDA levels in MCI patients' baseline group (n = 42), in MCI patients' group (n = 21) without EVOO treatment, and in MCI patients' group (n = 21) with EVOO treatment expressed as a mean value± SEM.

Table 5
Correlation between PAI-1 levels and the fibrinolytic factor tPA and different other AD and oxidative stress trademarks levels.

Correlation	Pearson r	P values
	PAI-1	
a2-antiplasmin	0.6886	<0.0001 (****)
tPA	−0.6555	<0.0001 (****)
Aβ1–42	−0.2357	0.0078 (**)
Aβ1–40	−0.4471	1.5347 (ns)
p-tau	0.0077	<0.0001 (****)
MDA	0.7456	<0.0001 (****)

The number of asterisks in the parenthese indicates the statistical significance of the PAI-1 levels correlation with the levels of all the other examined factors examined.

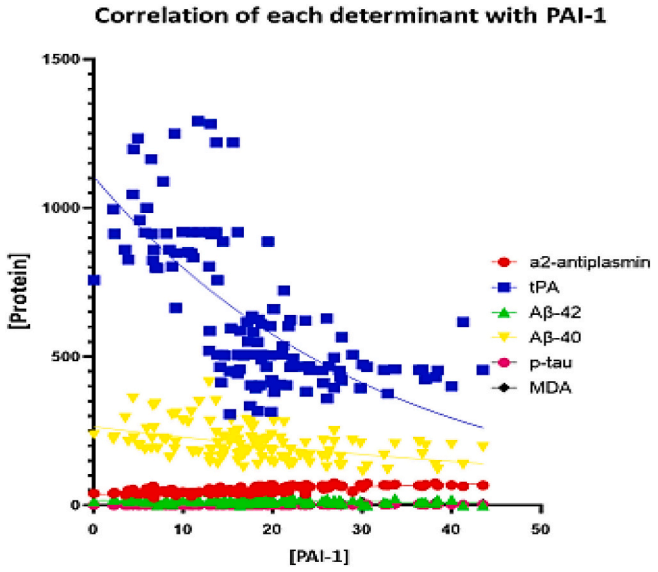


Fig. 8. Correlation of the various determinants including α2AP, tPA, α2AP, Aβ 1–42, Aβ 1–40, p-tau, and MDA.

MDA values it is concluded that starting from the MCI patients' group (baseline) the oxidative stress was almost doubled in the MCI patients' group which did not consumed EVOO compared to this of the MCI patients' group that annually consumed EVOO. It was also performed a multi-range Tukey test and the results are depicted in Table S7. All data are summarized in Fig. 7a, b and in Tables 3 and 4.

Table 6
Multiple linear regression of PAI-1, as a dependent variable, and independent variables of all the other biomarkers. P < 0.05 considered as significant.

Dependent Variable: PAI-1	Estimate	95% confidence interval	*P*-value	Statistical significance
β0 Intercept	9.472	2.094 to 16.85	0.0123	*
β1 A: a2-antiplasmin	0.1443	0.01829 to 0.2704	0.0252	*
β2 B: tPA	−0.01069	−0.01510 to −0.006268	<0.0001	****
β3 C: p-tau	3.607	1.807 to 5.407	0.0001	***
β4 D: MDA	0.9368	0.3350 to 1.539	0.0025	**

3.6. Correlations between the serum levels of PAI-1 and AD hallmarks

Correlations were attempted between the measured proteins and it was found that there was a significant correlation between serum PAI-1 and a2-antiplasmin levels ($r = 0.6886$, $P < 0.0001$), whereas serum PAI-1 levels was not correlating with Aβ1–40 levels ($r = -0.4471$, $P = 1.5347$) (Table 5). However, it should be noted that there was a strong correlation between serum PAI-1 levels with those of the Aβ1–42 fragment $r = -0.2357$, $P = 0.0078$ (**) (Table 5). At the other part, serum PAI-1 levels showed a significant correlation with the tPA protease ($r = -0.6555$, $P < 0.0001$), the phosphorylated tau (p-tau) ($r = 0.0077$, $P < 0.0001$) and MDA ($r = 0.7456$, $P < 0.0001$) as well, where the respective r and *P* values are in the parentheses) (Table 5, Fig. 8).

Conclusively, we found that the serum levels of the fibrinolytic factor PAI-1 are strongly correlated to the levels of AD hallmarks, those of the Aβ1–42 fragment and the phosphorylated tau as well as those of oxidative stress marker MDA.

3.7. Multiple linear regression of determinants of PAI-1 levels in the blood

With a view to determining which factor better contributes to the PAI-1 levels, a multiple linear regression analysis of PAI-1, as a dependent variable and independent variables all the other biomarkers, was performed with the backward elimination of less significant variables. The most significant variable for PAI-1 levels is tPA ($p < 0.0001$), followed by p-tau protein ($p = 0.0001$) and then by MDA ($p = 0.0025$).

More, we found that a2-AP affects to a lesser degree PAI-1 levels in serum ($p = 0.0252$). The results of all determinants are summarized in Table 6. The R^2 of this proposed model was 0.7013.

4. Discussion

Augmented PAI-1 expression/activity affects significantly Aβ accumulation during aging and in AD, probably through inhibition of plasminogen activation and thus Aβ clearance (Liu et al., 2011). A study investigated the association between plasma PAI-1, tPA/PAI-1 ratio, and MCI in Chinese type 2 diabetes mellitus (T2DM) patients, high PAI-1 levels and low tPA/PAI-1 ratio were significantly correlated with T2DM -associated cognitive impairment, especially memory function, in Chinese patients (Wang et al., 2018). PAI-1 inhibition restores tPA activity, rescues neurovascular coupling, diminishes amyloid deposition around blood vessels, and ameliorates cognition in a mouse model of Aβ accumulation demonstrating a previously unappreciated role of tPA in Aβ-related neurovascular dysfunction and in vascular amyloid deposition.

A small number of studies have investigated the role of PAI-1 on the AD onset, and more specifically they have been considered it as a diagnostic marker for AD (Barker et al., 2012; Ichimura et al., 2013; Jacobsen et al., 2008; Liu et al., 2011; Manley, 2013; Oh et al., 2014). Moreover, it is controversial whether there are different PAI-1 levels in blood between healthy humans and age-matched patients with vascular dementia or AD (Ban et al., 2009), in contrast to the report that blood levels of PAI-1 in humans with dementia were found higher in comparison with the healthy age-matched control group (Reed et al., 2017), while no data have been stated for the PAI-1 levels in participants with MCI (Ban et al., 2009).

Having bear in mind the aforementioned results, in this research, we investigated the effect of the annual EVOO consumption by the MCI patients of the clinical trial MICOIL on factors of the fibrinolytic system, and especially on the principal one PAI-1, on a2-antiplasmin, and moreover on the tPA protease, as well as on some well-established hallmarks biomarkers for the AD pathology. We found that the EVOO action abolished PAI-1-activity and, on the downside, tPA activity enhanced in EVOO-treated MCI patients. This outcome may result in plasmin activation and therefore in Aβ degradation. Our results come in agreement with a former study carried out in old rats demonstrating that the olive oil supplementation may decrease PAI-1 at the level of gene expression (Nassef et al., 2013).

Focusing on the fibrinolytic cascade, we measured the levels of a2AP in blood. We detected heavily reduced levels of a2AP in MCI group after one-year treatment with EVOO. This result reinforced our hypothesis that EVOO effect constitutes a hurdle to a2AP deposition, and thus, the levels in the blood of MCI patients group EVOO-treated, in turn, were found decreased.

We looked up on the evaluation of the neurological effects generated by EVOO previously examined by two tests: the Alzheimer's Disease Assessment Scale-Cognitive Subscale test (ADAS-Cog) and the Mini-Mental State Exam (MMSE). ADAS-Cog test is one of the most frequently used tests to measure cognition in research studies and clinical trials for new drugs and other interventions, more thorough than the MMSE, and it primarily measures language and memory. The MICOIL clinical trial (Tsolaki et al., 2020) demonstrated very recently significant improvement in ADAS-Cog score ($p = 0.001$) and MMSE ($p = 0.05$) in groups of patients after one year intervention received EVOO and mainly independent of the presence of *APOE* ε4, whereas the non-treated group displayed worse or similar to baseline performance in almost all domains. In detail, consuming groups had better outcomes in respect with ADAS-cog ($p = 0.003$), Digit Span ($p = 0.006$), and Letter fluency ($p = 0.003$). Moreover, MCI patients consuming EVOO exhibited better outcomes with regards to ADAS-cog ($p = 0.003$), Digit Span task a verbal short-term memory used to measure working memory's number storage capacity ($p = 0.006$), and Letter fluency test ($p = 0.003$), a kind of psychological test in which participants have to produce as many words as possible from a category in a given time (Tsolaki et al., 2020).

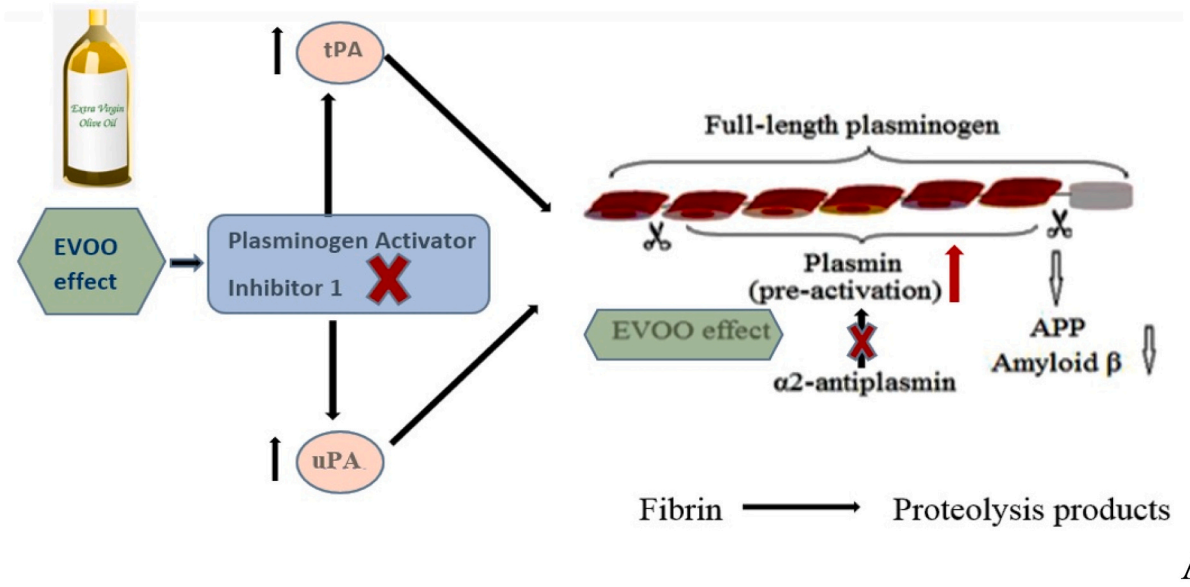
For this reason, we further examined the effect of EVOO on AD biomarkers (Aβ and p-tau), with a view to detecting an overall amelioration of AD symptoms via the regard of fibrinolysis-improvement. Our data in serum come in agreement with the report that plasma Aβ levels are obviously reduced in the different dementia stages of AD, and reduction in levels of Aβ1-40 and of Aβ1-42 (Pesaresi et al., 2006; Rembach et al., 2013) is associated with cognitive decline, suggesting that noteworthy alterations in Aβ metabolism happen later in the periphery, in comparison with the brain (Janelidze et al., 2016a). In our research the annual therapy with EVOO of the MCI patients group, molded a refinement as far as amyloid detecting levels. However it noteworthy to further examine, whether an exact ingredient of the EVOO, or the EVOO in total, exerts this effect on amyloidosis deposition.

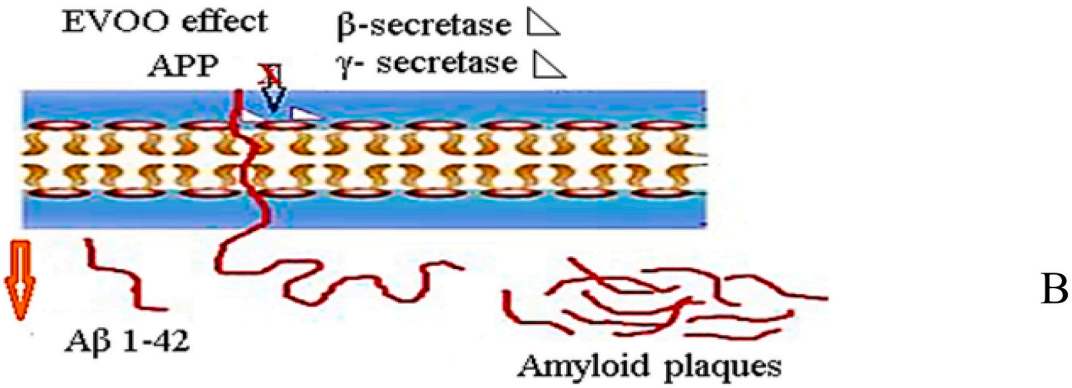
The decreased plasma levels of Aβ1–42 in MCI and AD patients could be owing to the severity brought by the progression of the disease, in contrast to CSF findings, where it is detected a decrease in Aβ1–42 levels, but not in Aβ1–40 during preclinical stages (Janelidze et al., 2016b). However, investigations that focus on assessing blood levels of Aβ1–42 in AD patients show conflicting results (Snyder et al., 2014). Contrastingly, some researches proposed that increased baseline plasma levels of Aβ relate the hazard of dementia trigger in the future; however, this hypothesis has not been confirmed by other investigations (Janelidze et al., 2016a). Blood Aβ levels reflect only to some extent the dysregulated Aβ metabolism and aggregation in the brain. The efflux of Aβ to peripheral blood accounts for 50% of total brain Aβ clearance in humans (Roberts et al., 2014), suggesting that the physiological Aβ clearance capacity of the peripheral system provides an important mechanism against Aβ accumulation in the brain.

Recently, plasma levels of Aβ1-42 and Aβ1-40 were analyzed in a cohort of 719 individuals (the Swedish BioFINDER study) and they were found reduced in AD dementia compared to all other diagnostic groups (Janelidze et al., 2016b; Lue et al., 2017), in contrast to the cerebrospinal fluid (CSF), where there is a clear decline in Aβ1-42, but not in Aβ1-40 already during preclinical stages. Moreover, there were weak positive correlations between plasma and CSF levels for both Aβ1-42 and Aβ1-40, and negative correlations between plasma Aβ1-42 and neocortical amyloid deposition (measured with PET) (Janelidze et al., 2016b). Other report correlates the lower plasma Aβ1-42 levels with incident dementia, but results are conflicting and few have investigated cognitive decline among non-demented elders (Hillen, 2019; Rembach et al., 2013; Yaffe et al., 2011).

Our outcomes of the clinical trial support that the annual EVOO consumption by the MCI patients group reduces the p-tau levels in their serum. It is already stated that under physiological conditions, kinases and phosphatases are counterbalanced in healthy individuals and as a result, phosphorylated and non-phosphorylated tau is found in a balance (Raskin et al., 2015). When the phosphorylation is increased, tau becomes insoluble and forms filaments and thus, neurofibrillary tangles are accumulated (Iqbal et al., 2005). A recent study investigated the effect of chronic supplementation of EVOO on the phenotype of a relevant mouse model of tauopathy, human transgenic tau mice (hTau). Consistent with our result, it was demonstrated that EVOO treatment resulted in a significant reduction of tau oligomers and phosphorylated tau at specific epitopes. Furthermore, EVOO directly improved synaptic activity, short-term plasticity, and memory, while decreasing tau neuropathology in the hTau mice (Lauretti et al., 2020). Recent conclusions of a cohort study demonstrated that plasma P-tau217 levels were augmented during the early preclinical stages of AD before the insoluble tau aggregates to be detectable by tau-PET, and proposed plasma P-tau217 as a good promised biomarker for early AD brain pathology (Janelidze et al., 2020).

Based on our outcomes concerning the measured reduction of the p-tau levels in serum of MCI patients' group, who were dedicated to the EVOO consumption for a duration of one year, EVOO administration





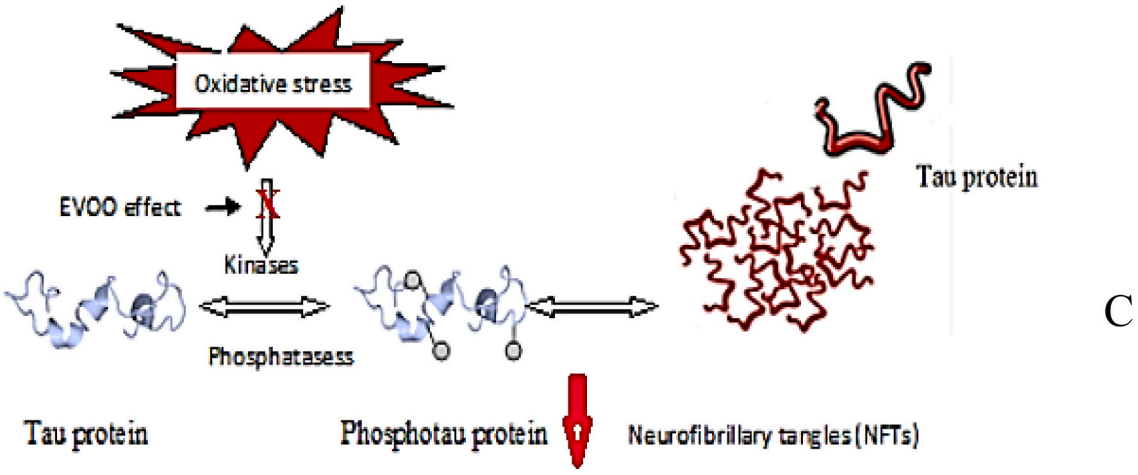


Fig. 9. A collective representative scheme of the fibrinolytic mechanism touched here, involving the influence of the intervention of EVOO. A) EVOO consumption by MCI patients diminishes serum PAI-1, tPA, and α2AP levels. PAI-1 normally regulates two proteases: tPA and uPA which activate plasminogen that in turn is converted to activated plasmin. The later reduces the Aβ peptide levels and cleavage in the middle of the Aβ peptide sequence which called α-site, excludes the formation of the amyloidogenic APP peptide. Plasmin activity is also regulated by another inhibitor, member of the serine proteases family, a2-antiplasmin (α2AP). B) Activation of PAI-1 affects the accumulation of Aβ in the elderly, suppressing plasminogen activation and, consequently, Aβ clearance. EVOO consumption by MCI patients affect amyloid clearance, and C) EVOO consumption by MCI patients diminishes oxidative stress reflecting in reduced p-tau protein.

may be proposed as a preventive therapy for fighting the tau protein hyperphosphorylation, a prodromal stage of AD, that is MCI, and consequently, to impede the further progression of AD. An extension of acceptance of these findings would turn therapies towards focusing on tau hyperphosphorylation inhibition. These results strengthen the healthy benefits of EVOO and further support the therapeutic potential of this natural product not only for AD but also for primary tauopathies. This finding certainly merits further investigation to approach and elucidate the involved mechanism.

Keeping in mind the direct correlation between the oxidative stress and the early onset of AD (Barbagallo et al., 2015; Butterfield et al., 2001; Chauhan and Chauhan, 2006; Sultana et al., 2009; Tillement et al., 2011; Valls-Pedret et al., 2015), EVOO seems to be implicated in many pathways of oxidative stress (Servili et al., 2014) affecting AD pathology. Firstly, oxidative stress takes place in the early stage of the disease, before the detection of the standard histological hallmarks of AD (Reddy, 2011). Secondly, oxidative stress and free radicals bring into action β-secretase expediting the cleavage of APP to Aβ (Reddy and Beal, 2008).

The primary oxidative stress-connected metabolic pathways in AD, including the generation and involvement of MDA, is recently reviewed (Chen et al., 2020), and our results that EVOO therapy reduced or abolished MDA levels in serum of MCI patients, dedicated to the EVOO therapy, due to the EVOO antioxidant properties thanks to biophenols and MUFA containing, are in line (Zorić et al., 2021). Furthermore, a study reinforced the antioxidant effect of olive oil on blood, brain, muscle and small intestine (Kouka et al., 2020).

This biochemical research aimed to approach in deep the possible mechanisms affected by an annual EVOO consumption through the measurement of biochemical markers. For this purpose, in this study, firstly we assessed factors involved in the fibrinolytic mechanism, secondly in the Aβ clearance (Aβ levels species in serum), and thirdly in oxidative stress. The obtained results may provide evidence that the one-year duration of EVOO administration as a natural therapy was enough and able to bring up its effect on the fibrinolytic system on PAI-1, a2-antiplasmin, and tPA levels and on AD hallmarks. This demonstrates that diet based on olive-oil should be considered as a useful tool in MCI patients or even in healthy elderly people for preventing the onset of AD pathology, but more research is needed to conclude in safe and clear results. Moreover, drug evolution against Aβ in the future may target on the clearance of Aβ from the circulation and might be a promising therapeutic approach for AD (Roberts et al., 2014). A collective image which conglomerates the EVOO effect and the measured markers are illustrated in Fig. 9.

Furthermore, we recently investigated the restoration of the neuroprotective BMI1 protein levels after the administration of early harvest extra virgin olive oil as a therapeutic strategy against AD (Tzekaki et al., 2021a). We are also advocated that EVOO or oleuropein itself effects on PAI-1 as binding molecule by implementing an in silico molecular docking tool. We also proved that EVOO treatment or oleuropein all alone may be displayed as a natural inhibitor of PAI-1 by incrementally destabilizing PAI-1 levels selectively in ER-/PR- breast cancer cells (Tzekaki et al., 2021b).

Targeting fibrinolytic system may constitute a novel strategy-especially micro-plasmin (μPlm) based therapeutics can be a practical candidate for developing an enzyme augmentation therapy for Aβ degradation. Restoration of tPA activity could be of therapeutic importance in diseases associated with the amyloid accumulation (Park et al., 2020). Very recently, by exploiting the involvement of plasmin in Aβ accumulation, there have been developed techniques to purify the catalytic domain of plasmin, μPlm, which can be used for an Aβ-clearance based AD therapy (Yang et al., 2020).

Among the strength of this study is that we used a well characterized, clinical population with different stage of cognitive decline. Many therapeutic options for fighting AD were reviewed including the dedication on the Mediterranean diet (MedDiet) (Abuznait et al., 2013; Batarseh and Kaddoumi, 2018; Cicerale et al., 2012; Lauretti et al., 2020; Martínez-Lapiscina et al., 2013; Qosa et al., 2015; Tsolaki et al., 2017). The beneficial effect of MedDiet on cognitive function was also previously assessed in 522 individual participants after one MedDiet nutritional intervention (supplemented with EVOO) versus a low-fat diet (control). Global cognitive performance examined by MMSE after 6.5 years of nutritional intervention, showed that participants allocated to the MedDiet + EVOO showed a higher mean MMSE score with significant differences versus control (adjusted difference: +0.62 95% CI +0.18 to +1.05, $p = 0.005$ for MMSE) (Martínez-Lapiscina et al., 2013). The recommended intake of olive oil estimated also previously from 25 to 50 ml on a daily basis (Abuznait et al., 2013). Considering that the phenolic content in EVOO varies significantly between varieties, regions, seasons, etc. (Diamantakos et al., 2021), the quantity for achieving expected beneficial effects varies too.

One of the main limitations of the randomized clinical trials is their constraint to therapeutic interventions that are alleged /hypothesized to have beneficial effects, and in this case, there is no further information for negative effects, for instance from the consumption of this EVOO quantity. A second constraint is that there are differences between the studied sample of individuals and the population of the physiological life. Taking part in a trial may influence the responses of a questionnaire and thus reflect in the result as in this case for instance of the MMSE. The sometimes-limited viewpoint of various trials abandons aside crucial details linked with the consequences of the intervention on costs of EVOO consumption, quality of life or pleasure.

Moreover, another of the main limitations of the randomized clinical trials, which are not funded by a big pharmaceutical company, is the small number of participants. A proposition-wish may include in establishing a disease management approach that may comprise a big number of patients with the execution of trials in real life with a long duration of follow-up to overcome some of these limitations.

Dropout rates in longitudinal surveys are dependent on three separate factors: failure to locate research participants, failure to contact participants, and failure to achieve cooperation. In this study, particular attention was given to existing knowledge on the correlates of dropout and strategies to limit the problem. To limit nonresponse, a total design approach was advocated with specific attention to each source. That was in order to limit both, noncontact and noncooperation, which was successful due probably to the small number of participants a problem that is amplified in big cohorts.

CRediT authorship contribution statement

E.E. Tzekaki: Methodology, Validation, Formal analysis, Investigation, Writing-original draft. **M.Tsolaki:** Conceptualization, recruitment of participants, clinical and neurological, neuropsychological, neuroimaging tests and cognitive and behavioral tests before and after one year of the EVOO intervention, resources. **G. Geromichalos:** Formal analysis, **E. Lazarou:** Investigation, Resources. **M. Kozori:** Investigation, Resources. **A.A. Pantazaki:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision. **Z. Sinakos:** Conceptualization, evaluation of the results of the fibrinolytic system factors.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

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