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Restoration of BMI1 levels after the administration of early harvest extra virgin olive oil as a therapeutic strategy against Alzheimer's disease

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ABSTRACT

Even though Alzheimer's disease (AD) is the most common cause of dementia, the mechanisms governing the establishment and progression of the disease remain largely unknown. Here, we investigated the implication of the neuroprotective protein BMI1 (B lymphoma Mo-MLV insertion region 1 homolog) in AD and the possibility to reverse the onset of the disease through the administration of extra virgin olive oil (EVOO) in Mild Cognitive Impairment (MCI) patients. For this purpose, we utilized a wide bank of MCI patient samples to examine the potential effects of EVOO. We found that while EVOO treatment increases BMI1 levels, p53 levels drop in MCI patient serum after EVOO treatment for 12 months. Additionally, AD-related biomarkers (p-tau, Aβ1–42 and Aβ1–42/Aβ-40 ratio) return to normal levels after administration of EVOO in MCI patients for 12 months. Moreover, we show that upon EVOO administration, BMI1-upregulation correlates with reduction of oxidative stress and inflammatory responses. In conclusion, we provide clinical trial evidence to confirm that restoration of BMI1 activity through EVOO administration in MCI patients constitutes a potential therapeutic approach against neurodegeneration leading to AD.

1. Introduction

AD comprises the most prominent cause of late-life dementia, as it affects over 50 million individuals in the world. Moreover, AD represents one of the leading causes of death worldwide, therefore it remains a subject of intense ongoing research (Dos Santos Picanco et al., 2018; Papaspyropoulos et al., 2020). Epigenetic gene silencing may be the driving force for AD onset (Lu et al., 2004), followed by cellular senescence (Savva et al., 2009), due to the accumulation of oxidative DNA damage (Raskin et al., 2015).

BMI1 is a component of the Polycomb Repressive Complex 1 (PRC1) promoting chromatin compaction and gene silencing through its E3-mono-ubiquitin ligase activity (Buchwald et al., 2006; Li et al., 2006). *Bmi1* deficiency in primary mouse embryonic fibroblasts induces premature senescence (Jacobs et al., 1999), while BMI1 downregulation in human fibroblasts also elicits replicative senescence (Itahana et al., 2003). BMI1 expression gradually diminishes in the neurons of aging mouse and human brains (Abdouh et al., 2012), and BMI1 inactivation in mice or in stem cell-derived human photoreceptors leads to retinal degeneration (Abdouh et al., 2012; Barabino et al., 2016). The equilibrium among Reactive Oxygen Species (ROS) and antioxidant factors is crucial to specify the extent of accumulated oxidative stress, and hence the subsequent impact on cellular and organism lifespan (Poljsak et al., 2013).

Bmi1 deficiency may amplify p53 activity during aging or in pathological conditions such as AD, thereby causing the acceleration of the aging process by promoting the accumulation of oxidative stress (Chatoo et al., 2009). In aged brains, p53 accumulates at the promoter of antioxidant response (AOR) genes, corresponds to a repressed chromatin state, downregulating AOR genes, and augmenting oxidative damages to lipids and DNA (Chatoo et al., 2009). *BMI1* knockout in human postmitotic neurons resulted in amyloidosis, p-tau accumulation, and ultimately, neurodegeneration (Flamier et al., 2018). BMI1 is a secretory protein, previously detected in cancer prostate patient blood (Siddique

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et al., 2013; Zhang et al., 2017).

A decreased risk of dementia has been strongly related to the direct effect of Mediterranean diet components on the metabolism of AD hallmarks (Knopman, 2009; Tsolaki et al., 2017); a substantial part of those positive effects has been attributed to EVOO consumption (Serra-Majem et al., 2004; Foscolou et al., 2018), as also shown by implementing relevant mouse models (Lauretti et al., 2017; Lauretti et al., 2020). Previous research has confirmed that participants who intensely consumed EVOO exhibited a decrease in cognitive decline in a 4-year timeline, in contrast to those whose intake of EVOO was rare or zero (Berr et al., 2009). High intake of EVOO and polyphenols decreased the rate of the onset of dementia by half in multivariate models (Lefèvre-Arbogast et al., 2018; Valls-Pedret et al., 2015; Martínez-Lapiscina et al., 2013), exhibiting a significantly positive impact on cognitive function.

Here, we show that BMI1 levels are found reduced in the serum of MCI and AD patients of the MICOIL clinical trial. Restoration of BMI1 levels in MCI patients occurred, at least partially, through a yearly EVOO natural product-based therapeutic approach. BMI1 increase after EVOO consumption correlated with a decrease in p53 and key AD marker levels, as well as in biomarkers of oxidative stress and inflammation, further confirming an overall prophylactic role of EVOO against neurodegeneration.

2. Materials and methods

2.1. ParticipantsT

This study included participants recruited into the MICOIL clinical trial (NCT03362996, MICOIL: Management of Mild Cognitive Impairment Patients With Extra Virgin Olive Oil). The study, which is still ongoing, initiated in December 2017 and is funded by Alzheimer Hellas, Thessaloniki, Greece and Yanni's Olive Grove Company providing the Early Harvest EVOO and EVOO from Potidea Chalkidiki, Greece.

The participants were from a Mediterranean area (Thessaloniki region, northern Greece). All subjects were white, community-dwelling individuals aged ≥65 years. Participants were literate and were not suffering from any debilitating diseases (e.g. cancer) as ascertained from their medical history, physical and neurological examination tests. 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; Plassmann et al., 2008; Kueper et al., 2018).

The present study includes a total of 80 individuals (aged 64–90) which are divided in: MCI patients consuming 50 ml EVOO daily (n = 20) who provided their sera 1 month past the beginning of the study (MCI EVOO – 1 month), and 12 months past the beginning of the study (MCI EVOO – 12 months); MCI patients not consuming EVOO for the respective period (n = 20), who provided their sera 1 month past the beginning of the study (MCI control – 1 month), and 12 months past the beginning of the study (MCI control – 12 months); AD (n = 20) and healthy individuals (n = 20) used as reference. The MCI EVOO – 1 month group was included for one main reason: to demonstrate that the effects of EVOO consumption are not rapid (samples acquired 1 month after the beginning of EVOO administration). The purpose of the MCI Control – 1 month group is to serve as the baseline group. The MCI Control group did not receive any treatment, including any other type of EVOO or placebo, and was naïve to the EVOO administered throughout the study. No group was asked to stop consuming olive oil during the 12-month duration of the study. All groups were allowed to continue their previous lifestyle, without dietary restrictions or additions, with the exception of the "EVOO" treatment group, which additionally received 50 ml of EVOO on a daily basis over the course of 1 year. The daily amount of raw EVOO (50 ml) administered to patients was divided equally over three meals throughout the day (main dish at lunchtime, side dish 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. One of the outcomes of the clinical trial concerns significant changes in body weight, which so far have not been observed as a consequence of EVOO administration. Patients with B12 and folic acid deficiency, hypothyroidism and other diseases connected with dementia were excluded from the study. The characteristics of the demographic data of the participants are shown in Tables 1 and 2. A full description of the MICOIL clinical trial can be found at: https://clinicaltrials.gov/ct2/show/NCT03362996. A full description of the EVOO content used in the MICOIL study is provided in Table S1.

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

Demographics	MCI control-1 month	MCI control-12 months	MCI EVOO-1 month	MCI EVOO -12 months	Healthy	AD
Participants	20	20	20	20	20	20
Age	72.30 ± 1.310	72.33 ± 1.497	70.73 ± 1.468	71.07 ± 1.316	76.56 ± 0.9441	81.40 ± 1.042
Gender male/ female	10M/ 10F	9M/ 11F	10M/ 10F	10M/ 10F	10M/ 10F	10M/ 10F
Education	9.300 ± 0.9151	9.810 ± 0.7826	9.538 ± 0.6731	9.821 ± 0.6321	8.813 ± 0.9670	6.900 ± 0.7101

Table 2
Statistical significance of demographic and clinical characteristics of the tested individuals.

p-Values	Healthy vs Alzheimer	Healthy vs MCI Control-1 month	Healthy vs MCI Control-12 months	Healthy vs MCI EVOO-1 month	Healthy vs MCI EVOO-12 months
Age	0.0567	0.1581	0.1563	0.0183	0.0263
Education	0.2895	0.9918	0.8524	0.9439	0.8172

2.2. Serum samples

Blood specimens from the participating individuals were centrifuged at 4000 rpm for 4 min to isolate the serum. Serum samples were kept at −80 °C for further analysis.

2.3. Chemicals

Polystyrene 96 well-plates were provided by Greiner BioOne (Greiner BioOne, Germany). *p*-Nitrophenyl phosphate, Tween-20, malondialdehyde (MDA), diethanolamine, thiobarbituric acid (TBA), trichloroacetic acid (TCA), were obtained by Sigma-Aldrich (St. Louis, MO, USA).

2.4. Enzyme-linked immunosorbent assay (ELISA)

Serum BMI1, p53, tau, p-tau, Aβ1–42, Aβ1–40, TNF-a, and IL-6 protein levels in individuals of all groups were measured using the ELISA method. ELISA experiments were performed using the following kits, according to the manufacturers' instructions: Human Polycomb complex protein BMI1(BMI1) ELISA kit (Cusabio, CSB-EL002726HU), Human P53 ELISA Kit (MyBioSource, MBS355295), Tau/MAPT (Human) ELISA Kit (BioVision, K4263), Human phospho Tau Protein ELISA Kit (MyBioSource, MBS164899), Human TNF alpha ELISA Kit (Abcam, ab181421), Human IL-6 Immunoassay Quantikine® ELISA (R&D Systems, D6050), Amyloid Beta 42 (Human) ELISA Kit (BioVision, E4288-100), Human Amyloid β (aa1–40) Immunoassay Quantikine® ELISA (R&D Systems, DAB140B).

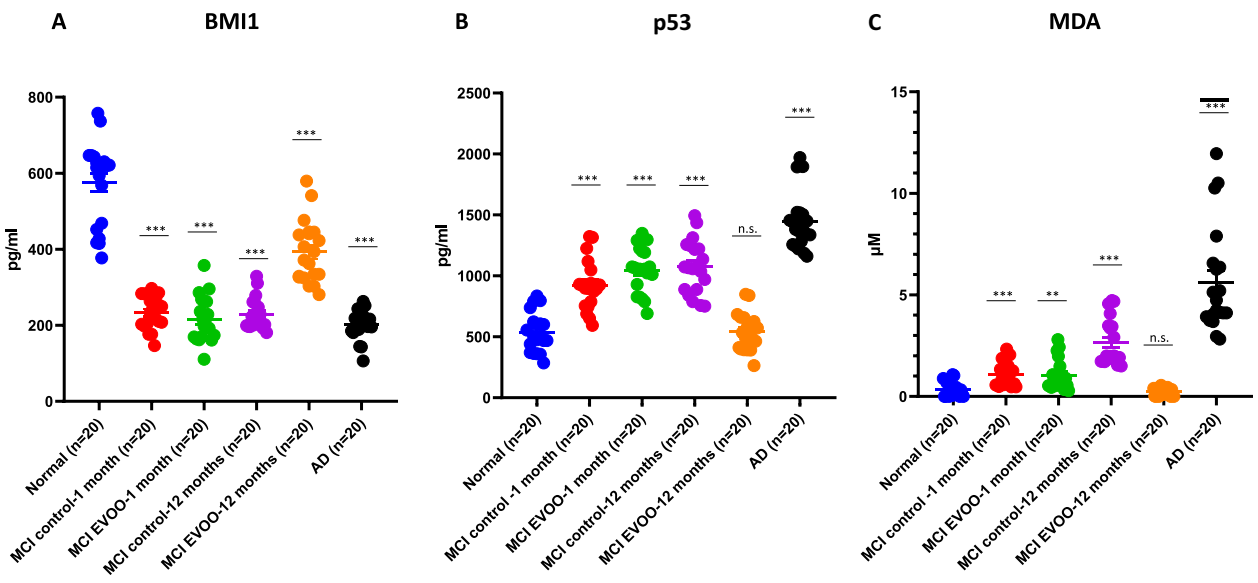

Fig. 1. EVOO treatment increases BMI1, and reduces p53 and oxidative stress levels.
A. Concentration of the BMI1 protein in the serum of indicated samples, estimated by ELISA. B. Concentration of the p53 protein in the serum of indicated samples, estimated by ELISA. C. MDA (in μM) concentration was measured in the serum of the indicated groups. All results are expressed as mean ± SEM. The statistical analysis was derived by comparing each patient group to healthy individuals (Normal). *** represents $p < 0.001$, ** represents $p < 0.01$, ns stands for non-significant and represents $p > 0.05$. See also Tables 3–5 for between groups statistical analyses.

2.5. Immunoblotting

Protein concentrations in serum samples were determined using a NanoDrop 2000 Spectrophotometer (Thermo Fisher). Western blotting was carried out as previously described (Papaspyropoulos et al., 2018), however signal intensity was assessed via alkaline phosphatase staining according to previously published protocols (Papaneophytou et al., 2012). The following primary antibodies were used at a concentration of 1:1000: β-actin (sc-130300); BMI1 (sc-390443, F-9) and p53 (sc-263, Bp53-12). Secondary goat anti-mouse IgG conjugated to alkaline phosphatase (A-2429) was purchased by Sigma-Aldrich (St. Louis, MO, USA). Nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and *p*-nitrophenyl phosphate were purchased by Sigma-Aldrich. Densitometry was carried out using the ImageJ 1.53a software.

2.6. MDA determination as oxidative stress biomarker before and after EVOO therapy

Malondialdehyde (MDA) derives from lipid peroxidation of polyunsaturated fatty acids and is a marker of oxidative stress in organisms. MDA levels in serum were measured as previously described (Chowdhury et al., 2017).

2.7. Statistical analysis

For all experiments reported in this manuscript, statistical significance was determined by Student's *t*-test. All values provided are expressed as the mean ± standard error of the mean (SEM). All analyses were performed using GraphPad Prism 8 and Microsoft Excel 2017. To minimize interpretation bias, randomization and allocation concealment were applied for sample selection. All raw data are available upon reasonable request.

3. Results

3.1. BMI1 levels are rescued after treatment with EVOO

Previous research showed a reduction of BMI1 levels in human AD brain (Flamier et al., 2018). We assessed the effect of EVOO supplemented diet on BMI1 by measuring the levels of BMI1 protein in the serum of all patient groups by ELISA. The AD patient group showed the lowest BMI1 serum levels compared to healthy individuals ($p < 0.001$; AD vs Normal), and in the MCI control-12 months group BMI1 levels were higher compared to the AD patient group ($p = 0.044$; MCI control – 12 months vs AD) (Fig. 1A and Table 3). By assessing serum BMI1 levels in MCI patients without and with EVOO consumption 12 months later, interestingly, we found that BMI1 levels in the MCI EVOO-12 months group were significantly higher compared to any other MCI patient group ($p < 0.001$) (Table 3). The effect of EVOO treatment on BMI1 levels in patient serum was also confirmed by Western blotting (Figs. 2 and S1).

Table 3
Between groups statistical analysis for BMI1 levels reported in Fig. 1A.

BMI1	MCI Control – 1 month	MCI EVOO – 1 month	MCI Control – 12 months	MCI EVOO – 12 months	AD
Normal	**p < 0.001**	**p < 0.001**	**p < 0.001**	**p < 0.001**	**p < 0.001**
MCI Control – 1 month		p = 0.283	p = 0.682	**p < 0.001**	**p = 0.019**
MCI EVOO – 1 month			p = 0.447	**p < 0.001**	p = 0.385
MCI Control – 12 months				**p < 0.001**	**p = 0.044**
MCI EVOO – 12 months					**p < 0.001**

Given the established neuroprotective role of BMI1 (Flamier et al., 2018), our results may imply a positive effect of EVOO conferred to the MCI treated groups through BMI1 restoration.

3.2. EVOO treatment reduces p53 and oxidative stress marker levels

It has been shown that BMI1 expression destabilizes p53 to exert a neuroprotective function in late-onset sporadic AD (Flamier et al.,

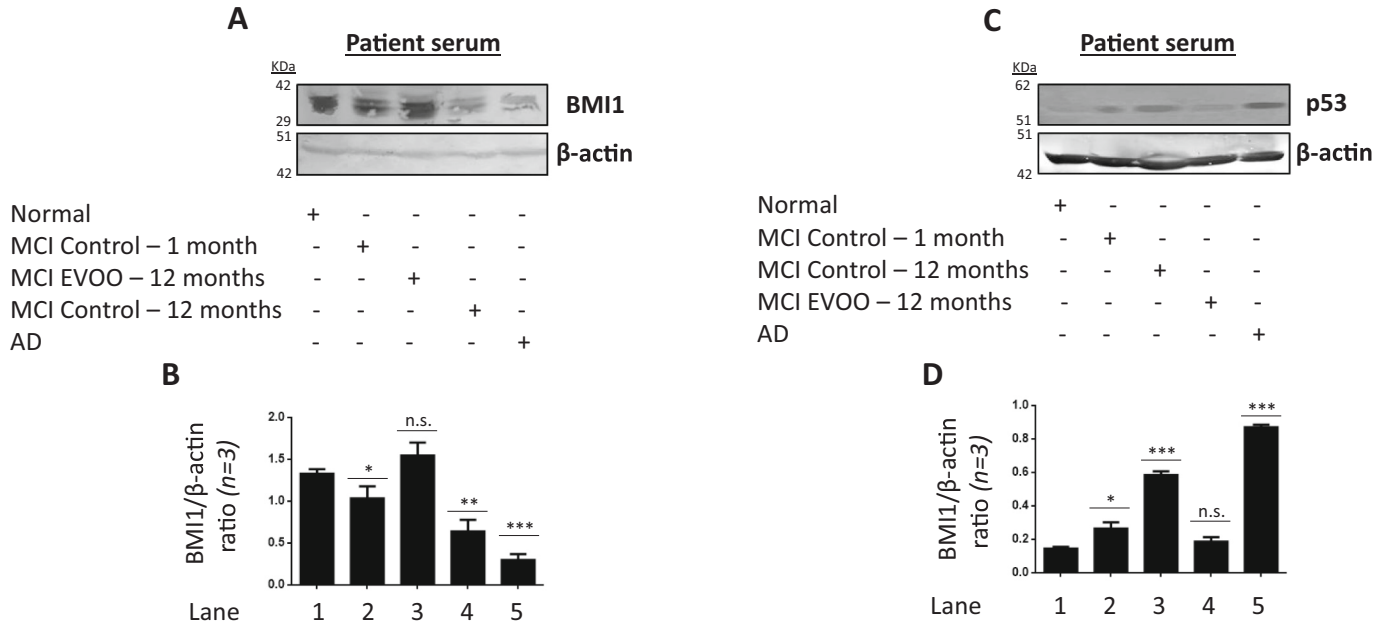

Fig. 2. Assessment of BMI1 and p53 levels in patient serum via Western blotting.
A. BMI1 protein levels in the serum of indicated samples, assessed by Western blotting. EVOO treatment for 12 months retained BMI1 levels at levels comparable to healthy individuals. B. Densitometry providing quantification for Western blots presented in A. C. P53 protein levels in the serum of indicated samples, estimated by Western blotting. EVOO treatment for 12 months maintained p53 levels low and comparable to healthy individuals. D. Densitometry providing quantification for Western blots presented in C. All results are expressed as mean ± SEM. Data shown are representative of 3 independent experiments (n = 3 subjects from each patient group). The statistical analysis was derived by comparing each patient group to healthy individuals (Normal). ***p < 0.001, **p < 0.01, *p < 0.05, and n.s. stands for non-significant (p > 0.05).

Table 4
Between groups statistical analysis for p53 levels reported in Fig. 1B.

P53	MCI Control – 1 month	MCI EVOO – 1 month	MCI Control – 12 months	MCI EVOO – 12 months	AD
Normal	**p < 0.001**	**p < 0.001**	**p < 0.001**	p = 0.966	**p < 0.001**
MCI Control – 1 month		p = 0.059	**p = 0.027**	**p < 0.001**	**p < 0.001**
MCI EVOO – 1 month			p = 0.601	**p < 0.001**	**p < 0.001**
MCI Control – 12 months				**p < 0.001**	**p < 0.001**
MCI EVOO – 12 months					**p < 0.001**

Table 5
Between groups statistical analysis for MDA levels reported in Fig. 1C.

MDA	MCI Control – 1 month	MCI EVOO – 1 month	MCI Control – 12 months	MCI EVOO – 12 months	AD
Normal	**p < 0.001**	**p = 0.001**	**p < 0.001**	p = 0.269	**p < 0.001**
MCI Control – 1 month		p = 0.881	**p < 0.001**	**p < 0.001**	**p < 0.001**
MCI EVOO – 1 month			**p < 0.001**	**p < 0.001**	**p < 0.001**
MCI Control – 12 months				**p < 0.001**	**p < 0.001**
MCI EVOO – 12 months					**p < 0.001**

2018). For this purpose, given that BMI1 levels were higher in the MCI EVOO-12 month group, we investigated the effect of EVOO consumption on p53 protein levels in the serum. We found that the MCI EVOO-12 month patient group displayed non-significant differences in p53 levels compared with the healthy individuals group (p = 0.966; MCI EVOO-12 month group vs Normal) (Figs. 1B, 2C, D and Table 4). In contrast, p53 serum levels were significantly higher in MCI patients receiving no EVOO therapy (p < 0.001; MCI Control-1 month and MCI Control-12 months vs Normal).

To assess the antioxidant effect of EVOO administration, the levels of the oxidative stress biomarker MDA were measured in serum using as readout spectrophotometry (Chowdhury et al., 2017) (Fig. 1C). MDA levels, before and after yearly EVOO consumption by MCI patients were assessed based on an MDA standard curve. We demonstrated that MCI patients had elevated MDA levels compared to healthy individuals (p < 0.001; MCI Control-1 month vs Normal), whereas MCI patients following one year EVOO therapy showed non-significant differences in MDA levels compared to healthy controls (p = 0.269; MCI EVOO – 12 months vs Normal) (Fig. 1C and Table 5). On the contrary, the MCI patient group receiving no EVOO had significantly higher levels of MDA one year later, compared to healthy controls (p < 0.001; MCI Control −12 months vs Normal) (Fig. 1C).

3.3. EVOO treatment reduces AD hallmark features

We next assessed the effect of EVOO-enriched diet on tau through the measurement of soluble protein levels and phosphorylated fractions in the serum. Tau levels were found elevated in AD patients, in line with other studies (Iqbal et al., 2005). As expected, the MCI patient group displayed an overall lower level of tau protein, compared to that of the AD patient group (p < 0.001; MDA Control – 12 months vs AD), which was not significant compared to normal individuals (p = 0.725; MDA Control - 12 months vs Normal) (Fig. 3A and Table 6). Tau levels of MCI patients on EVOO treatment were also not significantly different

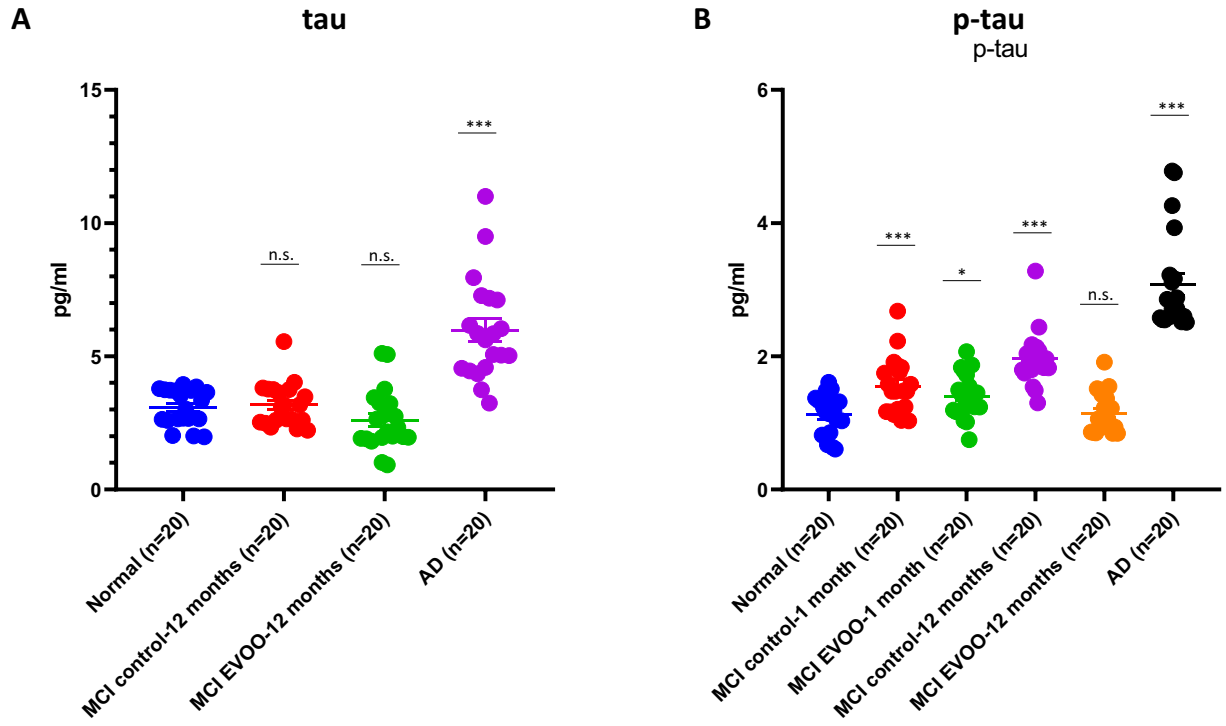

Fig. 3. P-tau levels are rescued in patients on EVOO treatment.
A. Concentration of total tau protein in the serum of indicated samples, estimated by ELISA. B. Concentration of the p-tau protein fraction in the serum of indicated samples, measured by ELISA. All results are expressed as mean ± SEM. The statistical analysis was derived by comparing each patient group to healthy individuals (Normal). *** represents $p < 0.001$, * represents $p < 0.05$ and ns stands for non-significant and represents $p > 0.05$. See also Tables 6 and 7 for between groups statistical analyses.

Table 6
Between groups statistical analysis for tau levels reported in Fig. 3A.

Tau	MCI Control – 12 months	MCI EVOO – 12 months	AD
Normal	p = 0.725	p = 0.102	**p < 0.001**
MCI Control – 12 months		p = 0.071	**p < 0.001**
MCI EVOO – 12 months			**p < 0.001**

Table 7
Between groups statistical analysis for p-tau levels reported in Fig. 3B.

p-Tau	MCI Control – 1 month	MCI EVOO – 1 month	MCI Control – 12 months	MCI EVOO – 12 months	AD
Normal	**p < 0.001**	**p = 0.018**	**p < 0.001**	p = 0.819	**p < 0.001**
MCI Control – 1 month		p = 0.218	**p = 0.003**	**p = 0.001**	**p < 0.001**
MCI EVOO – 1 month			**p < 0.001**	**p = 0.014**	**p < 0.001**
MCI Control – 12 months				**p < 0.001**	**p < 0.001**
MCI EVOO – 12 months					**p < 0.001**

compared to normal individuals (p = 0.102; MCI EVOO – 12 months vs Normal) (Fig. 3A and Table 6), implying that EVOO treatment may not directly affect tau levels in the serum.

Given that tau levels remained unchanged in response to EVOO treatment, we subsequently assessed p-tau protein levels in the serum, as tau hyperphosphorylation has been considered to be responsible for its loss of normal physiological function, gain of toxicity and its aggregation to form NFTs (Miao et al., 2019). P-tau levels were again found significantly increased in AD patients compared to healthy controls as expected (Fig. 3B). MCI control patients displayed increased p-tau levels ($p < 0.001$ in comparison with healthy controls), which were rescued in MCI patients receiving yearly EVOO (Fig. 3B and Table 7). Interestingly, p-tau levels in the MCI EVOO – 12 month group were not significantly different from healthy individuals (p = 0.819; MCI EVOO – 12 months vs Normal), implying that EVOO treatment may selectively reduce toxic p-tau rather than total tau levels. Of interest, EVOO administration for even 1 month conferred a significant decrease in p-tau levels in MCI patients compared to healthy individuals (p = 0.018; MCI EVOO-1 month vs Normal) (Fig. 3B and Table 7).

Previous research showed that reduction of Aβ1–42 and Aβ1–40 plasma levels was associated with cognitive decline, indicating AD manifestation (Janelidze et al., 2016). To further investigate the effects of EVOO on hallmarks of AD, we assessed amyloidosis in our clinical samples.

We found that Aβ1–42 and Aβ1–40 levels were elevated in normal individuals compared to any other group (Fig. 4A). In the serum of MCI EVOO – 12 months patients we observed a significantly higher concentration of Aβ1–42 species than MCI control counterparts ($p < 0.001$; MCI EVOO – 12 months vs MCI Control – 12 months) (Fig. 4A and Table 8). MCI Control and MCI EVOO groups displayed insignificant differences in Aβ1–40 levels (p = 0.597; MCI Control – 12 months vs MCI EVOO – 12 months) which were, however, significantly lower compared to healthy individuals ($p < 0.001$) (Fig. 4B and Table 9), while Aβ1–40 levels in the AD group were higher than those of the MCI groups (p = 0.002, AD vs MCI Control – 12 months; p = 0.003, AD vs MCI EVOO – 12 months) (Fig. 4B and Table 9). EVOO treatment, however, significantly restored the Aβ-42/Aβ-40 ratio in MCI patients, as indicated by

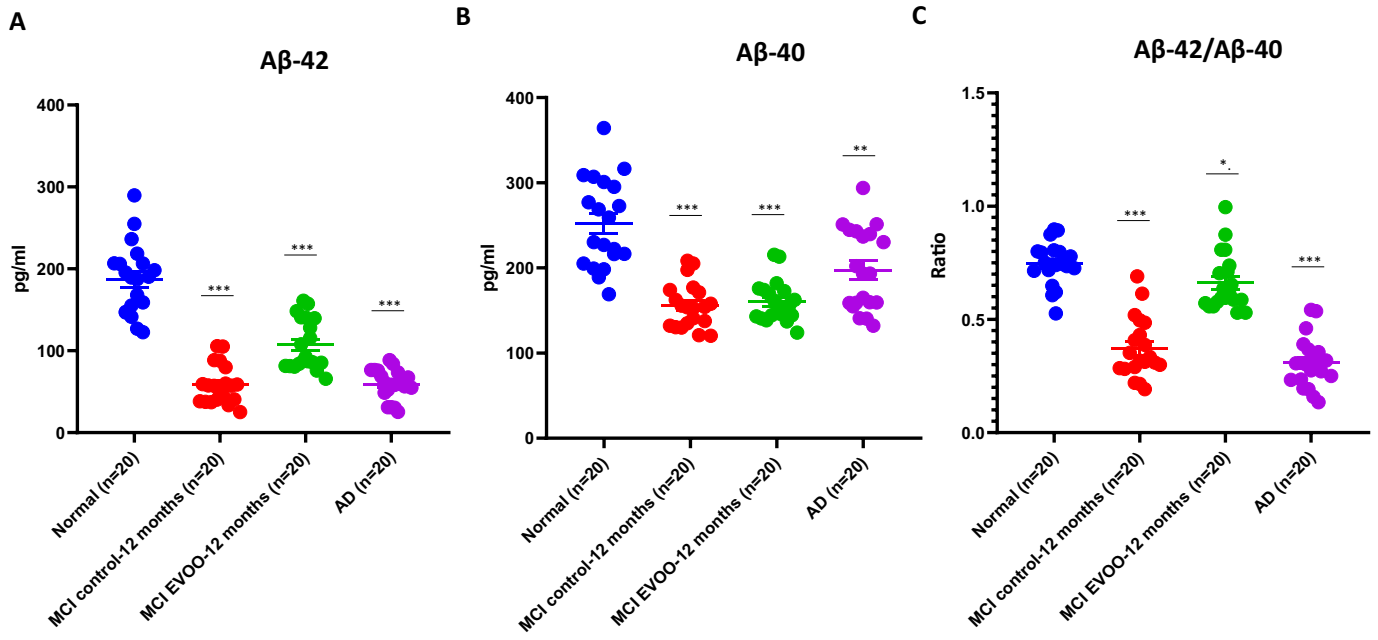

Fig. 4. EVOO treatment reverses key AD biochemical features.
A. Concentration of the Aβ1–42 protein in the serum of the tested patient groups measured by ELISA. B. As in A, but for the Aβ1–40 fragment. C. Calculation of the Aβ-42/Aβ-40 ratio for each patient group based on the values obtained in panels A and B. All results are expressed as mean ± SEM. The statistical analysis was derived by comparing each patient group to healthy individuals (Normal). *** represents $p < 0.001$, ** represents $p < 0.01$ and * represents $p < 0.05$. See also Tables 8–10 for between groups statistical analyses.

Table 8
Between groups statistical analysis for Aβ-42 levels reported in Fig. 4A.

Aβ-42	MCI Control – 12 months	MCI EVOO – 12 months	AD
Normal	**p < 0.001**	**p < 0.001**	**p < 0.001**
MCI Control – 12 months		**p < 0.001**	p = 0.926
MCI EVOO – 12 months			**p < 0.001**

Table 9
Between groups statistical analysis for Aβ-40 levels reported in Fig. 4B.

Aβ-40	MCI Control – 12 months	MCI EVOO – 12 months	AD
Normal	**p < 0.001**	**p < 0.001**	**p = 0.001**
MCI Control – 12 months		p = 0.597	**p = 0.002**
MCI EVOO – 12 months			**p = 0.003**

Table 10
Between groups statistical analysis for Aβ-42/Aβ-40 ratio levels reported in Fig. 4C.

Aβ-42/Aβ-40	MCI Control – 12 months	MCI EVOO – 12 months	AD
Normal	**p < 0.001**	**p = 0.022**	**p < 0.001**
MCI Control – 12 months		**p < 0.001**	p = 0.111
MCI EVOO – 12 months			**p < 0.001**

comparing the Aβ species ratio of MCI patients to that of healthy individuals ($p = 0.022$, MCI EVOO - 12 months vs Normal; $p < 0.001$, MCI Control – 12 months vs Normal) (Fig. 4C and Table 10), implying an overall ameliorating effect of the EVOO treatment on AD biochemical hallmark features. This is in accordance with studies supporting the ability of EVOO to facilitate Aβ clearance and decrease aggregate formation (Abuznait et al., 2013; Qosa et al., 2015b).

3.4. EVOO administration restricts inflammation in MCI patients

Previous research has shown that, contrary to Bmi1 downregulation, interleukin-6 (Il-6) expression was found elevated in the brain of aged versus young mice (Abdouh et al., 2012). Inflammation plays a critical role in the onset of neurodegenerative diseases (Chitinis and Weiner, 2017), and a hallmark of brain senescence is IL-6, a pro-inflammatory cytokine that participates in the pathogenesis of nearly all inflammation-associated diseases, including AD, where its levels are increased in patients (Rodier et al., 2009; Wyss-Coray, 2006).

It has been found that EVOO with high phenolic content may have the potential to act as an antioxidant and anti-inflammatory factor (Bayram et al., 2012; Rigacci et al., 2016). To this end, we measured two hallmarks of neuroinflammation, TNF-α and IL-6, in response to EVOO administration to MCI patients.

By implementing ELISA in patient serum, we confirmed that AD patients had the highest IL-6 levels compared to all other groups (Fig. 5A). Our analysis showed that MCI control patients had significantly elevated levels of IL-6 compared to healthy individuals ($p = 0.001$, MCI Control – 1 month vs Normal; $p < 0.001$, MCI Control – 12 months vs Normal), whereas in MCI patients subjected to EVOO treatment over a year, those levels were rescued ($p = 0.144$; MCI EVOO – 12 months vs Normal) (Fig. 5A and Table 11). Since IL-6 and TNF-α often show similar regulation in neurological disorders (Ng et al., 2018), we also assessed TNF-α levels in the same sample set. Indeed, we found that, following IL-6 regulation, MCI-EVOO patients had statistically lower TNF-α levels than the MCI control group 12 months after treatment ($p < 0.001$; MCI EVOO – 12 months vs MCI Control – 12 months) (Fig. 5B and

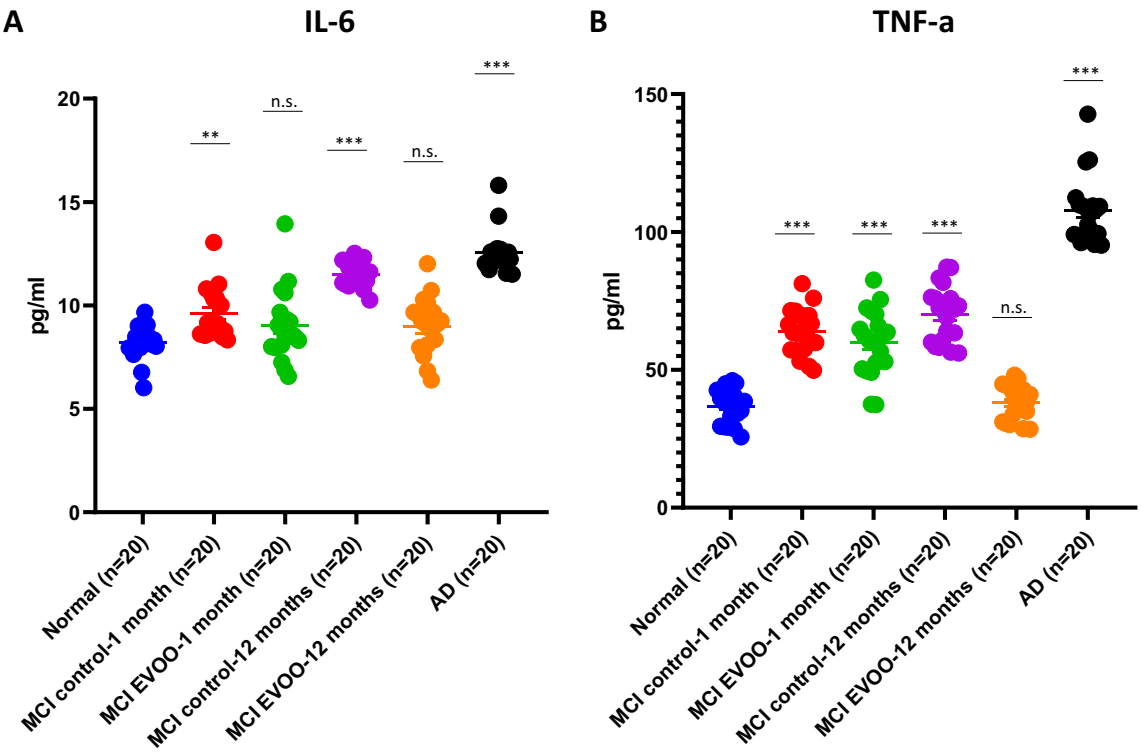

Fig. 5. Inflammation markers are reduced in patients receiving EVOO treatment.
A. Concentration of IL-6 protein levels assessed by ELISA in the serum of the indicated patient groups. B. Concentration of TNF-α levels assessed by ELISA in the serum of indicated samples. All results are expressed as mean ± SEM. The statistical analysis was derived by comparing each patient group to healthy individuals (Normal). *** represents $p < 0.001$, ** represents $p < 0.01$, n.s. stands for non-significant ($p > 0.05$). See also Tables 11 and 12 for between groups statistical analyses.

Table 11
Between groups statistical analysis for IL-6 levels reported in Fig. 5A.

IL-6	MCI Control – 1 month	MCI EVOO – 1 month	MCI Control – 12 months	MCI EVOO – 12 months	AD
Normal	**p = 0.001**	p = 0.177	**p < 0.001**	p = 0.144	**p < 0.001**
MCI Control – 1 month		p = 0.205	**p < 0.001**	p = 0.121	**p < 0.001**
MCI EVOO – 1 month			**p < 0.001**	p = 0.928	**p < 0.001**
MCI Control – 12 months				**p < 0.001**	**p < 0.001**
MCI EVOO – 12 months					**p < 0.001**

Table 12
Between groups statistical analysis for TNF-α levels reported in Fig. 5B.

TNF-α	MCI Control – 1 month	MCI EVOO – 1 month	MCI Control – 12 months	MCI EVOO – 12 months	AD
Normal	**p < 0.001**	**p < 0.001**	**p < 0.001**	p = 0.45	**p < 0.001**
MCI Control – 1 month		p = 0.203	**p = 0.044**	**p < 0.001**	**p < 0.001**
MCI EVOO – 1 month			**p = 0.005**	**p < 0.001**	**p < 0.001**
MCI Control – 12 months				**p < 0.001**	**p < 0.001**
MCI EVOO – 12 months					**p < 0.001**

Table 12). These results indicate that EVOO treatment may potentially exert neuroprotective effects in MCI patients additionally through suppression of inflammatory processes.

4. Discussion

Currently, discovery of less toxic or non-toxic phytochemicals targeting AD has attracted considerable attention in AD research. To the best of our knowledge, the MICOIL clinical trial is the first clinical trial that investigated the effect of one year administration of EVOO in MCI patients on several related parameters, including restoration of BMI1 levels. On the basis of our data, we showed that one year treatment of MCI patients with EVOO was accompanied by amelioration of biochemical hallmarks of neurodegeneration.

In this clinical study, we confirmed that BMI1 can be readily detected by ELISA not only in the serum of MCI and AD patients but also in that of healthy individuals, thus expanding its potential biomarker use in different disease settings. BMI1 levels decrease in MCI and AD patients compared to healthy individuals, in line with data suggesting that BMI1 is silenced in AD brains (Flamier et al., 2018). Our observations imply that EVOO treatment may actively reduce oxidative stress in MCI subjects, a finding of high importance considering the established correlation between oxidative stress and the early onset of AD (Raskin et al., 2015). Along these lines, our results may further strengthen the hypothesis that BMI1 activation associates with p53 down-regulation to exert a neuroprotective role (Flamier et al., 2018), as upon EVOO administration, BMI1 increase correlated with p53 decrease. Although additional studies are required to delineate the mechanism through which EVOO may exert its functions through BMI1, our data indicate that administration of EVOO, which is a completely natural treatment,

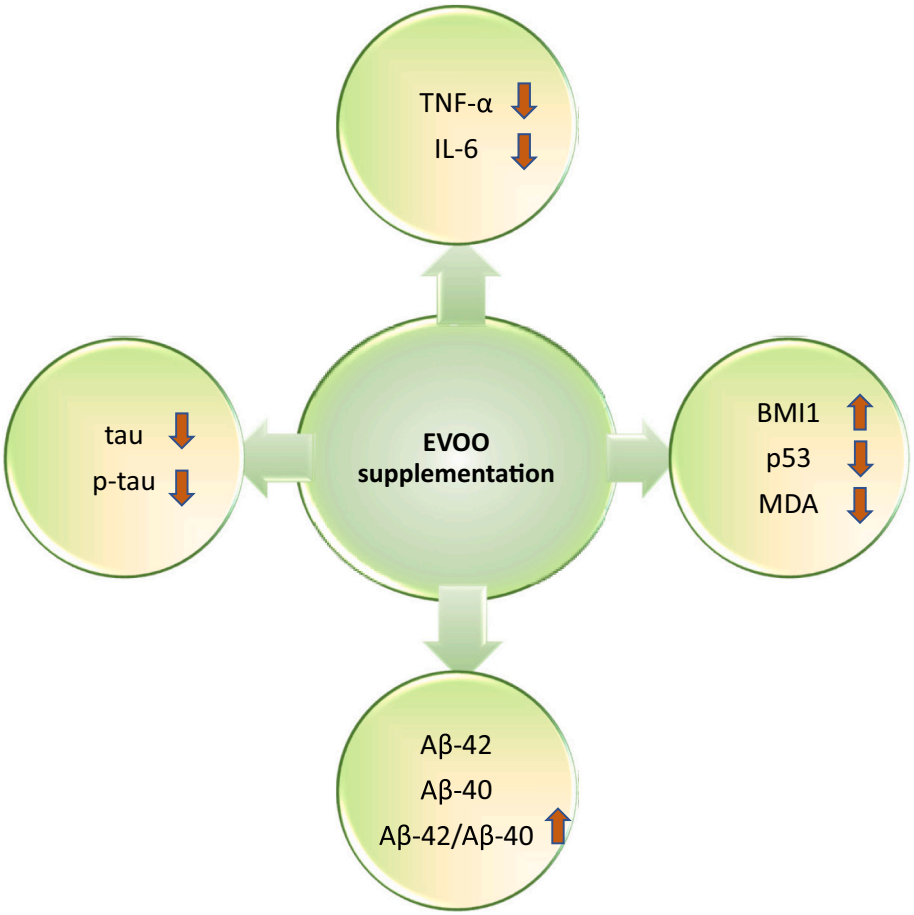

Fig. 6. Model depicting EVOO effect on BMI1 levels and neurodegeneration-related factors.
A 12-month EVOO treatment is sufficient to upregulate BMI1 levels in the serum of MCI patients, correlating with p53 downregulation and reduction of oxidative stress levels. Additionally, the EVOO treatment decreases inflammation factors as indicated by downregulation of key markers IL-6 and TNF-α.

may be sufficient to restore, at least partially, several markers of neurodegeneration and hallmarks of AD.

BMI1 is mechanistically required to repress microtubule-associated protein tau (MAPT) transcription and hinder p53 stabilization, thereby preventing neurodegeneration (Flamier et al., 2018). Additionally, *BMI1* inactivation in human post-mitotic neurons resulted in secretion and deposition of Aβ species, p-Tau accumulation, and ultimately, neurodegeneration (Flamier et al., 2018). The p53-mediated BMI1 activity against neuronal apoptosis may be closely connected to AD; while p53 expression is augmented in AD brains, and its transcription is activated by the intracellular levels of Aβ1–42, p53 inhibition impedes Aβ1–42-induced neuronal cell death (Zhang et al., 2002; Ohyagi et al., 2005). Our results showing that EVOO administration rescues the manifestation of AD-related factors, as quantified in patient serum, may suggest an underlying mechanism mediated by EVOO, which warrants further investigation.

As the detectable BMI1 and p53 serum levels may also derive from peripheral organs and nucleated blood cells, the EVOO-mediated regulation of BMI1 and p53 could be potentially investigated in tissues other than the brain. For example, BMI1 has been found to be secreted in cancer prostate patient blood (Siddique et al., 2013; Zhang et al., 2017). As vascular transport across the blood brain barrier has been shown to be the main route for rapid elimination of Aβ species in the brain (Shibata et al., 2000), this is likely the pathway through which EVOO may therapeutically target AD. Additionally, patients with early AD display leakage in the blood brain barrier, thus increasing the permeability of substance exchange between the CSF and blood (van de Haar et al., 2017), which explains the presence of AD biomarkers in the blood. P53 has been also shown to be transferred by exosomes between cells (Burdakov et al., 2018). Taken together, the observed BMI1 and p53 level changes in the blood in response to EVOO treatment should be, at least to a large extend, relevant to changes in the brain pathophysiology.

Mice that consumed EVOO for 3 months after the first appearance of Aβ plaques, showed diminished Aβ aggregation. EVOO potentially reduced brain Aβ by enforcing the Aβ clearance across the blood-brain barrier and by lowering Aβ production via a modulated process of APP (Qosa et al., 2015a; Qosa et al., 2015b).

Oxidative stress occurs in early stages of the disease, before the detection of the standard histological hallmarks of AD (Reddy, 2011). Increased BMI1 expression is highly neuroprotective resulting in activation of antioxidant defenses and suppression of ROS levels (Abdouh et al., 2012). Additionally, oxidative stress and free radicals bring into action β-secretase expediting the cleavage of APP to Aβ (Reddy and Beal, 2008). Thus, keeping in mind the direct correlation between oxidative stress and the early onset of AD (Raskin et al., 2015), EVOO seems to be implicated in many pathways of oxidative stress affecting AD pathology.

Published reports on Aβ plasma concentrations (Toledo et al., 2013; Yang et al., 2020) have shown that Aβ40 species are significantly more abundant than their Aβ42 counterparts in human serum. Our dataset demonstrates that Aβ42 levels are indeed lower than Aβ40 levels in MCI Control (~3 times on average) and AD patients (~4 times on average), as well as in healthy individuals (~1.5 times on average). Of note, Aβ40 levels have been reported to range from ~200 pg/ml (Fagan et al., 2009) to ~1000 pg/ml (Arvanitakis et al., 2002), whereas the respective Aβ42 levels have been shown to range from ~30 pg/ml (Fagan et al., 2009) to ~150 pg/ml (Roher et al., 2009), and the wide discrepancies in serum

concentrations have been attributed to the use of different antibodies and analytical platforms among research groups (Toledo et al., 2013). Our observed values for both amyloid markers mostly fall within previously reported concentration ranges in human serum.

Although BMI1 has been shown to be lost in the AD brain (Flamier et al., 2018), to our knowledge, quantification of BMI1 levels in human blood has never been used as a biomarker for AD. Similarly to BMI1, P53 serum levels have not been implemented as an AD biomarker, although unfolded P53 has been widely investigated in that context (Stanga et al., 2010). High MDA serum levels, on the other hand, have been widely linked to AD manifestation (Rani et al., 2017; Torres et al., 2011). Although observations vary, MDA concentrations have been roughly estimated around 1.5 μM in healthy individuals and around 2.5 μM in AD patients (Rani et al., 2017). In line with our observations, MDA concentrations in MCI patients are twice as high as those of healthy individuals, while AD patients display three-fold MDA concentrations compared to healthy controls (Torres et al., 2011).

Inflammation is a driving force of the onset of neurodegenerative diseases and a hallmark of brain senescence is considered to be interleukin-6 (IL-6), a secretory inflammatory cytokine, whose levels are indeed increased in AD (Rodier et al., 2009; Wyss-Coray, 2006) due to the Aβ plaques (Rubio-Perez and Morillas-Ruiz, 2012). We showed that a yearly EVOO treatment may prevent the increase of IL-6 and TNF-α levels, which again correlates with BMI1 restoration. It was previously reported that higher levels of Il-6, and lower levels of Bmi1, were found together in aged versus young mouse brains (Abdouh et al., 2012).

Our findings may imply that a natural product may potentially halt the progression of MCI towards AD (Fig. 6). A backbone study administering EVOO to MCI or AD patients for longer periods is expected to provide additional evidence regarding the long-term protective effects of EVOO against several forms of neurodegeneration. In line with our biochemical findings, our recent work investigating for the first time the effect of early harvest EVOO in MCI patients, with regards to cognitive function, showed that patients on EVOO treatment exhibited a better performance in almost all cognitive domains compared to MCI patients following only Mediterranean diet instructions (Tsolaki et al., 2020).

In conclusion, we suggest that this study has a considerable translational potential, however, warrants further investigation in a bigger cohort of human patients. Our study implies that EVOO, a completely natural product, may potentially act as an alternative therapy for preventing AD onset. Restoration of BMI1 activity by the proposed one year supplementation could represent a therapeutic modality for AD, hence having an important impact on the design of future therapeutic strategies for AD prevention.

CRediT authorship contribution statement

Elena E. Tzekaki: Methodology, Validation, Formal analysis, Investigation, Writing-original draft. **Angelos Papaspyropoulos:** Methodology, Validation, Formal analysis, Investigation, Writing-review & editing. **Magda Tsolaki:** Conceptualization, Resources, Supervision. **Eftychia Lazarou:** Investigation, Resources. **Mahi Kozori:** Investigation, Resources. **Anastasia A. Pantazaki:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision.

Declaration of competing interest

There are no conflicts of interest to declare.

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Appendix A. Supplementary data

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