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The Effect of Cretan High Phenolic Olive Oil on Fecal Calprotectin Levels in the Course of Multiple Sclerosis

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Abstract

Multiple sclerosis is a chronic immune-related inflammatory disease of the central nervous system that results in demyelination and lesions in the brain. Even though there is a massive research for the whole profile and treatment of the disease, to date, the etiopathology of multiple sclerosis is still unclear and there is no fully successful therapy. The urgent need to find new therapies led us to the search for the potential therapeutic effects of high phenolic early harvest extra virgin olive oil (HP-EH-EVOO) that interacts with the gut-brain axis. Recent research dictates that olive oil polyphenols can confront oxidative damage and inflammation through gut microbiota alterations. Therefore, we conducted a pilot study with relapsing-remitting multiple sclerosis (RRMS) patients. Patients in the experimental group, consumed Eliama Daily Value Gold - HP-EH-EVOO for 4 months, while patients in the control group did not. We used fecal calprotectin measurements at baseline, 2 and 4 months during the oil consumption to observe the inflammation status of the patients and found a decreased inflammation in the experimental group [$F(2,14) = 10.38$, $p = .002$]. Our results gave us an insight on how we can take advantage of our diet to treat multiple sclerosis and other inflammatory diseases. Nonetheless, it is important for future studies with bigger samples to replicate our results and extend the investigation in order for HP-EH-EVOO to be a proper candidate for clinical and therapeutic use.

Keywords: *Multiple Sclerosis; Calprotectin; Gut Microbiota; Extra Virgin Olive Oil; Polyphenols*

Introduction

Multiple Sclerosis (MS) is defined as a chronic inflammatory, degenerative, and demyelinating disease of the central nervous system (CNS), which results in lesions in the brain that gradually lead to motor and sensory deficits. MS is the most common primary neurological disorder of young adults in the western world and the main cause of disability in young and middle-aged people. It occurs with a double frequency in women than in men, even though men seem to have a worse prognosis [1].

To date, the aetiopathogenesis of the disease is still unclear. The role of oxidative stress (OS) is of great essence in MS. Brain is prone to OS due to its high demand for oxygen and a limited capability of obtaining antioxidants [2]. In the acute phase of MS, OS initiates the inflammatory process and in the chronic phase it sustains neurodegeneration. Redox process in MS is associated with mitochondrial dysfunction, dysregulation of axonal bioenergetics, iron accumulation in the brain, and impaired oxidant/antioxidant balance [2]. Moreover, Naviaux (2014) suggests that the aetiology of many chronic diseases may underlie in a dysfunction of the Cell Danger Response (CDR) [3]. The CDR is defined in terms of an ancient metabolic response to threat which encompasses inflammation, innate immunity, OS, and the endoplasmic reticulum stress response. The CDR can be maintained by extracellular nucleotide (purinergic) signaling. Given this information, an abnormal persistence of the CDR could explain MS.

Oxidative damage in MS is also closely associated with inflammation that occurs on the onset and development of the disease. Astrocyte activation by tumour necrosis factor-α (TNF-α) is involved in oligodendrocyte apoptosis and myelin vacuolation, following a primary

demyelination with lesions progressing and causing axonal damage, blood brain barrier (BBB) disruption, and significantly oligodendrocyte loss [4]. Another important cytokine is IFN-γ which seems to have a positive correlation with demyelinating lesions in the CNS of MS patients [5] and thus, this evidence supports a pathological role of IFN-γ in MS. Furthermore, a pilot study demonstrated an aggravation of clinical symptoms in some MS patients after IFN-γ administration [6], whilst antibodies against IFN-γ presented a decrease in clinical symptoms [7].

Nevertheless, there are some indications, taking in consideration a stage-specific role of IFN-γ in MS, that IFN-γ may have a protective role as well [8]. Another important factor is interleukin-6 (IL-6), having a crucial role in the regulation of IL-17-producing T helper 17 (Th17) cells and regulatory T cells (Treg), as Th17 promote the pathogenesis of autoimmune diseases and protect from bacterial infections whilst Treg confine excessive effector T-cell responses [9]. Additionally, it seems that IL-6 overpopulation can lead to several diseases through Th17 activity, such as MS [9]. Consequently, such cytokines with proinflammatory properties and their strong association with the development and pathogenesis of MS establish the central role of inflammation in the disease and the importance of targeting it in all our interventions.

Recent studies propose that there is an interaction between the gut microbiome and MS. It is estimated that the human intestine is a host to approximately $3,9 \times 10^{13}$ bacteria, forming most of the microflora and having a crucial role to the homeostasis of the human body [10] and the development and function of the immune system [11]. This interaction can be achieved through the gut-brain axis. More specifically, it seems that the gut microbiome can influence the processes of the synaptic genesis, the development of the dopaminergic system, the production of neurotransmitters, and it can also alter the permeability of the BBB [12]. On the other hand, the cerebrospinal system has the ability to form alterations on the gut microbiome through the Hypothalamus-Pituitary-Adrenal axis (HPA axis) and the autonomous nervous system. These neural processes may refer to alterations to the intestinal permeability, gastrointestinal tract motility and secretory activity [13,14].

In MS, an alteration in the levels of some archaea and bacteria in the gut microbiome is observed, already from the early stages of the disease, in comparison with healthy individuals [12]. More specifically, MS patients appear to have significantly decreased levels of bacteria potent to promote anti-inflammatory abilities [12,15-17] and a significant increase in bacteria that can promote proinflammatory abilities [16,18,19]. Moreover, some of the decreased bacteria in MS patients, can induce short chain fatty acids (SCFAs) [20] and aid in the maintenance of regulatory FoxP3+ CD4 T cells, which have anti-inflammatory properties [21]. Researchers using an animal model of MS, experimental autoimmune encephalomyelitis (EAE), observed the properties of fatty acids in the course of the disease [22]. It was found that short chain fatty acids (SCFAs) suppressed Th17 response and promoted Treg cell populations and therefore SCFAs ameliorate the course of the disease, whilst medium chain fatty acids (MCFAs) and long chain fatty acids (LCFAs) exert the opposite effect [22]. Therefore, these alterations in gut microbiota can be described as a dysbiosis between the host (human) and the microflora, intensifying the progress of MS. Furthermore, we need to keep in mind possible gut microbiota alterations in patients with MS due to their treatment [23].

Progress in gut microbiota research tells us that some of our dietary habits can have a crucial effect on the intestine microflora and the host's health. The products of bacterial metabolism may exhibit enhanced or more beneficial effects, or they may be degraded to inactive or toxic compounds, and polyphenols appear to play an important role in these processes [24]. Thereby, there is a need for the examination of dietary components and their potent therapeutic effects in several diseases such as MS. Mediterranean diet includes olives and olive oil (*Olea europaea* L.) as core products, which contain polyphenols such as tyrosol (TYR), hydroxytyrosol (HTY), oleuropein (OLE), and oleocanthal (OC) [25]. TYR, HTY and OLE appear to have antioxidant, anticarcinogenic, anti-inflammatory, antimicrobial and antihypertensive properties [26-29]. HTY is a highly efficient antioxidant based on its oxygen radical absorbance capacity, which is ten times higher than that of green tea and two times higher than the coenzyme Q10 [30,31]. OC appears to inhibit the human recombinant 5-lipoxygenase (5-LOX), which is responsible for the biosynthesis of proinflammatory leukotrienes [32] and therefore it serves as a non-steroidal anti-inflammatory drug (NSAID) candidate better than ibuprofen and other drugs due to their failure to inhibit 5-LOX and the

related adverse drug responses that can occur [33,34]. Moreover, OC is found to inhibit lipopolysaccharide (LPS)-mediated upregulation of proinflammatory signaling molecules such as interleukin-1β (IL-1β), IL-6, macrophage inflammatory protein-1α (MIP-1α), TNF-α, and granulocyte-macrophage-colony-stimulating factor (GM-CSF) [35,36].

Olive oil polyphenols seem to have neuroprotective effects as well, given that recent findings in animal models and humans show that polyphenols may have a role in regulating neurotrophic levels and in particular, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), suggesting that polyphenols may also induce their protective effects through the potentiation of neurotrophins' action [37]. NGF and BDNF, primarily known as biological mediators stimulating neuron growth, proliferation, survival and differentiation are recently studied also as metabotropic factors, acting on glucose and energy metabolism, pancreatic beta cells and cardiovascular homeostasis [37]. Additionally, there has been several indications that OC has the ability of counteracting inflammation in the brain by decreasing astrocytes activation and proinflammatory cytokines levels [38]. This evidence establishes the role of polyphenols in neuroprotection.

Given these alterations in the intestinal microflora, the inflammation induced in the gastrointestinal system, along with the information about the gut-brain axis, appears to be potentially associated with the neuroinflammation that is observed in MS. Subsequently, MS patients may experience gastrointestinal disorders, similar to the symptoms of Inflammatory Bowel Disease (IBD).

Fecal calprotectin levels correlate with endoscopic disease activity in IBD and are greater than C-reactive protein and clinical activity indices in noticing mucosal inflammation [39]. In fact, Kostas., *et al*. (2017) measured the fecal calprotectin levels of IBD patients at baseline and six months later. In their research, they found that IBD patients in remission had a large decrease in the fecal calprotectin levels six months after the baseline, indicating a decrease in inflammatory status in their body [39]. This evidence requires further studies and comparison with similar studies. Nevertheless, fecal calprotectin appears to be an essential marker of inflammatory intestinal disease, especially to decide on urgent endoscopy, and is highly useful to identify patients with IBD [39]. Considering all of the above, one could refer to fecal calprotectin to guide us as a biomarker in the diagnosis and development of MS.

The present research aims to assess the efficacy of HP-EH-EVOO as a potent therapy for the improvement, the stabilization, and the slower deterioration in patients with relapsing-remitting multiple sclerosis (RRMS), measuring fecal calprotectin levels to confirm the inflammation decrease. Provided with information from the literature so far, we hypothesize that a significant decrease in calprotectin levels will be noticed during the remitting phase of the disease.

Methods

Participants

Fifteen participants took part in the experiment. Participants were newly diagnosed (last 6 months) patients with RRMS. Participants' age ranged from 21y to 36y. The experiment included 6 male and 9 female patients, all residents of Cyprus. All patients gave their consent to participate in a research for the improvement of their lifestyle and nutrition and they were randomly assigned to either the experimental or control group. Participants completed the Expanded Disability Status Scale test (EDSS) plus the timed 25-foot walk and the 9-hole peg test and their performance in EDSS ranged from 0 to 2. The demographics of the patients are showed on table 1.

Baseline Characteristics	Experimental		Control	
	n	%	n	%
Sex				
Female	7	70	2	40
Male	3	30	3	60
Education				
Primary	0	0	0	0
Secondary	0	0	0	0
Tertiary	10	100	10	100
Age				
Below 25	3	30	2	40
Above 25	7	70	3	60

Table 1: *Sociodemographic characteristics of participants at baseline.*
Note: N = 15 (Experimental: n = 10, Control: n = 5). Participants were on average 28.13 years old (SD = 4.41) and participant age did not differ in the two groups (p = .97).

Participation credentials

Participants should be able to qualify for several credentials in order to take part in the experiment. A prior necessity was the diagnosis of RRMS in the last 6 months. Participants should also be able to have an abnormal brain MRI and lumbar puncture examination, indicating the presence of oligoclonal bands type 2 in their cerebrospinal fluid (CSF). Participants should also have ≥ 5 years of education, fluent language and require a study partner to accompany in visits and with whom the researchers would have 10+ hours of contact per week, either in person or through the phone.

Some exclusion criteria were assigned in order to prevent abnormalities and complications among the sample and the experimental processes. Therefore, individuals could not sign up for the research if they were enrolled in other research trial, had insufficient acuity for neuropsychological tests, a history of other major neurological or psychiatric illnesses or a presence of other diseases that could exclude enrolment including IBD, implants and devices not suitable for MRI scanning, dysplasia or other conditions that may complicate lumbar puncture. Use of immunosuppressant or immunomodulating agents, corticosteroids and other drugs, any significant or uncontrolled medical condition or emerging treatments, or some other clinically significant laboratory abnormalities could exclude participants from the enrolment as well.

Assessment/measures

Before the main examination, a series of tests were administrated in order to ensure the absence of some exclusion criteria. Those tests included the EDSS plus the timed 25-foot walk and the 9-hole peg test, the Frontal Assessment Battery (FAB) test, and the Multiple Sclerosis International Quality of Life questionnaire (MuSIQoL Greek 3.01 MS - SF-36). A questionnaire for nutritional habits was also administrated. For the measurements of fecal calprotectin, we used the Alegria® **Calprotectin. Alegria® Calprotectin is an ELISA-based, automated,** *in-vitro* test system for the quantitative determination of calprotectin in stool. It is used for the evaluation of IBD and for the differentiation of IBD and irritable bowel syndrome (IBS). By coating anti-calprotectin antibodies, it can calculate up to 1000 μg/g.

Procedure

Participants gave their written informed consent to take part in the research. They completed a questionnaire about their nutritional habits, participants were urged to have a low consumption on carbohydrates and saturated fat, and also try and quit smoking. Afterwards, five participants were assigned to the control group and ten to the experimental group, randomly. During the experiment, patients in experimental group consumed 50 ml (40gr) of Eliama Daily Value Gold - HP-EH-EVOO per day, while patients in control group were treated without any oil administration. Participants in experimental group consumed their daily dose by raw ingestion of 25ml of oil during the morning hours and 25 ml during the night hours. All participants were examined 2 and 4 months after the initiation of the experiment. Three measurements were taken on fecal calprotectin levels (baseline, 2-months, 4-months) since the calcium-binding protein has the ability of screening the activity of inflammation-related diseases. The baseline measurement was taken on the second day of the diagnosis. A 6-months measurement was not feasible since participants would then take a pharmacological treatment and therefore, an error for unreliable and invalid data would occur.

Results

Comparative and statistical analyses of the laboratory findings were carried out in relation to the possible reduction in the rate of disease progression and improvement of the patient's inflammation, in order to draw safe conclusions for olive oil's potential clinical therapeutic use.

First, a repeated measures analysis of variance (ANOVA) was conducted to examine the effect of HP-EH-EVOO consumption on inflammation reduction based on the three fecal calprotectin measurement periods in the experimental group (Table 2-4 and figure 1).

	Age		Cal - Baseline		Cal - 2 months		Cal - 4 months	
	Experimental	Control	Experimental	Control	Experimental	Control	Experimental	Control
Valid	10	5	10	5	10	5	10	5
Missing	0	0	0	0	0	0	0	0
Mean	28.100	28.200	57.300	53.200	39.300	55.000	33.000	55.200
Median	28.500	28.000	56.000	49.000	38.500	50.000	34.000	48.000
Std. Deviation	5.109	3.033	10.446	16.559	8.097	18.330	8.654	16.331
Minimum	21.000	24.000	42.000	39.000	27.000	38.000	19.000	42.000
Maximum	36.000	32.000	74.000	81.000	53.000	85.000	44.000	83.000

Table 2: *Descriptive statistics.*

	Sphericity Correction	Sum of Squares	df	Mean Square	F	p
Calprotectin	None	3180.600[a]	2.000[a]	1590.300[a]	34.737[a]	< .001[a]
	Greenhouse-Geisser	3180.600[a]	1.170[a]	2717.639[a]	34.737[a]	< .001[a]
Residual	None	824.067	18.000	45.781		
	Greenhouse-Geisser	824.067	10.533	78.235		

Table 3: *Repeated measures ANOVA of experimental group: within subjects effects.*

Note: Type III Sum of Squares.

[a]: Mauchly's test of sphericity indicates that the assumption of sphericity is violated (p < .05).

	Sum of Squares	df	Mean Square	F	p
Residual	1422.133	9	158.015		

Table 4: *Repeated measures ANOVA of experimental group: between subjects effects.*

Note: Type III Sum of Squares.

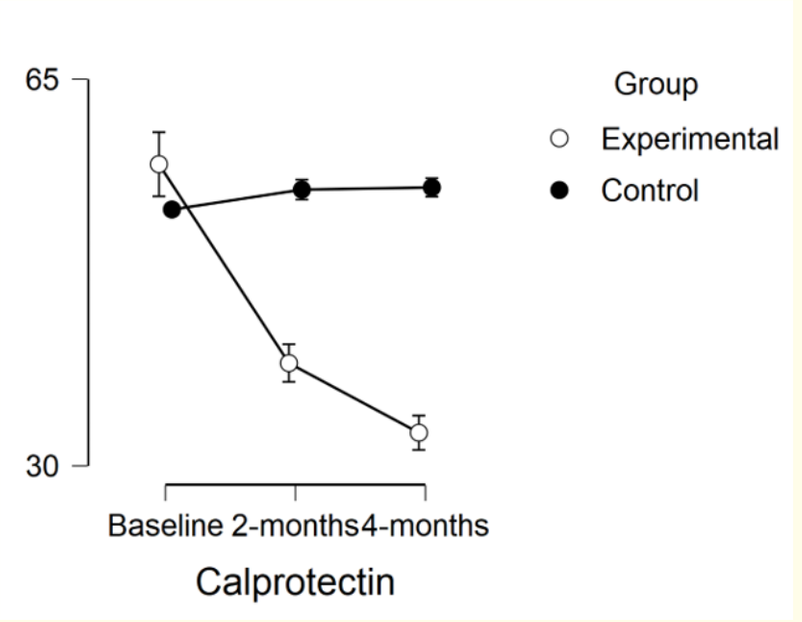

Figure 1: *The fecal calprotectin (mcg/gr) concentration in the control and experimental group during the three periods of measurement.*

There was a statistically significant difference among the measurements [F(1.17,10.53) = 34.74, p < 0.001]. Mauchly's test indicated that the assumption of sphericity had been violated [$\chi^2(2) = 9.87$, p = 0.007], therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity (ε = .59) (Table 5).

	Mauchly's W	Approx. X^2	df	p	Greenhouse-Geisser ε
Calprotectin	0.291	9.872	2	0.007	0.585

Table 5: *Repeated measures ANOVA of experimental group: test of sphericity.*

	t	df	p
Baseline - 2 months	4.901	9	< .001
Baseline - 4 months	6.870	9	< .001
2 months - 4 months	5.200	9	< .001

Table 6: *Paired samples T-test of experimental group.*
Note: Student's t-test.

	t	df	p
Baseline	0.592	13.000	0.564
2 months	-2.350	13.000	0.035
4 months	-3.503	13.000	0.004

Table 7: *Independent samples T-test of experimental and control groups.*
Note: Student's t-test.

Three paired samples t-tests were used to make post hoc comparisons among conditions. The first paired samples t-test indicated that there was a significant difference between baseline (M = 57.3 mcg/gr, SD = 10.5 mcg/gr) and 2-months (M = 39.3 mcg/gr, SD = 8.1 mcg/gr) measurements; t(9) = 4.9, p < 0.001. The second paired samples t-test indicated that there was a significant difference between baseline (M = 57.3 mcg/gr, SD = 10.5 mcg/gr) and 4-months (M = 33 mcg/gr, SD = 8.7) measurements; t(9) = 6.87, p < 0.001. The third paired samples t-test also indicated that there was a significant difference between 2-months (M = 39.3 mcg/gr, SD = 8.1 mcg/gr) and 4-months (M = 33 mcg/gr, SD = 8.7 mcg/gr) measurements; t(9) = 5.2, p < 0.001.

Secondly, independent samples t-tests followed to determine possible differences between the two groups on the three periods of fecal calprotectin measurements. At baseline, there was no statistically significant difference between the experimental (M = 57.3 mcg/gr, SD = 10.5 mcg/gr) and control group (M = 53.2 mcg/gr, SD = 16.6 mcg/gr); t(13) = 0.59, p = 0.564. At 2 months, the experimental (M = 39.3 mcg/gr, SD = 8.1 mcg/gr) and control group (M = 55 mcg/gr, SD = 18.33 mcg/gr) had a statistically significant difference; t(13) = -2.35, p = 0.035. At 4 months, there was also a statistically significant difference between the experimental (M = 33 mcg/gr, SD = 8.7 mcg/gr) and control group (M = 55.2 mcg/gr, SD = 16.3 mcg/gr); t(13) = -3.5, p = 0.004. Tests of normality (Shapiro-Wilk) and equality of variances (Levene's) were statistically non-significant for all t-tests (Table 8 and 9).

		W	p
Baseline	Experimental	0.961	0.796
	Control	0.852	0.201
2 months	Experimental	0.984	0.983
	Control	0.892	0.365
4 months	Experimental	0.938	0.530
	Control	0.811	0.100

Table 8: *Test of normality (Shapiro-Wilk) for independent samples T-test of groups.*
Note: Significant results suggest a deviation from normality.

	F	df	p
Baseline	0.534	1	0.478
2 months	3.079	1	0.103
4 months	1.432	1	0.253

Table 9: *Test of equality of variances (Levene's) for independent samples T-test of groups.*

We also conducted a Mixed ANOVA for a 2x2x2 factorial analysis to examine possible main effects and interactions between the groups, the sex of the participants, and the age of the participants, in each measurement period. Regarding age, participants were divided in two groups, below and above 25 years old. Post hoc tests were made possible using Holm and Bonferroni tests (Table 10-14).

	Sum of Squares	df	Mean Square	F	p
Calprotectin	494.467	2	247.233	6.553	0.010
Calprotectin ✻ Group	783.250	2	391.625	10.380	0.002
Calprotectin ✻ Sex	122.432	2	61.216	1.623	0.232
Calprotectin ✻ Age	12.443	2	6.222	0.165	0.850
Calprotectin ✻ Group ✻ Sex	110.303	2	55.151	1.462	0.265
Calprotectin ✻ Group ✻ Age	11.215	2	5.608	0.149	0.863
Calprotectin ✻ Sex ✻ Age	16.899	2	8.450	0.224	0.802
Calprotectin ✻ Group ✻ Sex ✻ Age	4.163	2	2.081	0.055	0.947
Residual	528.200	14	37.729		

Table 10: *Mixed ANOVA of 2x2x2 factorial analysis: within subjects effects.*
Note: Type III Sum of Squares.

	Sum of Squares	df	Mean Square	F	p
Group	1221.067	1	1221.067	8.303	0.024
Sex	1740.132	1	1740.132	11.832	0.011
Age	65.629	1	65.629	0.446	0.526
Group ✻ Sex	1057.909	1	1057.909	7.193	0.031
Group ✻ Age	835.102	1	835.102	5.678	0.049
Sex ✻ Age	333.348	1	333.348	2.267	0.176
Group ✻ Sex ✻ Age	1105.629	1	1105.629	7.518	0.029
Residual	1029.500	7	147.071		

Table 11: *Mixed ANOVA of 2x2x2 factorial analysis: between subjects effects.*
Note: Type III Sum of Squares

	Mauchly's W	Approx. X^2	df	p	Greenhouse-Geisser ε
Calprotectin	0.395	5.575	2	0.062	0.623

Table 12: *Mixed ANOVA of 2x2x2 factorial analysis: test of sphericity.*

Calprotectin	F	df1	df2	p
Baseline	2.374	7.000	7.000	0.138
2 months	1.227	7.000	7.000	0.397
4 months	1.380	7.000	7.000	0.341

Table 13: *Mixed ANOVA of 2x2x2 factorial analysis: test for equality of variances (Levene's).*

Calprotectin		Mean Difference	SE	t	p_{holm}	p_{bonf}
2-months	4-months	4.133	1.214	3.403	0.009	0.013
	Baseline	-11.400	3.478	-3.278	0.009	0.016
4-months	Baseline	-15.533	4.051	-3.835	0.005	0.005

Table 14: *Mixed ANOVA of 2x2x2 factorial analysis: Post Hoc comparisons - calprotectin.*
Note: Bonferroni adjusted confidence intervals.

The results of the Three-Way Mixed ANOVA indicated that there was a statistically significant main effect among the three fecal calprotectin measurements [F(2,14) = 6.55, p = 0.01], as well as a statistically significant interaction between the two groups and the three fecal calprotectin measurements [F(2,14) = 10.38, p = 0.002], supportive of the previous t-tests that were conducted. That is, no significant simple main effect was observed among the measurements of the control group and the baseline measurement of experimental group, whereas the 2-months (M = 39.3 mcg/gr, SD = 8.1 mcg/gr) and 4-months (M = 33 mcg/gr, SD = 8.7 mcg/gr) measurements of experimental group had statistically significant simple main effects, when compared with other conditions. The analysis also indicated a main effect between the gender of the participants [F(1,7) = 11.83, p = 0.011] and an interaction between the gender and the group as well [F(1,7) = 7.19, p = 0.031] revealing that males in the control group had significantly bigger scores in fecal calprotectin measurements, in comparison with the other conditions. Another interaction was observed between all three independent variables, group, gender and age [F(1,7) = 7.52, p = .029], revealing statistically significant simple main effects between males below 25 years old in the control group and females below 25 in the experimental group and between males below 25 in the control group and females above 25 in the experimental group (Table 15-18).

Calprotectin		Mean Difference	SE	t	p_{holm}	p_{bonf}
2-months, Control	2-months, Experimental	15.275	5.141	2.971	0.090	0.150
	4-months, Control	-0.750	4.063	-0.185	1.000	1.000
	4-months, Experimental	21.150	5.141	4.114	0.012	0.015
	Baseline, Control	1.625	4.063	0.400	1.000	1.000
	Baseline, Experimental	0.575	5.141	0.112	1.000	1.000
2-months, Experimental	4-months, Control	-16.025	5.141	-3.117	0.075	0.112
	4-months, Experimental	5.875	3.221	1.824	0.627	1.000
	Baseline, Control	-13.650	5.141	-2.655	0.149	0.280
	Baseline, Experimental	-14.700	3.221	-4.564	0.006	0.007
4-months, Control	4-months, Experimental	21.900	5.141	4.260	0.010	0.012
	Baseline, Control	2.375	4.063	0.585	1.000	1.000
	Baseline, Experimental	1.325	5.141	0.258	1.000	1.000
4-months, Experimental	Baseline, Control	-19.525	5.141	-3.798	0.021	0.029
	Baseline, Experimental	-20.575	3.221	-6.388	< .001	< .001
Baseline, Control	Baseline, Experimental	-1.050	5.141	-0.204	1.000	1.000

Table 15: *Mixed ANOVA of 2x2x2 factorial analysis: Post Hoc comparisons - calprotectin ✻ group.*
Note: Bonferroni adjusted confidence intervals.

Calprotectin		Mean Difference	SE	t	p_{holm}	p_{bonf}
Control, F	Control, M	-25.583	6.549	-3.906	0.029	0.035
	Experimental, F	0.833	5.752	0.145	1.000	1.000
	Experimental, M	-2.333	6.549	-0.356	1.000	1.000
Control, M	Experimental, F	26.417	5.193	5.087	0.009	0.009
	Experimental, M	23.250	6.064	3.834	0.029	0.039
Experimental, F	Experimental, M	-3.167	5.193	-0.610	1.000	1.000

Table 16: *Mixed ANOVA of 2x2x2 factorial analysis: Post Hoc comparisons - group *sex.*

Note: Bonferroni adjusted confidence intervals.

Calprotectin		Mean Difference	SE	t	p_{holm}	p_{bonf}
Control, Above 25	Control, Below 25	-12.750	6.549	-1.947	0.370	0.556
	Experimental, Above 25	2.083	5.193	0.401	0.700	1.000
	Experimental, Below 25	9.250	6.064	1.525	0.513	1.000
Control, Below 25	Experimental, Above 25	14.833	5.752	2.579	0.183	0.219
	Experimental, Below 25	22.000	6.549	3.359	0.073	0.073
Experimental, Above 25	Experimental, Below 25	7.167	5.193	1.380	0.513	1.000

Table 17*: Mixed ANOVA of 2x2x2 factorial analysis: Post Hoc comparisons - group ✻ age.*

Note: Bonferroni adjusted confidence intervals.

		Mean Difference	SE	t	p_{holm}	p_{bonf}
Control, F, Above 25	Control, F, Below 25	5.000	9.902	0.505	1.000	1.000
	Control, M, Above 25	-7.833	8.575	-0.913	1.000	1.000
	Control, M, Below 25	-38.333	9.902	-3.871	0.147	0.171
	Experimental, F, Above 25	2.333	7.670	0.304	1.000	1.000
	Experimental, F, Below 25	4.333	8.575	0.505	1.000	1.000
	Experimental, M, Above 25	-6.000	8.575	-0.700	1.000	1.000
	Experimental, M, Below 25	6.333	9.902	0.640	1.000	1.000
Control, F, Below 25	Control, M, Above 25	-12.833	8.575	-1.497	1.000	1.000
	Control, M, Below 25	-43.333	9.902	-4.376	0.081	0.091
	Experimental, F, Above 25	-2.667	7.670	-0.348	1.000	1.000
	Experimental, F, Below 25	-0.667	8.575	-0.078	1.000	1.000
	Experimental, M, Above 25	-11.000	8.575	-1.283	1.000	1.000
	Experimental, M, Below 25	1.333	9.902	0.135	1.000	1.000
Control, M, Above 25	Control, M, Below 25	-30.500	8.575	-3.557	0.204	0.259
	Experimental, F, Above 25	10.167	5.858	1.736	1.000	1.000
	Experimental, F, Below 25	12.167	7.002	1.738	1.000	1.000
	Experimental, M, Above 25	1.833	7.002	0.262	1.000	1.000
	Experimental, M, Below 25	14.167	8.575	1.652	1.000	1.000
Control, M, Below 25	Experimental, F, Above 25	40.667	7.670	5.302	0.031	0.031
	Experimental, F, Below 25	42.667	8.575	4.976	0.043	0.045
	Experimental, M, Above 25	32.333	8.575	3.771	0.160	0.195
	Experimental, M, Below 25	44.667	9.902	4.511	0.072	0.077
Experimental, F, Above 25	Experimental, F, Below 25	2.000	5.858	0.341	1.000	1.000
	Experimental, M, Above 25	-8.333	5.858	-1.423	1.000	1.000
	Experimental, M, Below 25	4.000	7.670	0.522	1.000	1.000
Experimental, F, Below 25	Experimental, M, Above 25	-10.333	7.002	-1.476	1.000	1.000
	Experimental, M, Below 25	2.000	8.575	0.233	1.000	1.000
Experimental, M, Above 25	Experimental, M, Below 25	12.333	8.575	1.438	1.000	1.000

Table 18: *Mixed ANOVA of 2x2x2 factorial analysis: Post Hoc comparisons - group ✻ sex ✻ age.*

Note. Bonferroni adjusted confidence intervals.

Discussion

The main results indicated a drop in fecal calprotectin levels 2 and 4 months after the administration of Eliama Gold - HP-EH-EVOO. These results not only support the literature about polyphenols' beneficial effects on health, but also seem to promote these benefits for a therapeutic and/or preventative use for not only MS but may also for other neurodegenerative and inflammatory diseases. We also ran some additional statistical analyses in order to collect more specific information about the decrease in fecal calprotectin levels, i.e. how patient's gender or age influence the effects of HP-EH-EVOO in inflammation. Although we found some statistically significant interactions between group-gender and group-gender-age, their interpretation is not feasible. This issue occurs frequently when we are dealing with small samples that are weak to exclude ambiguity. Thus, in future research it is necessary to support our results with greater sample.

It is also important to point out that even though no statistical significance was found, possibly due to the sample limitations, it was observed that the baseline calprotectin levels of patients above 25 years old (M = 56.3 mcg/gr, SD = 10.6 mcg/gr) were marginally greater than the ones observed in patients below 25 (M = 55.2 mcg/gr, SD = 16.8 mcg/gr); $t(13) = 0.13$, $p = 0.878$. The idea of dividing the sample in this way, is based on the possibility that in patients above 25, although newly diagnosed, the disease may have occurred earlier and thus its negative effects may be more severe, including inflammation. Also, overall calprotectin decrease for patients above 25 in the experimental group (M = 25.7 mcg/gr, SD = 11.3 mcg/gr) was greater than the one observed in the patients of the same group below 25 (M = 21mcg/gr, SD = 12.5 mcg/gr) but once again not statistically significant; $t(8) = 0.59$, $p = 0.573$. Therefore, although we have to be cautious on interpreting data with no statistical significance, we cannot ignore the indications that HP-EH-EVOO might be potent to not only reduce disease inflammation but also do it in such a manner that the greater the inflammation, the more it can reduce it.

Nevertheless, our results are partially supported by a recent research that, among others, observed the effects of Eliama Gold - HP-EH-EVOO administration for 2 months in rats with EAE [40]. Their findings revealed that HP-EH-EVOO ameliorated the oxidative damage induced in EAE and also decreased some markers of inflammation such as TNF-α and nitric oxide [40]. Correspondingly, the effects of HP-EH-EVOO are observed in MS too, as well as in other immune-related diseases such as rheumatoid arthritis and amyotrophic lateral sclerosis [41]. A review on the effects EVOO, revealed that TYR, HTY, OLE and OC can have some protective effects against oxidation and inflammation, and also some immunoregulatory effects regarding various chronic inflammatory diseases, including MS [42]. Just like the aforementioned review, we also feel the need to stress the fact that more research has to be done in order to confirm the beneficial indications of EVOO and consequently provide some accurate nutritional suggestions, i.e. proper consumption, for patients with such diseases that can not only milden the course of the disease but also improve their quality of life. An accurate measurement for the examination of some aspects that are able to provide information regarding the course of the disease is also needed.

A reliable indicator of the clinical course of various inflammation-related diseases is proven to be calprotectin [43,44]. Already activated in the acute phase of inflammation, it seems that calprotectin levels rise more than 100 folds [45]. Even though there are some evidence that indicate calprotectin as a histopathological marker of monocyte activation, the literature is divided on whether it can depict disease activity in MS. Floris., *et al*. (2004) supported that calprotectin, from serum samples, did not correlate with the disease activity of RRMS [46]. On the other hand, Berg-Hansen., *et al*. (2009) found that CSF levels of the multiprotein complex could represent the disease activity in MS, although it could not differentiate between MS and other inflammatory conditions [47]. This conflict dictates that we need a reliable extraction of calprotectin samples that can offer a disease activity reflection. An association among MS and IBD is supported by their mutual epidemiological and immunological standards [48] and their co-morbidity [49,50]. In IBD, fecal calprotectin sampling is proven to be a better indicator for the evaluation of inflammation, in comparison to blood tests and it seems there are potentials to be used as a diagnostic, detecting, and monitoring tool for intestinal inflammation [51]. Keeping in mind the alterations of the gut microbiome in patients with MS and their inflammatory impact, fecal calprotectin sampling can be of good use as an indicator of the inflammation observed in MS, thus explaining why we chose our particular method of inflammation measurement and encouraging future research to adopt this technique.

The main limitation of our research occurs due to the small sample of patients that were participating in the two groups. This is a problem because even if our hypothesis is supported, we do not know if these results will be replicated with a bigger number of participants and therefore our results might be misleading, as indicated by Simpson's Paradox [52].

Conclusion

MS, as a disease of the CNS, not only impacts one's health but also their quality of life to a large extent. Cognitive deterioration hinders the functional levels of the individual and oxidative stress worsens their state. Oxidative stress is conspicuous in MS and it is strongly associated with inflammation, another main aspect in MS. Recent research data have provided us with information that the intestine microflora interacts with the host's health. It seems that there is a connection between the intestinal microflora and the CNS through the HPA axis and the autonomous nervous system. As a result, the alterations observed in certain gut microbiota species are potent to explain the inflammation in MS and the disease course.

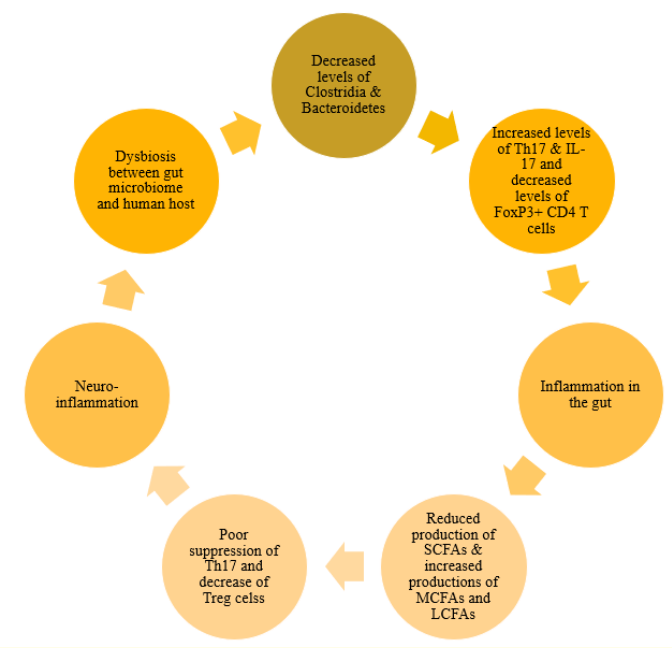

Figure 2: *Schematic representation of gut microbiome alterations and their associated gut-brain interactions in MS that create a vicious cycle which exacerbates the inflammation in both gut and CNS.*

Other recent findings stressed the importance of dietary components in our health such as olive oil polyphenols (TYR, HTY, OLE, OC). The offering of these polyphenols to an individual's health extends to neuroprotection as well, by decreasing astrocytes activation and proinflammatory cytokines levels. Therefore, an intervention on the dietary habits of patients with MS can provide research with essential information, aiding the process of addressing successfully the symptoms of the disease. Our check-up of the fecal calprotectin levels of our participants seems to agree with the literature indications. MS patients consuming HP-EH-EVOO had significantly reduced fecal calprotectin levels 2 and 4 months after the initiation of the oil consumption, in comparison with patients that did not. Thus, the results of the experiment can potentially lead to further clinical trials and enhance the future research for a therapy in MS and other inflammatory diseases, since safer conclusions can be drawn with a larger sample research.

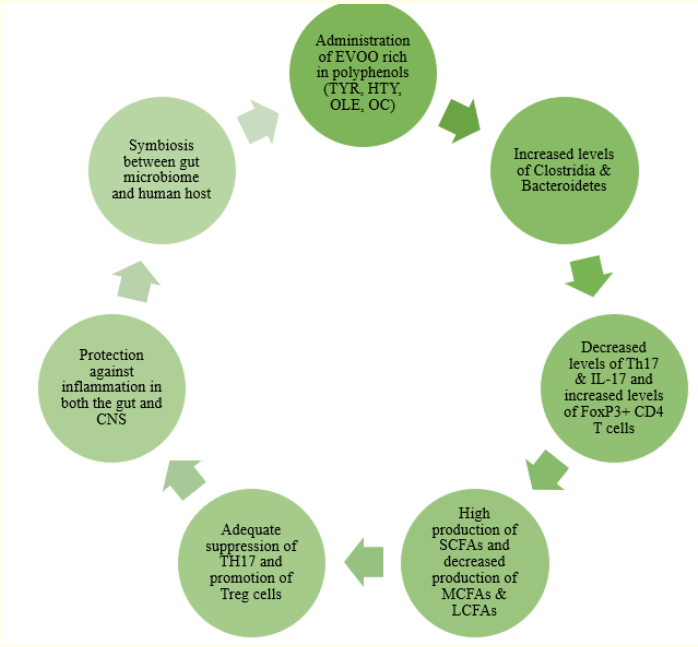

Figure 3: *Schematic representation of EVOO rich in polyphenols effects in gut microbiome and its consequence in gut-brain interactions, establishing protective effects for various inflammatory diseases, including MS.*

Our future direction heads towards the evaluation of the effects of olive oil in the course of neurological and other diseases and consequently we encourage future research to turn its interest on nutritional neuroscience and how our dietary habits can be of good use to address several diseases, without largely altering the every-day routine of patients and thus affecting their quality of life less. This can be done by examining proper and optimal doses of HP-EH-EVOO to address the disease and investigate as well other dietary substances, potent to aid in the treatment of diseases that afflict the humankind.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

Consent to Participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the Declaration of Helsinki 1964. All the participants gave informed consent prior to their inclusion in the study.

Consent for Publication

All the authors have consented for the publication of the study

Availability of Data and Material

Data available upon duly justified request. A chemical analysis of the product Eliama D.V. Gold was done by an approved chemical lab, the polyphenols analysis from the Pharmacology Department of National Kapodistrian University of Athens, Greece.

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