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The Anti-Neuroinflammatory Effect of Extra-Virgin Olive Oil in the Triple Transgenic Mouse Model of Alzheimer's Disease

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Abstract.
Background: Chronic intake of extra virgin olive oil is beneficial for brain health and protects from age-related cognitive decline and dementia, whose most common clinical manifestation is Alzheimer's disease. Besides the classical pathologic deposits of amyloid beta peptides and phosphorylated tau proteins, another frequent feature of the Alzheimer's brain is neuroinflammation.
Objective: In the current study, we assessed the effect that extra virgin olive oil has on neuroinflammation when administered to a mouse model of the disease.
Methods: Triple transgenic mice were randomized to receive a diet enriched with extra virgin olive oil or regular diet for 8 weeks. At the end of this treatment period the expression level of several inflammatory biomarkers was assessed in the central nervous system.
Results: Among the 79 biomarkers measured, compared with the control group, mice receiving the extra virgin olive oil had a significant reduction in MIP-2, IL-17E, IL-23, and IL-12p70, but an increase in IL-5. To validate these results, specific ELISA kits were used for each of them. Confirmatory results were obtained for MIP-2, IL-17E, IL-23, and IL-12-p70. No significant differences between the two groups were observed for IL-5.
Conclusions: Our results demonstrate that chronic administration of extra virgin olive oil has a potent anti-neuroinflammatory action in a model of Alzheimer's disease. They provide additional pre-clinical support and novel mechanistic insights for the beneficial effect that this dietary intervention has on brain health and dementia.

Keywords: Alzheimer's disease, cytokines, extra-virgin olive oil, Mediterranean diet, neuroinflammation, transgenic mice

INTRODUCTION

The extra virgin olive oil (EVOO) is one of the most peculiar components of the Mediterranean diet, whose health benefits have been widely demonstrated in human studies as well as preclinical investigations.

In humans, the reported health benefits of EVOO range from a reduction in cardiovascular diseases and related events and morbidities, to a longer life [1, 2]. Interestingly, in recent years emerging evidence has also demonstrated that adherence to the Mediterranean diet, which among other components

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includes daily EVOO intake, confers significant benefits for brain health which is associated with better cognitive performance and a reduced risk to develop Alzheimer's disease (AD) and related dementias [3].

The benefit of EVOO on brain health is also well documented by studies showing that a chronic daily intake of this food prevents cognitive decline, improves functional brain connectivity and classical AD peripheral biomarkers [4]. Additionally, there is a large body of preclinical research showing that chronic administration of EVOO has ameliorating effects on the pathologic phenotype of various animal models of AD and premature aging. Thus, EVOO-treated mice had better results in learning and memory tests, lower brain amyloid-β peptides load, and tau phosphorylation when compared to untreated ones [5]. Other studies have demonstrated that in these very animal models EVOO also improves blood-brain barrier function, learning and memory deficits, reduces oxidative stress and activation of glia cells that have been implicated in the dysregulated neuroinflammatory response that characterize the neuropathology of AD [6, 7].

However, the effect of chronic dietary administration of EVOO on the neuroinflammatory biomarker signature of an AD brain has not been well characterized so far. In the current study, by implementing a microarray analytical approach we assessed the effect of EVOO on the expression levels of a large number of inflammatory biomarkers, which included cytokines, chemokines and some of their receptors, in the brain of a mouse model of AD that develops amyloid beta plaques and tau tangles [8].

MATERIALS AND METHODS

Experimental design

Five-month-old triple transgenic mice (3xTg-AD) were randomized in two groups one receiving standard chow diet (PicoLab Rident 20, Labdiet, St. Louis, MO) ($n = 7$; 2 males and 5 females), and the other receiving the same diet enriched with EVOO (50 mg/Kg diet) ($n = 7$; 3 males and 4 females). The two diets were always matched for calories. Fresh diet was made available to the animals 3 times per week. After 8 weeks treatment mice were euthanized. After perfusion, brains were harvested form each mouse, and cortex separated and immediately stored at –80°C for biochemical analysis.

Table 1
Phenolic composition of EVOO (mg/kg)*

	EVOO
Hydroxytyrosol (3,4-DHPEA)	2.4 ± 0.1
Tyrosol (p-HPEA)	2.6 ± 0.0
Vanillic acid	0.6 ± 0.0
Oleacein (3,4-DHPEA-EDA)	494.7 ± 0.5
Oleocanthal (p-HPEA-EDA)	153.2 ± 0.4
(+)-1-Acetoxypinoresinol	31.1 ± 0.0
(+)-Pinoresinol	20.6 ± 0.6
Oleuropein aglycone (3,4-DHPEA-EA)	178.3 ± 0.4
Listroside aglycone	21.8 ± 0.8
Total phenol	905.2 ± 1.3

*The results are the mean of two determinations ± the standard deviation.

Table 2
Fatty acid profile (%) of EVOO*

	EVOO
Myristic acid	0.01 ± 0.0009
Palmitic acid	11.83 ± 0.30
Palmitoleic acid	0.45 ± 0.03
Heptadecanoic acid	0.07 ± 0.003
Heptadecenoic acid	0.08 ± 0.004
Stearic acid	2.51 ± 0.10
Oleic acid	76.79 ± 0.68
Linoleic acid	6.91 ± 0.18
Linolenic acid	0.68 ± 0.01
Arachidic acid	0.37 ± 0.02
Eicosenoic acid	0.31 ± 0.01
Behenic acid	n.d.
Lignoceric acid	n.d.

*The results are the mean of two determinations ± the standard deviation.

This study was approved by the Institutional Animal Care & Use Committee of Temple University according to the Laboratory Animal Welfare Guidelines of the American Veterinary Medical Association.

EVOO used for the study

Olive fruits (*Olea europaea* L.) from a Coratina cultivar used to produce the EVVO added to the study's diet were from an olive grove located in the municipality of Andria, Puglia region, Italy. The product showed low levels of free fatty acid (0.21%), peroxides (4.0), and extinction indices (K232 : 1.655; K270 : 0.136; ΔK: –0.002), and high concentration of natural bioactive phenols such as Oleacein, Oleocanthal, and Oleuropein as reported in Table 1. In addition, it contained high level of oleic acid with more than 70% of the overall fatty acid composition, as reported on Table 2 [9, 10].

Tissue preparation and cytokine array

Radioimmunoprecipitation assay (RIPA) buffer extracts from brain cortices were used for Quantibody Mouse Inflammation Array Q 1 kit, and Quantibody Mouse Cytokine Array Q 4 kit (Ray Biotech Life, Inc.), which detect 40 inflammatory biomarkers each. Considering that one of them is present in both kits the final total number of biomarkers assayed by the two kits was 79.

Briefly, protein was extracted in RIPA (Invitrogen, catalog #FNN0011) supplemented with a protease inhibitor (SCBT, catalog # sc-29130) and phosphatase inhibitor (Thermo Scientific, catalog # 78427) according to manufacturer's instructions. RIPA, 8 times in volume to the tissue weight, was added to the samples. Tissue was briefly sonicated followed by centrifugation at 17000 × g for 20 min. Supernatants were subjected to BCA analysis to determine protein concentration before using them to assess the levels of different biomarkers. Samples were shipped in dry ice to Ray Biotech Life, Inc., Peachtree Corner, GA, where the analysis was performed. Data obtained from the arrays was normalized to protein concentration before subjecting to statistical analysis by Raybiotech Life Inc. Significance analysis of microarrays (SAM analysis) was used to identify differentially expressed biomarkers (delta = 1.2) between the two groups. The fold change between the groups was calculated as the ratio of the mean or median.

ELISA analyses

To validate the cytokines that were altered in the array analysis we used different specific ELISA kits from commercial vendors. IL-17E kit was from Invitrogen (catalog # BMS6046), IL-12-p70 kit was from Bio-techne R&D Systems (catalog # M1270), IL-23 kit was from Invitrogen (catalog # BMS6017), IL-5 kit was from Bio-Techne R&D Systems (catalog # M5000), MIP-2 kit was from Abcam (catalog # ab204517). Each assay was performed by following the manufacturer's instructions.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). The two groups were always compared by using the unpaired two-tailed t test. The statistical significance was set at $p < 0.05$. The GraphPad software version 9 : 00 was used for the analyses.

RESULTS

Randomization and effect of EVOO-rich diet on body weight

Starting at five months of age, 3xTg-AD mice were randomized to receive regular chow diet, or a diet enriched with EVOO for 8 weeks. No significant body weight differences were observed between the two groups at the beginning of the study (control: 36.14 ± 1.41 g; EVOO: 36.84 ± 1.50 g; $n = 7$ mice per group). Similarly, no significant differences were noted at the end of the study between the mice on the regular diet versus the ones receiving the EVOO-rich diet (control: 35.65 ± 1.40 g, EVOO: 36.76 ± 1.50 g; $n = 7$ mice per group).

Cytokine array

At the end of the 8-weeks treatment, mice were euthanized and brain harvested, cortices isolated and used for biochemical analyses. Initially a total of 79 cytokines were assessed in brain cortex extracts from 3xTg-AD mice by using 2 commercially available kits from Ray Biotech Inc. (Mouse inflammation array Q1 and cytokine array Q4). Among them a total of 31 inflammatory biomarkers were differentially expressed based on statistical comparison, although they did not make the original cut-off (Figs. 1 and 2). No significant differences in these results were noted when we looked at the results separating males from females (not shown). To control for false positive results, we added a constraint of 2-fold minimum for increase and a 0.5-fold minimum for decrease in levels to our analytes. With this new approach we found that only IL-17E, IL-12p70, IL-23, MIP-2, and IL-28 were downregulated, while IL-5 and Leptin were elevated upon treatment with EVOO.

Cytokine validation

Individual commercially available ELISA kits were used to validate the results of 6 out of the 7 identified biomarkers. IL-28 was not included in the validation study due to the unavailability of a commercial ELISA kit for this cytokine. As shown in Fig. 3, compared with controls mice receiving diet enriched with EVOO had a significant reduction in the brain levels of IL-17E, MIP-2, IL-12p70, and IL-23. These findings confirmed the array results. By contrast with the finding of the array for IL-5 and leptin, the levels of these biomarkers measured by the

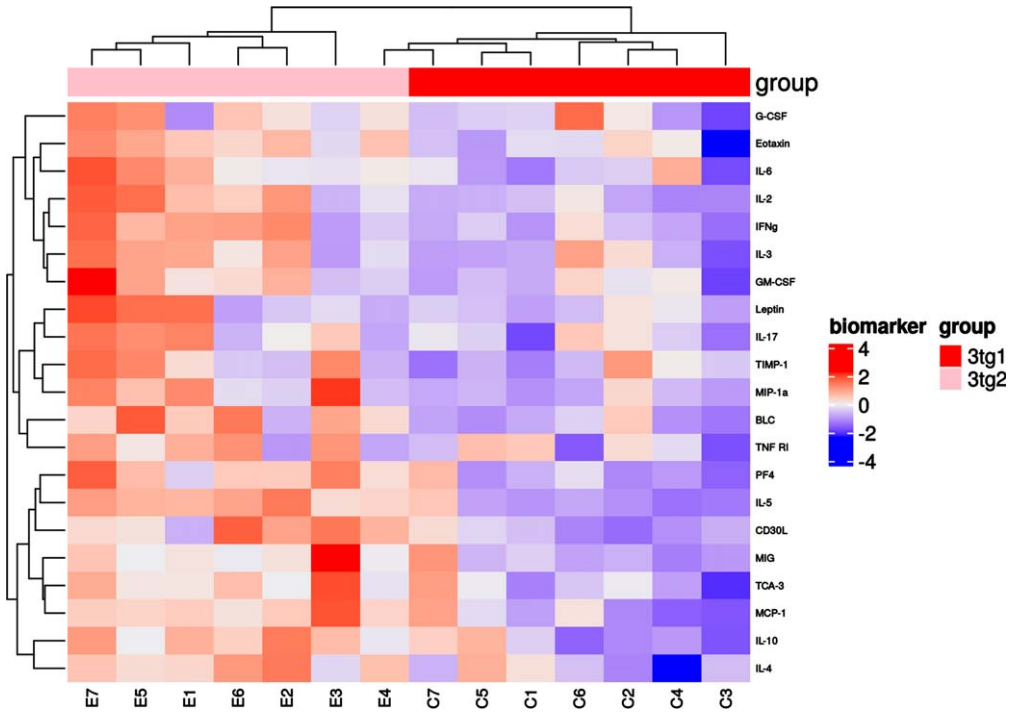

Fig. 1. Heatmap of 21 differentially expressed biomarkers between the control group (3xtg1) and EVOO-treated group (3xtg2).

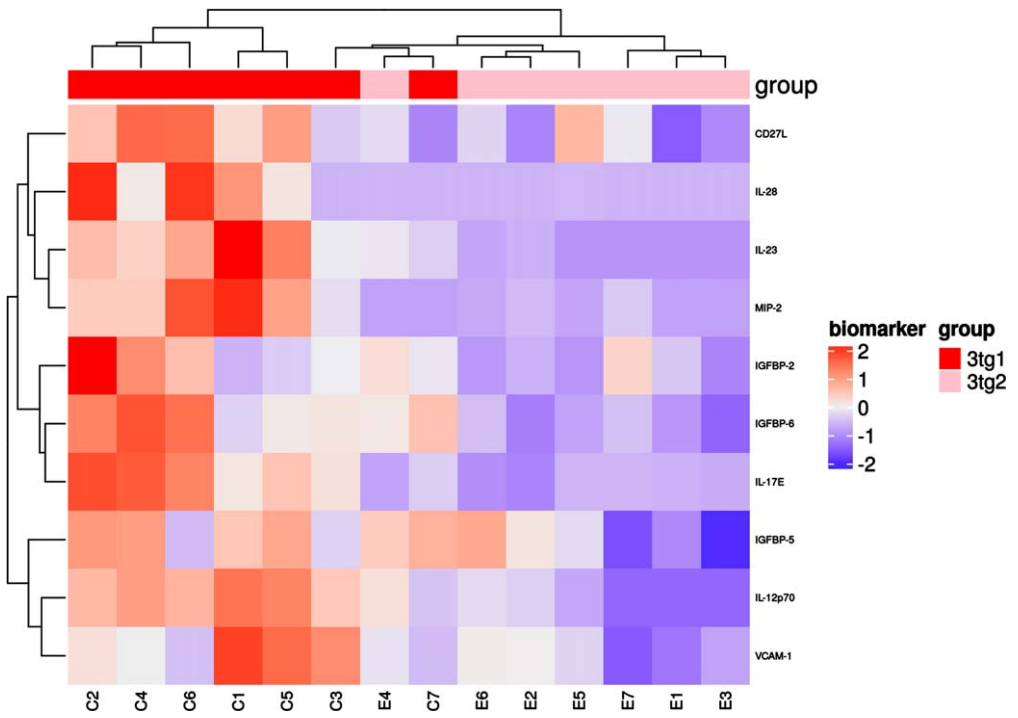

Fig. 2. Heatmap of 10 differentially expressed biomarkers between the 3xTg-AD control group (3tg1) and EVOO-treated group (3tg2).

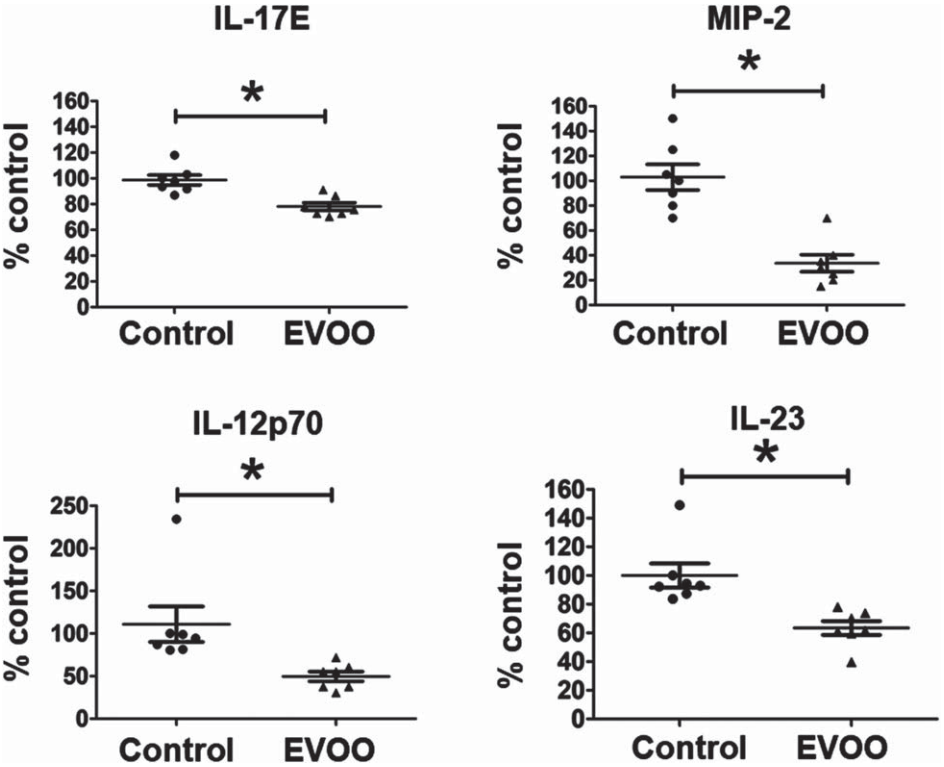

Fig. 3. Levels of IL-17E, MIP-2, Il-12p70 and IL-23 in brain cortex homogenates from 3xTg mice control or 3xTg-AD mice chronically treated with EVOO (*$p < 0.05$).

specific and sensitive ELISA kit in the EVOO-treated mice compared with the controls were not different (not shown).

DISCUSSION

EVOO is a mainstay of the Mediterranean diet which in recent years has gained attention and popularity among scientists and physicians because of its proven beneficial effects spanning from cardiovascular to brain health. Interestingly, a recent study with more than 35,000 patients followed for 28 years looked at all causes of mortality among the subjects enrolled who had high consumption of olive oil compared with the ones who did not. The results showed that olive oil intake was associated with a 19% reduction in cardiovascular diseases mortality, 17% lower risk of cancer mortality, 18% reduction in respiratory diseases mortality and 29% less risk of neurodegenerative diseases mortality [11]. Furthermore, other studies comparing groups of individuals chronically intaking EVOO versus groups that do not, have shown significant beneficial effect on cognitive decline and the risk to develop age-related dementia like AD [12]. We recently published a randomized clinical trial showing that 6-months treatment with EVOO in subject with mild cognitive impairment was sufficient to improve cognition and behavioral rating scores, blood-brain barrier permeability and enhance brain functional connectivity, an anatomical substrate for memory formation, retention, and retrieval [4].

Preclinical studies using mouse models of AD have also confirmed those beneficial effects on brain health and neurodegeneration. Thus, chronic EVOO intake improved brain amyloidosis both in the parenchyma and the vasculature, a condition commonly observed in human AD and known as cerebral amyloid angiopathy [5–7]. We reported that EVOO dietary administration had beneficial effect on the development of the behavioral impairments and tau neuropathology of a mouse model of human tauopathy (h-tau mice) [13]. Additionally, a similar dietary approach had a significant beneficial effect on the overall AD-like pathologic phenotype of the 3xTg-AD mice [14]. Among the various targets of EVOO *in vivo*, there are reports showing that it significantly reduces the reactive gliosis that is associated with neurodegenerative diseases like AD. This effect has

been considered functionally relevant and with therapeutic implications since neuroinflammation has been linked to the onset and the progression of AD. Interestingly, among the various genetic risk factors recently identified for AD onset by GWAS studies, some of them are directly or indirectly linked to the neuroinflammatory cascade [15–17]. However, which of the several potential inflammatory biomarkers is directly affected by EVOO remains to be elucidated.

In the current study we were interested in assessing the effect that its chronic dietary administration had on the inflammatory biomarker profile in the central nervous system (CNS) of the 3xTg-AD mice at an age before the development of the full neuropathology [18]. To reach this goal we randomized the mice to a diet enriched with EVOO or a regular chow diet for 8 weeks. The initial analysis performed using a commercially available microarray platform showed that compared with controls, cortices of mice receiving EVOO were different in at least 31 of these biomarkers. To reduce the possibility of false positivity, we reanalyzed the results by setting more stringent parameters such that only the measures that were 2-fold higher or 0.5 lower were considered significant. By using this approach, we reduced the initial value of 31 to a total of 7 molecules: IL-17E, IL-12p70, MIP-2, IL-23, IL-28, IL-5, and leptin. Among these inflammatory biomarkers we were able to validate only 6 since we could not find a commercially available ELISA kit for IL-28. The validation analysis confirmed the changes in the same direction for four of them, but for the remaining two, IL-5 and leptin, we could not confirm the changes observed with the microarray.

The finding that EVOO significantly decreased IL-17E is novel and has relevance for AD pathogenesis. Thus, IL-17 family of cytokines are generally considered important modulators of host defense against pathogens and inflammatory responses. Among them IL17-E, known also as IL-25, is produced by cells such as activated T helper type 2 (Th2) cells, alveolar macrophages, and lung epithelial cells. IL-25 mRNA is expressed in the CNS and in brain endothelial cells [19]. While there is abundant data in the literature regarding IL-17A and AD, very little is available about IL-17E. Recent evidence suggests that pharmacological blockade of the signaling of this mediator could be used as an anti-inflammatory approach in several autoimmune diseases [20, 21]. Our study is the first one showing that this cytokine can be targeted by chronic administration of EVOO in the CNS of a model of AD as part of its potent anti-inflammatory action *in vivo*. Another inflammatory biomarker affected by the chronic administration of EVOO was MIP-2, also known as CXCL2, a well characterized chemokine involved in the recruitment of immune cells at the site of inflammation or tissue injury [22]. Studies have shown that MIP-2 activates the NLRP3 inflammasome via a G-protein-coupled receptor and by doing so can sustain a chronic pro-inflammatory condition [23]. Interestingly, previous studies have reported that this mediator is upregulated in two AD mouse models: the APP/PS1 and the 3xTg-AD [24, 25], whereas an increase in blood levels were reported in patients with cognitive impairment and brain amyloidosis [26].

The powerful action of EVOO in modulating the immune/inflammatory systems in the CNS of this AD mouse model was further strengthened by the significant reduction in the levels of IL-12 and IL-23. These cytokines share a common subunit called p40 which is responsible for their intracellular pro-inflammatory action. Previously, it was demonstrated an increased production of this subunit by microglia cells and that genetic ablation of IL-12/IL-23 signaling resulted in a significant reduction of brain amyloidosis in an AD mouse model [27]. Since the same authors observed that the levels of p40 were significantly elevated in the cerebrospinal fluid of AD patients, they concluded that reduction of these cytokines could be effective against AD pathology [27, 28]. Our findings are in line with this observation and add novel insights into the potential molecular mechanism responsible for the beneficial effect of EVOO in AD and its preclinical models.

Overall, the effect that chronic EVOO assumption had on specific inflammatory biomarkers has potential clinical implication and relevance for the pathophysiology of AD. In fact, considering that all the markers we found affected by our dietary regimen have been already implicated in the pathogenesis of AD, we speculate that our findings provide further support for the well described beneficial effect that EVOO has both on cognitive decline and the risk of AD onset.

Our study does not identify the active compounds of the EVOO we administered to the AD mouse model that could be responsible for the observed anti-neuroinflammatory effect *in vivo*. While we can speculate that based on previous work oleuropein could be considered a candidate for it, other compounds highly represented in the EVOO we used such as oleacein should not be dismissed [29–31].

Future studies will address this very important biologic question by looking at the array of biologically active compounds that form the final product EVOO.

Conclusions

In conclusion, our study demonstrates that chronically administered EVOO acts as a potent anti-neuroinflammatory agent by significantly reducing the levels of specific inflammatory biomarkers which are implicated in AD pathogenesis. Our findings provide additional pre-clinical support and novel mechanistic insights for the beneficial effect that this dietary intervention has on brain health and AD prevention.

AUTHOR CONTRIBUTIONS

JianGuo Li (Investigation; Methodology; Writing – original draft); Yamini Mutreja (Data curation; Formal analysis; Methodology); Maurizio Servili (Conceptualization; Writing – original draft; Writing – review & editing); Alessandro Leone (Conceptualization; Writing – original draft; Writing – review & editing); Domenico Pratico (Conceptualization; Supervision; Writing – original draft; Writing – review & editing).

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CONFLICT OF INTEREST

Domenico Praticò is an Editorial Board Member of this journal but was not involved in the peer-review process nor had access to any information regarding the peer-review.

The authors declare no conflict of interest.

DATA AVAILABILITY

The datasets generated during the current study are available from the corresponding author upon reasonable request.

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